SERUM SOMATOMEDIN AND SOMATOMEDIN GENERATION BY THE

PERFUSED LIVER IN PROTEIN MALNOURISHED RATS

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the Faculty of Medicine, University of Cape Town.

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ABSTRACT

The studies presented in this thesis represent an attempt to characterise some aspects of the abnormalities of somatomedin physiology in protein-energy malnutrition.

A suitable bioassay to study somatomedin was found in that of Van den Brande and Du Caju (1974) which makes use of uniform discs of cartilage punched from slices of immature porcine costal cartilage. The handling of the cartilage discs was made easy by the specially designed incubation rack. Preincubation increased sensitivity of the cartilage, and post incubation with radioactive tracer reduced the potential interference of varying sulphate content of assay samples. Serum samples were subjected to formal multipoint parallel line bioassay and data analysed by a computer programme designed to examine such assays. The bioassay was found to be sensitive to a concentration of serum of 5% and to have an index precision of less than 0.30 (and usually less than 0.20).

An experimental model of protein-energy malnutrition (Stead and Brock) 1972) was adopted which made use of weanling rats fed semi-synthetic diets (ad lib) containing either 4% casein (to produce malnutrition) or 20% casein (to produce wellnourished age-matched controls). The diets were isocaloric and provided known and adequate amounts of vitamins, minerals and trace elements. Untreated weanlings were used as additional weight-matched wellnourished controls. The protein deficient diet produced a syndrome in the rats which had many features in common with human protein-energy malnutrition: failure to gain weight, failure of linear growth, hypoproteinaemia, hypoalbuminaemia and periportal fatty infiltration of the liver.

Histologically the epiphyseal growth plates of long bones (especially that of the upper tibia) were found to be narrow and inac-

tive in malnourished animals. The cartilagenous ground substance of malnourished cartilage was found to stain normally by a variety of histochemical techniques. The incorporation of $^{35}SO_4$ into cartilage, 24 hours after the intraperitoneal administration of $Na_2{}^{35}SO_4$, was found to be reduced in protein malnourished animals, autoradiography being used to examine long bones and scintillation counting for costal cartilage.

Serum somatomedin activity was found to be reduced to nearly hypopituitary levels in protein malnourished animals. There was no evidence for the presence of an inhibitor in mixing experiments and dose response curves of protein malnourished and costal sera were parallel. Growth hormone was found to be normal by radioimmunoassay and in this respect the situation in the rat differs from that in man where the growth hormone levels are elevated. Nevertheless this represents a situation not dissimilar to that in humans in that the somatomedin activity is inappropriately low for the level of growth hormone present. Hypoinsulinaemia was confirmed in this model of experimental protein malnutrition and this may be a factor responsible for the reduced somatomedin activity. The administration of pharmacological doses of growth hormone (from species known to be biologically active in the rat) failed to stimulate weight gain, linear growth, tibial width or serum somatomedin activity.

When the cartilage of malnourished and age-matched control animals was examined in an in vitro incubation system both were found to incorporate $^{35}SO_4$ similarly under serum-free conditions and to be equally stimulated in the presence of normal rat serum, while serum from malnourished animals was poorly stimulating to both types of cartilage. These observations suggested that an end organ resistance of cartilage to somatomedin's action was not a

major factor in the reduced growth rate, narrow epiphyses and reduced in vivo incorporation of $^{35}SO_4$ in protein-energy malnutrition.

An isolated in situ liver perfusion technique based on that of Hems, Ross, Berry and Krebs (1966) was developed to study the generation of somatomedin by the livers of malnourished and wellnourished animals. The viability of the liver preparation was determined as being satisfactory by the following criteria: macroscopic appearance, histological appearance, perfusate flow rate, bile secretion rate, normal wet/dry weight ratios of perfused livers, oxygen consumption, adequate and linear gluconeogenesis from lactate and, in a slightly modified system, sensitivity to glucagon-induced glycogenolysis in the physiological range. The livers of protein malnourished animals were found to generate less somatomedin activity than control livers in the absence of hormonal stimulus or when stimulated by growth hormone, insulin, or a combination of growth hormone and insulin. The slightly greater relative sensitivity of protein malnourished livers to insulin may be an indication of the importance of insulin in the maintenance of normal somatomedin activity (in vivo, protein malnourished animals were noted to be hypoinsulinaemic). The reduced liver generation of somatomedin activity by the livers of protein malnourished animals may be a mechanism whereby the low serum somatomedin activity in this condition occurs. Reduced somatomedin generation itself may represent a non-specific effect of deranged liver function or an adaptive homeostatic mechanism by means of which the direct actions of growth hormone might be made possible without concomitant stimulation of growth, at a time that growth may be disadvantageous to the survival of the organism.

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CONTENTS

		Page No.
ABSTRACT		i-iii
ACKNOWLEDGEMENTS		1
CONTENTS		3
CHAPTER 1	Scope and objectives	4
CHAPTER 2	Review of the literature	6
CHAPTER 3	The bioassay of somatomedin activity	38
CHAPTER 4	Experimental protein malnutrition	68
CHAPTER 5	In vivo $^{35}SO_4$ incorporation into cartilage and cartilage histology and histochemistry	82
CHAPTER 6	Serum somatomedin activity (sulphation factor) in experimental protein malnutrition in the rat	110
CHAPTER 7	In vitro $^{35}SO_4$ into cartilage	124
CHAPTER 8	Somatomedin activity generation by the isolated perfused liver in experimental protein malnutrition	135
CHAPTER 9	Summary of conclusions	176
APPENDIX A	Histological and histochemical techniques	181
APPENDIX B	Chemical and biochemical methods	187
APPENDIX C	Statistical methods	207
REFERENCES		221

CHAPTER ONE

SCOPE AND OBJECTIVES

"He who has begun has half done. Dare to be wise; Begin"

Horace

In the last quarter of the 20th century malnutrition remains a major problem for a majority of the population of our planet. The problem is especially one for the under-developed or Third World countries. Unbridled population growth, increasing shortages of resources of all types, the advance of deserts into previously arable land on every continent and poor and increasingly expensive distribution of food reserves, all give little reason for optimism. The University of Cape Town Medical School has been in the unusual position of having sophisticated technology and skilled staff available to study protein-energy malnutrition which is a disease all too frequently encountered in the Cape peninsula. Professor J.F. Brock and his coworkers were responsible for defining many of the clinical and biochemical abnormalities of protein-energy malnutrition. The endocrine adjustments and abnormalities which occur in this condition have been studied in considerable detail by Professor B.L. Pimstone and this thesis represents the work done to explore some of the endocrine changes which may be responsible for failure of linear growth in protein-energy malnutrition and the mechanism whereby these changes occur. Specifically these changes are those which are related to somatomedin (growth hormone dependent sulphation factor). Because the cartilagenous end-organ (the site of action of somatomedin), and the liver, which is a major site of somatomedin generation, are not readily accessible to study in man, it was decided to use a rat model of protein-energy malnutrition.

Chapter 2 reviews the literature on somatomedin and related growth factors.

Chapter 3 discusses the bioassay of somatomedin activity and describes the methodology and validation of the bioassay which was used to explore the problems of somatomedin in protein-energy malnutrition.

Chapter 4 deals with animal models of protein-energy malnutrition, the relevance of such models to the human syndrome and the reasons for choosing the rat model of Stead and Brock (1972). The diet, animals (protein malnourished and control) and the morphologic and biochemical characterisation of the model are fully recorded.

Chapter 5 describes the histological and histochemical features of growth cartilage as well as the $^{35}SO_4$ incorporation in vivo in protein-energy malnutrition.

Chapter 6 deals with the measurement of somatomedin activity in the serum of protein malnourished rats and the failure to demonstrate obvious inhibitors. Growth hormone and insulin, both of which have a role in somatomedin generation, were also measured by radioimmunoassay and the relationships between growth hormone, insulin and somatomedin activity are discussed.

Chapter 7 describes the behaviour of cartilage from protein malnourished and control rats in an in vitro incubation system with special emphasis on the responsiveness of such cartilage to normal and protein malnourished serum.

Chapter 8 covers a discussion of the role of the liver in the generation of somatomedin activity and the suitability of liver perfusion systems, the development and validation of a liver perfusion system and its uses in studying the generation of somatomedin activity by the livers of protein malnourished and control animals in response to growth hormone and insulin.

Chapter 9 provides a summary of the main conclusions reached in this study.

CHAPTER TWO

REVIEW OF THE LITERATURE

"Listening to both sides of a story will convince you that there is more to a story than both sides" *Frank Tyger*

The purpose of this review is to provide a comprehensive but not exhaustive survey of the literature of somatomedin including an introductory coverage of those areas which are the special brief of this thesis. The literature relating to the bioassay of somatomedin, the production of experimental models of protein-energy malnutrition, cartilage histology, serum somatomedin and cartilagenous end-organ sensitivity in protein-energy malnutrition and somatomedin generation by the isolated perfused liver is dealt with in greater depth in the separate chapters on these subjects.

A Introduction

The very name "growth hormone" or "somatotropin" implies that this hormone acts directly on skeletal tissue to cause growth and this was long held to be one of its major functions (Becks, Simpson, Evans, Ray, Li and Asling, 1946). In a search for an improved in vitro bioassay for growth hormone Salmon and Daughaday (1956) performed a series of experiments which to this day provide the basis for the somatomedin hypothesis of growth hormone's action on skeletal tissue and all subsequent work in this field. It was found that utilising the incorporation of $^{35}SO_4$ into hypophysectomised rat costal cartilage as the bioassay preparation, hypophysectomised rat serum (which would be free of growth hormone) was less stimulatory to $^{35}SO_4$ incorporation than normal rat serum but that the addition of growth hormone to hypophysectomised rat serum would not result in further stimulation. In contrast to the in vitro situation the administration of growth hormone in vivo to hypophysectomised rats res-

tored the ability of serum from these animals to stimulate $^{35}SO_4$ incorporation. The initial name given to this stimulatory activity was "sulphation factor" but when it was found that sulphation factor activity was but one action (see Table 2.1), the more all embracing term "somatomedin" was coined (Daughaday, Hall, Raben, Salmon, Van den Brande and Van Wyk, 1972). This term indicates by "somato" the relationship to somatotropin or growth hormone, and by "medin" that it is the mediator for growth hormone's action on skeletal tissue.

The somatomedin hypothesis which resulted from the work of Salmon and Daughaday (1956) states that growth hormone does not act directly to cause growth of tissues (especially skeletal tissue) but stimulates the generation of secondary factor(s) known as somatomedin(s) which acts on peripheral tissue to cause growth. For over 20 years this has remained only an hypothesis, in that in spite of a large body of supportive evidence direct proof in the form of growth stimulation by a somatomedin preparation in a hypophysectomised animal has been lacking. In 1978, however, almost certain proof of the hypothesis has been provided by the demonstration of highly significant somatic growth in Snell dwarf (hereditary hypo somatotrophic) mice by a somatomedin preparation from human serum (Van Buul, Dumoleijin, Korteland, Van de Klundert and Van den Brande 1978).

B The bioassay of somatomedin

Somatomedin(s) has diverse biological actions which are shown in Table 2.1. All of these biological actions have potential use as bioassays but most practical techniques depend on $^{35}SO_4$ incorporation into hypophysectomised rat costal cartilage (^3H Methyl thymidine incorporation may be determined simultaneously or in place of $^{35}SO_4$

TABLE 2.1

METABOLIC EFFECTS OF SOMATOMEDIN (modified from Daughaday, Phillips and Herington, 1975)

(A) Stimulation of:-

1. Cartilage

   i) Chondroitin sulphate synthesis - ^{35}S sulphate incorporation [a-f]

   ii) Chondromucoprotein synthesis - ^{14}C leucine incorporation [afg]

   iii) Collagen synthesis - ^{14}C proline converted to collagen ^{14}C hydroxyproline

   iv) RNA synthesis - ^{3}H uridine incorporation [f]

   v) DNA synthesis - ^{3}H thymidine incorporation [fij]

2. Other tissues

   i) Protein synthesis in muscle - ^{14}C leucine incorporation

   ii) Glucose oxidation in adipose tissue (NSILA)[k]

   iii) HeLa cell growth in tissue culture[1]

   iv) DNA synthesis in human glial cells - ^{3}H thymidine incorporation [o]

   v) DNA synthesis in chick embryo fibroblasts - ^{3}H thymidine incorporation [p]

   vi) In vitro growth of human serum fibroblasts [q]

(B) Inhibition of:-

1. Glycerol release in epinephrine stimulated epididymal fat pads [m]
2. ^{125}I insulin binding to adipocytes, chondrocytes and liver cell membranes.

a Salmon, W.D. and Daughaday, W.H. (1957) J.Lab.Clin.Med. 49, 825.

b Almqvist, S. (1961) Acta Endocrinol. 36, 31.

c Yde, H. (1968) Acta Endocrinol. 57, 557.

d Adamson, L.F. and Anast, C.S. (1966) Biochim. Biophys. Acta 121, 10.

e Hall, K. (1970) Acta Endocrinol. 63, 338.

f Salmon, W.D. and Du Vall, M.R. (1970) Endocrinology 86, 721.

g Salmon, W.D. and Du Vall, M.R. (1970) Endocrinology 87, 1168.

h Daughaday, W.H. and Mariz, I.K. (1962) J.Lab.Clin.Med. 59, 741.

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

j Garland, J.T., Lottes, M.E., Kozak, S. and Daughaday, W.H. (1972) Endocrinology 90, 1086.

k Hall, K. and Uthne, K. (1971) Acta Med. Scand. 190, 137.

l Salmon, W.D. and Hosse, B.R. (1971) Proc. Soc. Exp. Biol. Med. 136, 805.

m Underwood, L.E., Hintz, R.L., Voina, S.J. and Van Wyk, J.J. (1972) J. Clin. Endocrinol. Metab. 35, 194.

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

o Fryklund, L., Uthne, K., Sievertsson, H. and Westermark, B. (1974) Biochem. Biophys. Res. Commun. 61, 950.

p Cohen, K.L., Short, P.A. and Nissley, S.P. (1975) Endocrinology 96, 193.

q MacGillivray, M.H., Hastings, C. and Brown, J.A. (1975) J. Clin. Endocrinol. Metab. 40, 62.

but provides less precision) (Daughaday, Phillips and Herington, 1975), $^{35}SO_4$ incorporation into cartilage of chick embryo pelvic rudiments (Hall, 1970) or $^{35}SO_4$ into porcine costal cartilage (Van den Brande and Du Caju, 1974) (See also Table 3.1). Bioassays are subject to non-specific stimulation and also to inhibitors which may interact with the somatomedin present to give reduced values (Daughaday, Phillips and Herington, 1975).

There are as yet no international standards for serum or for a purified somatomedin preparation, each laboratory using its own reference serum, usually a pool (of variable size) obtained from normal subjects, with a nominal potency of 1 U/ml. This situation makes the direct comparison of data between different laboratories difficult (Beaton, 1976).

C <u>Somatomedin activity in health</u>

i) The effect of age on somatomedin activity

Reports on somatomedin levels in cord blood and maternal serum and the changes occurring in early life vary greatly. Somatomedin activity of cord blood has been reported as being equal to that of maternal serum (Daughaday, Salmon and Alexander, 1959; Chesley, 1962; Hintz, Seeds, Johnsonbaugh, 1974) or higher (Tato, Du Caju, Prévôt and Rappaport, 1975). Cord blood levels of somatomedin A (by radio receptor assay) and somatomedin B (by radioimmunoassay) however are lower than maternal blood levels (Svan, Hall, Ritzen, Takano and Skottner, 1977) as is bioassayable non-suppressible insulin-like activity-soluble (NSILA-S) (Franklin, Pepperel, Burger and Cameron, 1978). Similar low levels have been observed with an NSILA competitive protein binding assay (Heinrich, Draznin, Johnson and Schalch, 1978) and a somatomedin bioassay (Ashton, Matheson, Vesey and Francis, 1978). Tato, Du Caju, Prévôt and Rappaport

(1975) have reported a rise in somatomedin activity from very low levels in the newborn to the normal adult range by the fourth day of life and a fall between 23 days and 15 months. Conversely, most workers have shown that after the first months of life a slow rise in somatomedin activity occurs, reaching adult levels by four to eight years of age (Daughaday, Salmon and Alexander, 1959; Almqvist, Ikkos and Luft, 1961; Almqvist and Rune, 1961; Kogut, Kaplan and Shimizu, 1963; Van den Brande and Du Caju, 1973). A similar rise has been observed for somatomedin C by radio receptor assay (D'Ercole, Underwood and Van Wyk, 1977) and radioimmunoassay (Furlanetto, Underwood, Van Wyk and D'Ercole, 1977) from hypopituitary levels in the newborn to near adult levels over a similar time span.

No correlation between birth weight or labour stress and cord blood somatomedin activity could be found by Giordano, Foppiani, Minuto and Perroni (1976) or with somatomedin A (radio receptor assay) and somatomedin B (radioimmunoassay) (Svan, Hall, Ritzen, Takano and Skottner, 1977). In contrast Gluckman and Brinsmead (1976) found somatomedin activity to correlate positively with birth weight, length and head circumference, independently of gestational age. NSILA has also been found to correlate with birth weight and gestational age (Heinrich, Draznin, Johnson and Schalch, 1978) as has somatomedin activity (Ashton, Matheson, Vesey, and Francis, 1978). A number of conditions characterised by foetal hypopituitarism are associated with normal or only slightly reduced foetal size at birth (Blizzard and Alberts, 1956; Reid, 1960). Somatostatin levels in cord blood do not correlate with growth hormone (Gluckman and Brinsmead, 1976; Giordano, Foppiani, Minuto and Perroni, 1976), but placental lactogen (somatomammotropin) is equipotent with growth hormone in causing weight gain in hypophysec-

tomised rats, binds to growth hormone receptors in the liver with high affinity (Chan, Robertson and Friesen, 1976) and has been implicated in the generation of foetal somatomedin activity (Hurley, D'Ercole, Handwerger, Underwood, Furlanetto and Fellows, 1977). Foetal somatomedin activity has not been systemically studied in man, but in the rat, in spite of high growth hormone and insulin levels, somatomedin activity was scarcely detectable until birth after which it rose to adult levels within 11 days (Stuart, Lazarus, Moore and Smythe, 1976). Beaton (1976) found a progressive rise in somatomedin activity in the rabbit from prenatal to pubertal animals. In children, Hall and Filipsson (1975) have shown a correlation between dental maturity and growth rate with somatomedin A. Daughaday (1971) reported no change in somatomedin activity at the time of puberty while Rothenberg, Hintz and Van Camp (1977) found a significant increase in girls in late puberty. The changes with age described above have not been specifically related to puberty. Somatomedin activity persists into old age (Almqvist and Rune, 1961).

That growth should be most rapid at times that somatomedin activity of serum is low is apparently paradoxical but the explanation lies in the sensitivity of the cartilage of young animals to the stimulus of somatomedin. Dziewiatkowski (1954) showed that the ability of the rat to incorporate $^{35}SO_4$ into the cartilage fell with age, Herbai (1970) found the incorporation of $^{35}SO_4$ into costal cartilage in the mouse to correlate with weight gain in the preceding week. In an elegant longitudinal study Beaton (1976) has shown that incorporation of $^{35}SO_4$ and thymidine into rabbit costal cartilage is related to the growth velocity of the animal from which they were taken.

Cartilage responsiveness to stimulation by somatomedin is greatest in young animals (Phillips, Herington and Daughaday, 1974)

(but cartilage from the foetal rat was not stimulated by human serum) (Heins, Garland and Daughaday, 1970) while the cartilage responsiveness in foetal and post-natal rabbits was related to growth velocity (Beaton, 1976).

ii) Somatomedin activity in pregnancy

Early reports suggest that somatomedin was unchanged by pregnancy (Chesley, 1962; Daughaday, Salmon and Alexander, 1959; Hintz, Seeds and Johnsonbaugh, 1974), while Tato, Du Caju, Prévôt and Rappaport (1975) and Giordano, Foppiani, Minuto and Perroni (1976) reported low somatomedin activity in pregnancy falling to hypopituitary levels after delivery. Reduced somatomedin A (by radio receptor assay) and increased somatomedin B (by radioimmunoassay) have been found by Svan, Hall, Ritzen, Takano and Skottner,(1977).

NSILA-S (by bioassay) has been found to rise progressively during pregnancy and to fall from high levels at delivery (2.49 U/ml) to normal within 48 hours (1.10 U/ml) (Franklin, Pepperel, Burger and Cameron, 1978).

iii) Circadian variation

Daughaday, Salmon and Alexander (1959) were unable to show significant circadian variation. A suggestion of cyclical variation, inversely related to plasma cortisol concentration has been reported (Du Caju and Van den Brande, 1976). In the intact animal the incorporation of $^{35}SO_4$ (Simmons, 1968) and the mitotic index (Simmons, 1964) of the epiphyseal growth cartilage of mice have been shown to have a significant diurnal variation.

iv) Except for the special circumstances which obtain in pregnancy sexual dimorphism of somatomedin activity have not been reported (Daughaday, Salmon and Alexander, 1959; Almqvist and Rune,

1961; Van den Brande, Van Buul, Heinrich, Van Roon, Zurcher and Van Steirtegem, 1975).

D Somatomedin activity in disease

Somatomedin activity has been measured in a wide variety of disease states, especially those in which there are growth abnormalities. The findings fall into three broad groups, that in which growth hormone and somatomedin are concordant, that in which low somatomedin activity is associated with an inappropriately elevated growth hormone level and finally where somatomedin is normal or elevated in the face of low or absent growth hormone levels (see Table 2.2).

i) Conditions associated with concordance of growth hormone and somatomedin

a) Conditions associated with low or absent growth hormone levels:

Hyposomatotropism due to isolated growth hormone deficiency, panhypopituitarism or hypophysectomy is associated with low somatomedin activity (usually in the range 0.20 - 0.40 μ/ml) (Daughaday, Salmon and Alexander, 1959; Hall, 1970), and the administration of exogenous growth hormone results in the restoration of serum somatomedin activity (Hall and Olin, 1972; Schimpff, Donnadieu, Gourmelen and Girard, 1974). The therapeutic response to exogenous growth hormone in children with growth hormone deficiency, as measured by growth velocity, has been shown to correlate well with the serum somatomedin activity (Hall and Olin, 1972; Schimpff, Donnadieu, Gourmelen and Girard, 1974). After intravenous administration of growth hormone (1 mg) an increase of somatomedin activity was detectable within three hours (Hall, 1971), while after subcutaneous or intramuscular injections the increase was detectable within six hours and peaks between 24 and 30 hours (Daughaday, 1975) and

Table 2.2 RELATIONSHIP BETWEEN SOMATOMEDIN ACTIVITY AND GROWTH HORMONE IN HEALTH AND DISEASE

	Condition	Somatomedin Activity	Growth Hormone	Remarks
Conditions in which SMA and GH are congruent.	Normal	N	N	
	Acromegaly	↑	↑ to ↑↑	SMA and GH return to normal with successful therapy.
	Isolated growth hormone deficiency	↓	↓	GH therapy restores SMA.
	Panahypopituitarism	↓	↓	GH therapy restores SMA.
	African pygmies	N	N	Probable end organ resistance to SMA.
	Hypoparathyroidism	N	N	Probable end organ resistance to SMA.
	Cerebral gigantism	N or slight ↓	N	Cause still obscure.
	Turners syndrome	N	N or slight ↑	Probable end organ resistance to SMA.
Conditions in which SMA is reduced in spite of normal or increased GH.	Kwashiorkor	↓	↑↑	SMA and GH return to normal with refeeding.
	Marasmus	↓	↑	SMA and GH return to normal with refeeding.
	Liver disease	↓	N or ↑	
	Renal disease	↓	N or ↑	Some cases with dialysis or transplant SMA increases.
	Oestrogen therapy	↓	N or slight ↑	
	Corticosteroid therapy	↓	N	Alternate day steroids causes less SMA depression.
	Cushings syndrome	↓	N	
	Laron's dwarfism	↓	↑	Probable end organ resistance to GH.
Conditions in which SMA is normal in spite of low or absent GH.	Post-surgical growth in craniopharyngioma	N	0 or ↓	This phenomenon occurs in those patients with hyperphagia and hyperinsulinism.
	Obesity	N	↓	Increased food intake and increased insulin levels.

and complete normalisation of serum somatostatin requires two to four days of continuous growth hormone therapy (Daughaday, 1975). Children suffering from psychosocial dwarfism or the emotional-deprivation short stature syndrome, who have retarded skeletal maturation, short stature and who show catch-up growth when hospitalised or when home circumstances improve, have been shown to have low growth hormone levels and somatomedin activity which return to normal in the phase of catch-up growth (Van den Brande, Van Buul, Heinrich, Van Roon, Zurcher and Van Steirtegem, 1975; Powell, Brasel and Blizzard, 1967). The mechanism for the depressed growth hormone levels in this syndrome remains obscure.

b) Conditions associated with elevated growth hormone levels:

In acromegaly, somatomedin activity is usually elevated but there is poor correlation between the immunoreactive growth hormone levels and somatomedin activity (Yde, 1969). There is a fair correlation between the clinical assessment of disease activity and somatomedin (Stuart and Lazarus, 1975).

c) Conditions associated with normal growth hormone and normal somatomedin activity with a disorder of growth:

A number of conditions associated with short stature fall into this group: Turner's syndrome in which growth hormone is normal or slightly elevated (Saenger, Schwartz, Wiedemann, Levine, Tsai and New, 1976), African pygmies (Daughaday, 1969) and pseudohypoparathyroidism (Urdanivia, Mataverde and Cohen, 1975). It is postulated that short stature in these conditions is due to an end-organ resistance to somatomedin at cartilage level. Proof of this hypothesis requires the demonstration of resistance to exogenously administered somatomedin in vivo or the failure of cartilage to bind labelled somatomedin, or to respond to somatomedin by $^{35}SO_4$ uptake

in vitro.

Soto's syndrome or cerebral gigantism is characterised by a typical facies, advanced skeletal maturation, rapid and excessive growth from birth, large hands and feet, mental retardation and dilated cerebral ventricles, and has been found to have normal or low normal somatomedin activity and normal growth hormone levels (Van den Brande and Du Caju, 1973; Saenger, Levine, Wiedemann, Schwartz and New, 1976; Lecornu, 1973). The mechanism for the excessive growth in this syndrome remains undetermined.

ii) Conditions associated with low somatomedin activity in the presence of elevated or normal growth hormone levels

There exists a number of clinical situations in which the normal relationship between growth hormone and somatomedin no longer obtains.

Laron's dwarfism is a syndrome characterised by the phenotypic features of growth hormone deficiency but grossly elevated immunoreactive growth hormone levels and low somatomedin activity (Laron, Pertzelan and Mannheimer, 1966). The condition is especially common in Jews of Asiatic origin but has been reported in other groups; the inheritance appears to be as a Mendelian recessive trait. Although the initial hypothesis was that the syndrome resulted from biologically inactive growth hormone, serum growth hormone from patients was found to behave identically to human growth hormone on electrophoresis and immunologically (Eshet, Laron, Brown and Arnon, 1973; Elders, Garland, Daughaday, Fisher, Whitney, and Hughes, 1973) and to bind normally in a growth hormone radio receptor assay (Jacobs, Sneid, Garland, Laron and Daughaday, 1976). The exogenous administration of growth hormone even in large doses could not increase serum somatomedin (Daughaday, Laron, Pertzelan and

Heins, 1969; New, Schwartz, Parks, Landey and Wiedemann, 1972) nor does growth hormone induce nitrogen and phosphorus retention, calcium excretion, lipolysis or skeletal growth (Laron, Pertzelan, Karp, Kowadlo-Silbergeld and Daughaday, 1971). The hypothesis of Daughaday and Garland (1972) is that there is an end-organ resistance to growth hormone, not only for its direct effects (e.g. lipolysis) but also for the induction of somatomedin generation.

Liver disease, especially cirrhosis, has been found to be associated with high growth hormone levels and low somatomedin activity (Wu, Grant, Hambley and Levi, 1974; Stuart and Lazarus, 1975). Children with type VI glycogen storage disease (phosphorylase deficiency) have growth retardation with normal growth hormone and low somatomedin which does not rise after the administration of exogenous growth hormone in pharmacological doses (Levine, Wiedemann, Saenger, Schwartz and New, 1976).

The growth failure occurring in children with severe renal disease is associated with normal or elevated growth hormone levels and low somatomedin activity (Stuart and Lazarus, 1975; Saenger, Wiedemann, Schwartz, Korth-Schutz, Lewy, Riggio, Rubin, Stenzel and New, 1974). In those cases where treatment by dialysis or transplantation resulted in improved growth rate, serum somatomedin activity was found to increase. Children treated with glucocorticoids have normal growth hormone levels and low somatomedin activity with poor growth (Elders, Wingfield, McNatt, Clarke and Hughes, 1975). Glucocorticoids may act to reduce somatomedin generation (Hill, Francis, Conroy and Ashton, 1977; Mosier, Jansons, Hill and Dearden, 1976) and to reduce $^{35}SO_4$ uptake into cartilage in vitro (Keret, Schimpff and Girard, 1976; Mosier, Jansons, Hill and Dearden, 1976). Alternate day glucocorticoid therapy has been found to improve growth rate in children and somatomedin activity

has been found to be normal on the day in which glucocorticoids are not administered (Elders, Wingfield, McNatt, Clarke and Hughes, 1975). The doses of hydrocortisone administered for the treatment of congenital adrenal hyperplasia have no effect on somatomedin activity (Rappaport and Prévôt, 1977).

Oestrogens have been used to treat acromegaly since they reduce hypercalciuria, hydroxyprolinuria, the increased radiocalcium bone accretion rate and hyperphosphataemia in spite of the fact that growth hormone levels are unchanged or even elevated. The mechanism for these effects is a reduction of somatomedin activity (Wiedemann and Schwartz, 1972; Saenger, Schwartz, Wiedemann, Levine, Tsai and New, 1976). Exogenously administered glucagon has been found to reduce serum somatomedin without an effect on growth hormone levels (Binoux, Schimpff and Donnadieu, 1977).

Protein-energy malnutrition in which growth failure is a major feature is associated with grossly elevated non-suppressible growth hormone levels (Pimstone, Wittmann, Hansen and Murray, 1966; Pimstone, Barbezat, Hansen and Murray, 1967) and in general growth hormone levels are higher in kwashiorkor than marasmus (Pimstone, Barbezat, Hansen and Murray, 1967). Somatomedin activity is reduced in protein-energy malnutrition and returns to normal with refeeding, as does the growth hormone (Grant, Hambley, Becker and Pimstone, 1973; Van den Brande and Du Caju, 1973; Hintz, Suskind, Amatayakul, Thanangkul and Olson, 1978). Frequently the dose response of serum from children with untreated protein malnutrition is not parallel to that of normal serum (Grant, Hambley, Becker and Pimstone, 1973) and this may be due to an inhibitor (Van den Brande and Du Caju, 1973); with recovery parallelism of the dose reponse returns.

Many of the above clinical situations are in keeping with a

postulated feedback loop in which somatomedin activity may act at pituitary or hypothalamic level to inhibit growth hormone secretion (Chochinov and Daughaday, 1976).

iii) Conditions associated with normal or increased somatomedin activity in the presence of reduced or absent growth hormone levels

A variety of factors other than growth hormone may operate to induce somatomedin activity generation.

Catch-up growth can occur in children following surgery for craniopharyngioma, in the absence of immunoreactive growth hormone in the serum, whilst normal somatomedin activity is found (Kenny, Guyda, Wright and Friesen, 1973; Finkelstein, Kream, Ludan and Hellman, 1972; Costin, Kogut, Phillips and Daughaday, 1976). Similar results have been reported with hypothalamic tumours other than craniopharyngiomas (Kenny, Iturzaeta, Mintz, Drash, Garces, Susen and Askari, 1968). Elevated prolactin has been postulated as the cause for the normal somatomedin activity (Kenny, Iturzaeta, Mintz, Drash, Garces, Susen and Askari, 1968) and ovine and rat prolactin have been shown to stimulate the generation of somatomedin activity from the isolated perfused rat liver (Francis and Hill, 1975; Hill, 1977). It seems more likely however that the hyperphagia which may result from hypothalamic injury in such cases causes hyperinsulinism which stimulates somatomedin generation (Kenny, Guyda, Wright and Friesen, 1973; Costin, Kogut, Phillips and Daughaday, 1976). Insulin has been shown to have growth promoting effects (Salter and Best, 1953) and to stimulate somatomedin generation by the isolated perfused rat liver (Daughaday, Phillips and Mueller, 1976). Simple obesity is another situation in which growth hormone levels tend to be low, insulin levels high and somatomedin activity normal (Van den Brande and Du Caju, 1973).

Patients with the clinical features of active acromegaly with normal growth hormone and insulin levels but elevated somatomedin activity have been reported (Hoffenberg, Howell, Epstein, Pimstone, Fryklund, Hall, Schwalbe and Rudd, 1977). Hypophysectomised rats infested with the spargana stage of the cat tapeworm (Spirometra mansonoides) grow in spite of the total absence of growth hormone. This phenomenon is due to the elaboration of a "worm factor" or pherocercoid growth factor by the worms (Garland, Ruegamer and Daughaday, 1971). The factor appears able to induce most of the metabolic effects of growth hormone (Steelman, Morgan, Cuccaro and Glitzer, 1970) including an increase in ornithine decarboxylase (Sogani, Matsushita, Mueller and Raben, 1972). That the pherocercoid growth factor induces somatomedin in a similar way to growth hormone is suggested by the decrease in pituitary content of growth hormone of rats infested with spargana (Garland and Daughaday, 1972) and that the factor displaces ^{125}I-labelled human growth hormone from receptors on liver cell membranes (Tsushima, Friesen, Chang and Raben, 1974). After several weeks antibodies are produced in the infested rat which block the biological effects of the worm factor and inhibit further somatomedin activity generation and further growth ceases (Daughaday, 1971).

E <u>The site of origin of somatomedin activity</u>

i) The presence of somatomedin activity in body fluids

Apart from the original observation of somatomedin being present in serum (Salmon and Daughaday, 1957) and being purified from outdated human plasma (Hall, Takano, Fryklund and Sievertsson, 1975; Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975), somatomedin activity has also been found in cerebrospinal fluid (Beaton, Sagel and Distiller, 1975), lymph (Cohen and Nissley, 1975),

amniotic fluid (bioassayable, Bala and Smith, 1976; somatomedin C by radio receptor assay, Chochinov, Mariz, Hajek and Daughaday, 1977), urine (somatomedin B by radioimmunoassay, Yalow, Hall and Luft, 1975a), and aqueous humor (unpublished personal observations).

ii) The presence of somatomedin activity in tissue

Somatomedin differs from other hormones in that no tissue contains more than plasma itself. Aqueous extracts of muscle, liver and brain have been shown to stimulate the sulphation of chick cartilage (Hall and Bozovic, 1969).

iii) The role of the liver in the generation of somatomedin activity

Considerable evidence has accumulated indicating that the liver is a major site for the generation of somatomedin activity. Partial hepatectomy in the rat results in a fall in serum somatomedin activity (Uthne and Uthne, 1972). Chronic liver disease is associated with reduced serum somatomedin activity (Wu, Grant, Hambley and Levi, 1974; Giordano, Foppiani, Minuto, Perroni and DiCicco, 1975), and a positive transhepatic gradient of somatomedin activity has been demonstrated in the dog (Schimpff, Donnadieu, Glasinovic, Warnet and Girard, 1976). In vitro somatomedin activity has been generated by a variety of liver preparations including crude microsomal fractions (Hall and Uthne, 1971) and the isolated perfused rat liver (McConaghey and Sledge, 1970). Less somatomedin activity is released from the livers of hypophysectomised than intact animals (McConaghey, 1972) and growth hormone stimulates somatomedin release from the perfused liver (McConaghey and Sledge, 1970; Phillips, Herington, Karl and Daughaday, 1976).

Insulin (Daughaday, Phillips and Mueller, 1976) and prolactin (Francis and Hill, 1975; Hill, 1977) are other stimulants for

hepatic release of somatomedin. A cloned rat liver cell tumour grown in culture releases a somatomedin-like peptide into the medium (Smith and Temin, 1974). While the liver is probably the major site of somatomedin generation, the kidney may play a role as somatomedin activity has been found to be generated in an isolated perfused rat kidney system (McConaghey and Dehnel, 1972).

iv) Somatomedin activity in plasma occurs as a high molecular weight complex in which the small molecular weight somatomedin is associated with a protein MW > 50000 daltons (Van den Brande, Van Wyk, Weaver and Mayberry, 1971). The carrier protein is presumably of liver origin in that somatomedin activity generated by isolated perfused rat livers occurs as a large molecular weight complex (Dehnel, McConaghey and Francis, 1974). The binding to plasma proteins may account for the long half life of serum somatomedin activity, 3-4 hours in the rat (Daughaday, 1971) and $\sim$ 12 hours in man (Almqvist and Falkheden, 1961).

F The isolation and characterisation of somatomedins and other growth factors

i) Introduction

The availability of suitable bioassay systems enabled workers to follow the concentration of growth factors through purification procedures. As somatomedin activity is not concentrated in any organ, serum and plasma were the starting materials chosen and initial purification procedures were applied to small volumes of serum (normal human, acromegalic human and rat). Somatomedin activity was found to be retained by dialysis tubing (Salmon and Daughaday, 1958) and similar findings were reported with membrane ultrafiltration giving an exclusion point of 50,000 daltons (Van Wyk, Hall, Van den Brande and Weaver, 1971). The high molecular

weight of somatomedin has been found to be due to its association with binding proteins (Hall, Takano, Fryklund and Sievertsson, 1975; Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975; Froesch, Schlumpf, Heimann, Zapf, Humbel and Ritschard, 1975), and some of these binding proteins themselves have been shown to be growth hormone dependent (Zapf, Kaufmann and Froesch, 1977; Zapf and Froesch, 1978; Draznin, Schalch, Heinrich and Schlueter, 1978; Enberg, Fryklund and Hall, 1978). Acid-ethanol extraction or heating, with or without gel chromatography, resulted in the dissociation from binding proteins and purification of somatomedin by up to 100 to 500 fold but with the low recoveries only very small amounts of the material were isolated. It was only when blood fractions representing starting material equivalent to several tons of out-dated plasma were subjected to industrial scale preparative technology that highly purified somatomedins were isolated in any meaningful quantity.

ii) Peptides with cartilage-stimulating properties

a) Somatomedin A

Large scale purification of modified Cohn fraction IV derived from out-dated human plasma was undertaken using the embryonic chick cartilage bioassay to monitor the concentration of somatomedin activity. Initial purification schemes consisted of acid-ethanol extraction, Sephadex G-75 gel chromatography, Dowex 50 W-X8 ion exchange chromatography and high-voltage paper electrophoresis at pH 6.5, 3.6 and 2.0, resulting in a 1 to 2 million-fold concentration of activity with a recovery of 0.10% (Hall, 1972). More recent schemes involved acid-ethanol extraction, Sephadex G-75 and G-50 gel chromatography, cellulose column electrophoresis at pH 7.5 and pH 5.0 and final Sephadex G-50 gel chromatography (Sievertsson,

Fryklund, Uthne, Hall and Westermark, 1975). The purified material is a neutral peptide having an isoelectric point of 7.1-7.5 and molecular weight of 7,600. Gel electrophoresis revealed two bands, equipotent in the bioassay and having somewhat different amino acid compositions (Sievertsson, Fryklund, Uthne, Hall and Westermark, 1975; Fryklund, Uthne and Sievertsson, 1974).

A peptide with not dissimilar properties has been isolated from bovine plasma (Liberti, 1975).

b) Somatomedin C

Commencing with Cohn fraction IV, but using the rat costal cartilage bioassay to monitor the purification process, and following a somewhat different purification scheme, a basic peptide with an isoelectric point of 8.4-9.2 and molecular weight of 7,600 has been isolated. The purification scheme consisted of acid-ethanol extraction, gel chromatography on Sephadex G-75 and G-50, continuous isoelectric focusing between pH 3-10 and pH 7.0-10, SP Sephadex chromatography, Sephadex G-50 gel chromatography, preparative gel electrophoresis (pH 2.3 in 6M urea) and final Sephadex G-50 chromatography. A peptide isolated from rat plasma has similar properties (Chochinov, Mariz and Daughaday, 1977; Daughaday, Mariz, Daniels, Jacobs and Rubin, 1978).

c) Non-suppressible insulin-like activity-soluble (NSILA-S)

When insulin bioactivity of serum was measured by the conversion of ^{14}C-U-glucose into $^{14}CO_2$ or the incorporation of ^{14}C-U-glucose into glycogen in rat diaphragm in the presence of an excess of anti-insulin serum, 90-95% of the insulin-like activity in the serum remained and the term non-suppressible insulin-like activity (NSILA) was applied to this activity (Froesch, Bürgi, Müller, Humbel, Jakob and Labhart, 1967). The NSILA activity was found to reside

in a variety of different proteins. NSILA-S is the term applied to those fractions which are soluble in acid-ethanol (Froesch, Schlumpf, Heimann, Zapf, Humbel and Ritschard, 1975). Large scale purification from out-dated human plasma of Cohn fraction III was by means of acetone extraction of lipids, acid ethanol extraction, Sephadex G-75 gel chromatography, CM cellulose and DEAE ion exchange chromatography, Sephadex G-50 gel chromatography and final CM cellulose ion exchange chromatography. This resulted in a 270,000 fold concentration of the peptide and on polyacrylamide gel electrophoresis this was found to have two major components, both bioactive. The amino acid composition of the material has been determined (Froesch, Schlumpf, Heimann, Zapf, Humbel and Ritschard, 1975; Rinderknecht and Humbel, 1976), and the amino acid sequences and secondary structures have been found to show considerable homology with proinsulin. This has resulted in the new term insulin-like growth factor (IGF) being applied to the peptides. The two peptides IGF-I and IGF-II have been shown to have slightly different stimulatory potencies in rat fat pad, rat costal cartilage and chick cartilage assays (Humbel and Rinderknecht, 1977).

Another major NSILA component is that which is insoluble in acid ethanol (NSILA-P) (Jakob, Hauri and Froesch, 1968; Poffenbarger, 1975). This material has a molecular weight of > 100,000 and may be a major fraction of the total NSILA activity of serum.

d) Multiplication-stimulatory activity (MSA)

Calf serum or tissue culture medium in which a cloned liver cell tumour (derived from a buffalo rat) was cultured, has been shown to contain factors which stimulate the multiplication of chick embryo fibroblasts in culture. Calf serum was subjected to ion exchange chromatography on Dowex 50 W-X8, Biogel P-100 chromatography and preparative polyacrylamide gel electrophoresis which

resulted in an 8000-fold concentration of activity (Pierson and Temin, 1972). The same process was used to concentrate MSA from serum-free rat liver cell tumour conditioned medium. The major component of MSA has a molecular weight of 10,000 but no clearcut isoelectric point (Smith and Temin, 1974).

iii) Tissue growth factors not related to cartilage growth

a) Somatomedin B

An acidic fraction from the initial electrophoretic step in the preparation of somatomedin A was found to stimulate thymidine uptake into human glia-like cells and human foetal lung fibroblasts but to have no sulphation factor activity (Uthne, 1973). Further purification by electrophoresis at pH 5 and Sephadex G-50 chromatography yielded a peptide with a molecular weight of 5,000 of known amino acid composition (Sievertsson, Fryklund, Uthne, Hall and Westermark, 1975). The full amino acid sequence of 44 amino acids has been determined and shows considerable homology with certain protease inhibitors. The physiological reasons for a protease inhibitor which is under partial growth hormone control is as yet not known (Fryklund, Skottner and Forsman, 1978).

b) Nerve growth factor (NGF)

A polypeptide extracted from the submandibular gland of the mouse was found to stimulate the outgrowth of nerve fibre processes from chick sympathetic ganglia in culture and in vivo (Levi-Montalcini and Angeletti, 1968). A structurally similar peptide has been isolated from cobra venom (Cohen, 1959). Both peptides show structural homology with proinsulin (Hogue-Angeletti, Bradshaw, and Frazier, 1975). NGF has not been shown to have sulphation factor activity (Hambley, Howell and Grant, 1974).

c) Epidermal growth factor (EGF)

An extract of male mouse submandibular gland has been isolated which causes precocious eyelid opening and tooth eruption in new-born mice (Cohen, 1962). The factor is a polypeptide, molecular weight 6045, and is a single chain of 53 amino acid residues and has been shown to stimulate amino acid uptake, protein synthesis and RNA synthesis of cell cultures of ectodermal origin, as well as to stimulate the growth of fibroblasts in culture. Like NGF the material is present in periductal cells and is stimulated by testosterone (Cohen, Carpenter and Lembach, 1975). Urogastrone, an inhibitor of gastric acid secretion and a stimulator of mucosal proliferation, has been isolated from human urine and shown to have a structure closely homologous to EGF (Gregory, 1975).

d) Granulocytosis-inducing factor was the third peptide derived from mouse submandibular gland and stimulates the proliferation of granulocyte precursors in culture (Angeletti, Salvi, Capani and Frati, 1965).

e) Fibroblast growth factor (FGF)

Extracts of pituitary glands or brain have yielded a peptide, molecular weight 13,000, which stimulates growth or a variety of cells in culture: 3T3 cells, mouse and human fibroblasts, Y1 adrenal cells, L6 myogenic cells, foetal calf myoblasts, human amniotic cells and early passages of rat chondrocytes. The preparation was free of known pituitary hormones and proteases (Gospodarowicz, Rudland, Lindstrom and Benirschke, 1975).

f) Erythropoietin stimulates the proliferation of erythrocyte precursors both in vivo and in vitro (Gordon, 1971) and is under partial growth hormone control (Peschle, Rappaport, Sasso, Gordon and Condorelli, 1972). Liver, spleen and kidney appear to be the main sites of synthesis.

g) Thymic growth factors

A variety of factors have been extracted from calf thymus which include thymosin, thymopoietin I and II and a number of other less well characterised factors. These factors stimulate the proliferation and immunocompetence of lymphocytes and other components of the immune system (White and Goldstein, 1975).

h) Relaxin

A factor isolated from porcine corpora lutea which relaxed the pubic symphysis of oestrogen primed guinea pigs was termed relaxin. It has been shown to have homology with proinsulin (James, Niall, Kwok and Bryant-Greenwood, 1977; Schwabe and McDonald, 1977). In spite of a major locus of its action being skeletal tissue, sulphation factor activity of the peptide has not yet been examined (Niall, personal communication, 1977).

iv) Comparison of biological actions of growth factors (Table 2.3)

Four well defined peptides meet the criteria which make them true somatomedins: somatomedin A, somatomedin C, NSILA-S (IGF-I and II) and MSA. The first three are all of similar molecular weight and are derived from human plasma where they are bound to a high molecular weight protein. All are bioactive in the rat, pig and chick cartilage assays except NSILA-S which appears to have no activity in the porcine cartilage assay (Hall, Takano, Fryklund and Sievertsson, 1975; Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975; Froesch, Zapf, Meuli, Mader, Waldvogel, Kaufmann and Morell, 1975). MSA has mitogenic effects on chick fibroblasts, sulphation factor activity and NSIL activity (Moses, Nissley, Rechler, Short and Podskalny, 1978). Fibroblast growth is stimulated by somatomedin C (Van Wyk, Underwood, Baseman, Hintz, Clemmons

Table 2.3. PARTIAL LIST OF BIOLOGICAL CHARACTERISTICS OF SOME PURIFIED GROWTH FACTORS

Growth factor	Source material	MW (Daltons)	Isoelectic focussing point	Growth hormone dependence	Sulphate incorpo-ration	Mito-genic activity	Membrane receptors			Plasma protein carrier	Insulin-like action on fat cells
							Placenta	Fibro-blast	Liver		
Somatomedin A	Human plasma	7,600	7.1 - 7.5	+	+	-	+	-	?	+	+
Somatomedin B	Human plasma	5,000	5.9 - 6.2	+	-	+	-	?	?	+	-
Somatomedin C	Human plasma	7,600	8.4 - 9.2	+	+	+	+	+	+	+	+
NSILA-S	Human plasma	7,600	7.5 - 8.2	+	+	+	+	+	+	+	+
MSA	Calf serum	10,000)	not clearly defined	Partial	+	+	+	+	+	?	+
MSA	Rat liver tumour cell conditioned culture medium	10,000)		Partial	+	+	+	+	+	?	+
Insulin	Pancreas	6,000	5.3	-	+ (high dose)	+ (high dose)	+	+	+	-	+

and Marshall, 1975) and NSILA-S (Froesch, Zapf, Meuli, Mader, Waldvogel, Kaufmann and Morell, 1975) but probably not somatomedin A (Wasteson, Westermark and Uthne, 1975). The two components of NSILA-S (IGF-I and II) whilst both having NSILA, sulphation and mitogenic activity, have them in slightly different proportions (Zapf, Schönle and Froesch, 1977). Somatomedin A (Hall, Takano, Fryklund and Sievertsson, 1975) and somatomedin C (Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975) both have NSILA properties. There is evidence that the action of somatomedins is associated with an inhibition in adenylate cyclase activity of their target tissues (Hall, Takano, Fryklund and Sievertsson, 1975; Froesch, Zapf, Meuli, Mader, Waldvogel, Kaufmann and Morell, 1975; Tell, Cuatrecasas, Van Wyk and Hintz, 1973).

In vivo somatomedin A injected into hypophysectomised rats was found to increase tibial epiphyseal width and incorporation of amino acids into diaphragmatic muscle (Uthne, 1975) and to stimulate sulphation of skin and cartilage (Fryklund, Skottner and Forsman, 1978). In the intact rat NSILA-S stimulates the incorporation of ^{14}C-U-glucose into the glycogen of diaphragmatic muscle and the lipids of adipose tissue. The hypoglycaemic effects of NSILA-S is more prolonged than that of insulin (Froesch, Schlumpf, Heimann, Eigenmann and Zapf, 1975) and there is evidence to suggest that NSILA-S acts via a receptor distinct from that of insulin to activate the same glucose transport mechanism as insulin itself (Meuli and Froesch, 1977).

G <u>Receptor binding of somatomedins and radioreceptor assays</u>

The availability of well characterised purified somatomedins opened the door for the study of the interaction of these peptides with cell membranes. Radioreceptor assays using radioiodinated somatomedins or insulin binding to receptor sites on cell surfaces

from various tissues have been developed. In broad outline, NSILA-S, somatomedins A and C and MSA bind to their own receptor(s) with high affinity as well as to insulin receptors with lower affinity. Growth promoting effects may be mediated by the former and the insulin-like effects by the latter (which at physiological concentration is of minor importance). The binding and radioreceptor assays for non-somatomedin growth factors (EGF, NGF, FGF, etc.) lie outside the scope of this review.

Somatomedin A

^{125}I-Somatomedin A binds specifically to membranes prepared from various tissues of rat and monkey including lung, kidney, liver, thymus, brain, spleen, pancreas, heart and fat (Takano, Hall, Fryklund and Sievertsson, 1976), cartilage and human placenta (Hall, Takano, Fryklund and Sievertsson, 1975). Placental membranes have been used as a ligand in a radioreceptor assay which is not completely specific in that similar concentrations of somatomedin A or C (Hall, Takano, Fryklund and Sievertsson, 1975) or IGF-I and IGF-II (Hall, Brant, Enberg and Fryklund, 1978) will displace ^{125}I-Somatomedin A bound to the membranes as will very high concentrations of insulin. No data on displacement by MSA have been reported.

Somatomedin C

^{125}I-Somatomedin C binds to membranes from rat liver, embryonic chick cartilage, human placenta, liver, lung, kidney, brain, muscle and thymus (Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975; Hintz, Clemmons, Underwood and Van Wyk, 1972). Using placental membranes as ligand and ^{125}I-Somatomedin C as tracer, a radioreceptor assay has been developed. Crossreaction occurs with somatomedin A, MSA and with insulin in very high concentrations

but not with the non-somatomedin growth factors NGF and EGF (Van Wyk, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975). This radioreceptor assay has been used to monitor the isolation of a somatomedin C-like peptide from amniotic fluid (Chochinov, Mariz, Hajek and Daughaday, 1976).

NSILA-S (IGF)

^{125}I-NSILA-S binds to rat adipose tissue, rat liver, chick embryo fibroblasts and transformed human lymphocytes (Froesch, Zapf, Meuli, Mader, Waldvogel, Kaufmann and Morell, 1975; Magyesi, Kahn, Roth, Neville, Nissley, Humbel and Froesch, 1975; Waldvogel, Schalch and Froesch, 1975). Impure preparations of somatomedin A and C, insulin and proinsulin poorly displace ^{125}I-NSILA-S from liver cell membranes while MSA is almost as potent as NSILA-S itself.

In addition to its receptors on cell membranes NSILA-S has a highly specific high affinity serum binding protein which has been utilised as a ligand for a radioligand assay (Zapf, Waldvogel and Froesch, 1975).

MSA

^{125}I-MSA binds to rat liver membranes, chick embryo fibroblasts, transformed human lymphocytes (Rechler, Podskalny and Nissley, 1976) and human placental membranes (Chochinov and Daughaday, 1977). MSA and NSILA-S are equipotent in their ability to displace each other from liver cell membranes but are poorly displaced by insulin (Zapf, Mader, Waldvogel, Schalch and Froesch, 1975; Rechler, Podskalny and Nissley, 1976). ^{125}I-MSA bound to chick embryo fibroblasts and placental membranes is easily displaced by insulin while ^{125}I-MSA is even more readily displaced from transformed human lymphocytes by insulin than by MSA itself, indicating that these cells have only

insulin receptors of low affinity and no high affinity MSA receptors (Rechler, Podskalny and Nissley, 1976).

H Radioimmunoassay of Somatomedins

The development of radioimmunoassays for the somatomedins, was dependent on the availability of pure or semi-pure somatomedin preparations for the immunisation of suitable animals to generate antisera of adequate specificity and titre, for radioiodination to produce a label and for standards. Radioimmunoassay with its potential for specificity, sensitivity and the ability to deal with large numbers of samples, offers enormous advantages in the study of somatomedin and will be a major area of study in the years to come.

i) Somatomedin A (Hall, Brant, Enberg and Fryklund, 1978)

A radioimmunoassay has recently been developed for somatomedin A using an antibody raised in chickens, human somatomedin A labelled with ^{125}I by the chloramine-T method as tracer and highly purified human somatomedin as standard; bound from free tracer was separated by polyethylene glycol (or charcoal). The assay was not completely specific in that it crossreacted with insulin-like growth factor (IGF-I and IGF-II).

ii) Somatomedin C (Furlanetto, Underwood, Van Wyk and D'Ercole 1977)

A radioimmunoassay for this peptide has been developed using an antiserum raised in rabbits to a human somatomedin C - ovalbumin complex, chloramine-T iodinated human somatomedin as label (5,800 U/mg, 90% pure, < 1 part per 10,000 immunoreactive insulin) and a pool of normal human serum from three healthy adult males as standard with an arbitrary value of 1 U/ml; bound from free tracer was

separated by double antibody precipitation. Because of competition between serum binding proteins and antibody, the dose-response curves for standard serum and purified somatomedin C were not parallel under equilibrium conditions of assay. This non-parallelism could be overcome by a non-equilibrium assay procedure. The antiserum bound 70% of the label at 1:10,000 dilution and had a Ka of 4.6×10^{10} litres/mole. There was no crossreactivity with pituitary hormones or MSA. There was slight crossreactivity with insulin at very high concentrations and somatomedin A crossreacted 3% on a weight basis. NSILA was not tested.

In normal humans radioassayable somatomedin C rose dramatically from birth (0.38 U/ml) to four years (1.30 U/ml) after which it was fairly constant (adults 1.50 U/ml). In acromegaly mean levels were 6.28 U/ml while in hypopituitarism mean levels were 0.2 U/ml, the difference between these two being greater than with any other assay of somatomedin.

Sera from dog, baboon, guinea pig, lamb and chicken were all found to produce parallel displacement in the assay, rat serum and purified rat somatomedin caused non-parallel displacement, while fish serum produced no displacement.

iii) Somatomedin B (Yalow, Hall and Luft, 1975b)

This peptide was the first of the somatomedin group to be measured by radioimmunoassay. The assay makes use of an antiserum raised in guinea pigs to a human somatomedin B - guinea pig albumin conjugate, human somatomedin B iodinated by the chloramine-T technique as tracer and highly purified human somatomedin B as standard. Bound tracer was separated from free by a precipitating second antibody or by uncoated charcoal.

Somatomedins A and C and NSILA-S did not crossreact in the assay. The assay was able to discriminate between acromegalic

(19.3 ± 2.3 μg/ml) and hypopituitary (6.6 ± 0.5 μg/ml) sera and a fall in serum somatomedin B could be demonstrated after the successful treatment of acromegalics. Immunoreactive somatomedin B was only present in the sera of monkeys and man and not of guinea pig, mouse, rat, cow, dog, sheep, pig or rabbit.

Low serum somatomedin B levels have been reported in liver and renal disease (Eversmann, Glockner, Gottsmann, Weidl and Werder, 1977). Radioimmunoassayable somatomedin B has been detected in urine (Yalow, Hall and Luft, 1975a).

iv) Rat somatomedin (Daniels, Mariz and Daughaday, 1977)

An as yet unperfected assay has been reported using an antibody raised in rabbits to rat somatomedin, a tracer of chloramine-T labelled rat somatomedin and rat somatomedin standards. There was no crossreaction with human somatomedin A and C, human insulin, proinsulin or NSILA-S. The assay cannot yet be applied to unextracted rat serum due to problems with competition from serum proteins for tracer. Extracts of hypophysectomised serum contained 20% of the immunoreactive material of normal serum (100%), whilst serum from rats bearing growth hormone secreting tumours contained 1000%.

v) NSILA-S (Reber and Liske, 1976)

A radioimmunoassay for this peptide using an antiserum raised in rabbits against human NSILA-S, chloramine-T radioiodinated highly purified human NSILA-S as label and highly purified human NSILA-S as standard; bound from free tracer was separated by the second antibody precipitation method. The assay was sensitive to 30 pg/tube. Insulin, glucagon and growth hormone did not crossreact in the assay but NSILA related growth factors were not examined.

vi) NSILA-P (Poffenbarger, 1978)

A radioimmunoassay for this peptide has been reported using an antibody raised in rabbits to human NSILA-P. The assay does not crossreact with NSILA-S (IGF-I and II) somatomedin C or MSA

CHAPTER THREE

THE BIOASSAY OF SOMATOMEDIN ACTIVITY

"And in a glas he hadde pigges bones" *Geoffrey Chaucer*

A Introduction

i) General and statistical considerations

The diverse actions of somatomedin on a variety of tissues has been shown in Table 2.1. A practical bioassay making use of one or more of these actions was required. Such an assay had to provide acceptable sensitivity, precision and reproducibility for a reasonable expenditure of time, effort and money. A large number of assay systems have been described which lay claim to these ideals (see Table 3.1).

Bioassays have the great advantage of measuring a physiological, as distinct from an immunological or chemical, property of a hormone but often lack precision. Non-specific stimulation must be rigorously guarded against and inhibitors may interact to give reduced values (which may of course represent the biologically relevant net activity). Because of interassay variability in the responsiveness of the bioassay preparation and the techniques in handling it, a standard must be assayed on each occasion. Standard and unknown sample are assumed to be qualitatively similar but may differ quantitatively from each other. Thus standard and unknown should dilute in parallel for valid potency ratios to be calculated. The absence of an international reference standard of either serum or a purified somatomedin preparation (Bangham, 1976, personal communication) has resulted in each laboratory using its own reference standard (usually a pool of "normal" human serum) and makes comparison of data between laboratories difficult.

Table 3.1

SOME REPORTED APPROACHES TO THE BIOASSAY OF SOMATOMEDIN

Author	Tissue	Isotope	Total incubation times (h)	Range of linear response (% serum)	Index of precision
Salmon & Daughaday, 1957	Hypox. rat costal cartilage	$^{35}SO_4$	24	2.5 - 17%	0.26
Daughaday et al, 1959	Hypox. rat costal cartilage	$^{35}SO_4$	24	1.7 - 10%	0.25 - 0.28
Almqvist, 1961	Hypox. rat costal cartilage	$^{35}SO_4$	24	1 - 20%	0.14*
Daughaday & Reeder, 1966	Hypox. rat costal cartilage	^{3}H thymidine	24	2 - 17%	-
Van Wyk et al, 1969	Hypox. rat costal cartilage	^{3}H methyl thymidine	48	0.1 - 8.3%	0.47
Van den Brande et al, 1971	Hypox. rat costal cartilage	$^{35}SO_4$, ^{3}H methyl thymidine	48	0.1 - 8.3%	0.38
Yde, 1968	Intact fasted rat costal cartilage	^{3}H methyl thymidine	48	0.25- 18%	0.3
Alford et al, 1972	Intact fasted rat costal cartilage	$^{35}SO_4$	24	5 - 15%	0.209*
Hall, 1970	Chick embryo pelvic leaflet	$^{35}SO_4$	6	2.5 - 40%	0.20
Van den Brande & Du Caju, 1974	Immature porcine costal cartilage	$^{35}SO_4$	48 or 73	5.6 - 45.2%	0.15
		^{3}H methyl thymidine	48 or 73	5.6 - 45.2%	0.19
Phillips, Herington & Daughaday, 1974	Immature porcine costal cartilage	$^{35}SO_4$	24 (no pre-incubation)	5 - 40%	0.20 (0.15-0.27)
Fujisawa, 1964	Hypox. puppy costal cartilage	$^{35}SO_4$	88	5 - 20%	0.18
Bala et al, 1975	Intact (or hypox) immature rabbit costal cartilage	$^{35}SO_4$	72	0.125-6.25%	0.24 (0.12-0.36)
		^{3}H thymidine	72	0.125-6.25%	0.25 (0.12-0.46)
Garland et al, 1972	Chick embryo pelvic leaflet isolated chondrocytes	^{3}H methyl thymidine	24	0.6 - 6.0%	-
Uthne, 1973	Human glia-like cells (622CG)	^{3}H thymidine	18	1 - 10 %	-
Wateson et al, 1973	Human foetal lung fibroblasts (460 Hel)	$^{35}SO_4$	6 - 50	3 - 50 %	-
Garland et al, 1976	Chick embryo pelvic leaflet isolated chondrocytes	^{3}H methyl thymidine	18	1 - 10 %	-

*After rejection of invalid assays.

Hypox. = Hypophysectomized.

- = Data not quoted in reference.

The usual treatment of assay data is to express the assay response (e.g. $^{35}SO_4$ incorporation) as a function of the logarithm of dose of somatomedin (serum), to select linear portions of the dose-response lines for standard and unknowns, and to calculate the least square regression lines of these dose responses. Only if the dose response regression lines of unknowns are linear and parallel to those of the standard to within an acceptable probability limit (usually arbitrarily chosen as $p < 0.05$) can a valid potency ratio be calculated. The potency ratio is derived from the horizontal distance between the least square regression lines of standard and unknown, the precision being expressed as confidence or fiducial limits.

The index of precision or residual mean square per slope (λ) for the standard provides a general idea of the reliability of a bioassay. When the λ of an assay is 0.3 - 0.4, it will provide semi-quantitative data on large differences in potency ratios, while with λ of 0.2 - 0.3 more quantitative data on smaller differences can be obtained. A more detailed discussion of the statistics of parallel dose-response bioassays based on the work of Finney (1964) appears in Appendix C.

ii) An appraisal of commonly used somatomedin bioassays

a) Hypophysectomised rat costal cartilage bioassays: Such assays were the first for somatomedin and they have evolved little since that of Salmon and Daughaday (1957). The major advantage of such assays is their sensitivity, 0.5% serum or less, (Daughaday, Phillips and Herington, 1975) (see Table 3.1) but they show a high λ. A number of techniques have been applied to improve the index of precision including a single rat source of cartilage (Wiedemann and Schwartz, 1972) which suffers from the defect that each rat only

provides enough cartilage for the determination of a standard and one (or at most two) unknown sera. The use of a serine-free buffer (Daughaday, Salmon and Alexander, 1959) increases the precision of the assay at the expense of a potential loss of specificity, in that serine even in the absence of serum stimulates $^{35}SO_4$ incorporation. Other amino acids which have been shown to stimulate rat cartilage are glycine, glutamine, threonine and valine (Koumans, and Daughaday, 1963; Almqvist, 1961; Salmon and Daughaday, 1958; Boström, Roden and Westermark, 1955). Chick cartilage was found to be stimulated by glutamine and serine (Hall, 1970). Homocysteine which stimulates cartilage may be responsible for the skeletal abnormality of homocysteinuria (Dehnel and Francis, 1972). All these amino acids and a large range of other amino acids are present in the incubation and dilution media advised for somatomedin assays, the concentrations being similar to those found in normal human serum (Daughaday and Reeder, 1966; Hall, 1970, Van den Brande, Du Caju, 1974; Phillips, Herington and Daughaday, 1974). To date no attempts have been made to adjust amino acid composition of media to that of the serum of the species or condition being assayed (Van den Brande, Kootte, Tielenburg, van der Wilk and Huyser, 1974; Shapiro and Pimstone, 1977). As in most assays serum is considerably diluted, any changes in amino acid composition probably do not result in significant effects providing the diluting medium contains adequate concentrations of stimulatory amino acids. The glucose added to all assay media provides a source of energy and substrate for mucopolysaccharide synthesis (Herington, Adamson and Bornstein, 1972).

When comparing $^{35}SO_4$ to ^{3}H Methyl-thymidine incorporation the latter is generally found to be as sensitive as $^{35}SO_4$ incorporation but to have consistently greater λ values (see Table 3.1). In

general, the high sensitivity and low precision of the hypophysectomised rat costal cartilage assays makes them best suited to the monitoring of somatomedin purification or production by isolated organ or tissues (Daughaday, Phillips and Herington, 1975).

b) Intact-rat costal cartilage bioassays: Such assays using intact male rats aged 21-27 days fasted for 48 hours were originally described by Almqvist (1961) and improved by Yde (1968) and Alford, Bellair, Burger and Lovett (1972). All use a serine free buffer (with its potential lack of specificity) and provide a sensitivity similar or slightly less than that of hypophysectomised rat costal cartilage, with λ values of 0.3 or less. The use of intact rats is advantageous in that they are more readily available, cost far less and more easily maintained than hypophysectomised rats. The assay of Alford, Bellair, Burger and Lovett (1972) is interesting in that the cartilage is dissolved by papain and the mucopolysaccharide precipitated on filter paper strips by acid-ethanol before counting; which allows the re-use of scintillation fluid (scintillation fluid can be a major expense in somatomedin bioassays).

c) Embryonic chick pelvic cartilage bioassays: Adamson and Anast (1966) noted that serum stimulated $^{35}SO_4$ incorporation into the pelvic rudiments of chick embryos. Hall (1970) used such pelvic rudiments for the bioassay of somatomedin. This assay provides a sensitivity as low as 2.5% serum with λ values of 0.3 or less, in addition embryonated hens' eggs can be obtained at relatively low cost, the tissue preparation is not difficult and the resultant cartilage fragments are uniform in size, shape and weight for a given embryonic age. Such assays are considered satisfactory for the monitoring of somatomedin purification and for clinical studies (Daughaday, Phillips and Herington, 1975).

(d) Porcine costal cartilage bioassays: Such assays have been described by Van den Brande and Du Caju (1974) and Phillips, Herington and Daughaday (1974) and have the advantage that a single animal provides the cartilage for the assay of 8 or more unknown sera. Other advantages are the fact that the uniform discs punched out of the porcine cartilage do not have to be individually weighed and a λ value less than 0.3 is consistently achieved. In spite of this high precision, the assay is less sensitive in that it does not respond to serum concentrations less than 5%. The assay of Van den Brande and Du Caju (1974) uses a phosphaline buffer and examines the incorporation of $^{35}SO_4$ and/or ^{3}H thymidine while Phillips, Herington and Daughaday (1974) using $^{35}SO_4$ incorporation and a Tris-HCl-amino acid buffer claim a steeper and more consistent dose response curve and omit the preincubation step. This assay is particularly suited for the assay of large numbers of samples.

(e) Bioassays using cartilage from other sources: A variety of other cartilagenous tissues have been used, these include; hypophysectomised puppy cartilage (Fujisawa, 1964) which is not practical because of the complex surgery required, the limited availability and cost of laboratory dogs; monkey costal cartilage; human neonatal articular cartilage (Van den Brande, Kootte, Tielenburg, Van der Wilk and Huyser, 1974) and immature ovine costal cartilage (Daughaday, Phillips and Herington, 1975). All compare unfavourably with the porcine costal cartilage assay in that tissue is either not easily available (man and monkey) or results are less satisfactory (lamb cartilage). An assay using normal immature rabbit costal cartilage (Bala, Hankins and Smith, 1975) makes use of only one animal per assay and provides satisfactory sensitivity and precision.

(f) Bioassays utilising cell cultures: Isolated chick pelvic rudiment chondrocytes have been used by Garland, Lottes, Kozak and Daughaday (1972). This provides an assay sensitive to as low as 0.5% serum and having a λ value less than 0.4, but many sera are inhibitory unless heat treated (56°C for 30 minutes) and it has not been found to consistently distinguish normal from hypopituitary serum. The main potential use of this assay is in the isolation of somatomedin-rich fractions from plasma.

Uthne (1973) used thymidine incorporation into a culture of human glia-like cells obtained from a brain biopsy to monitor the purification of a stimulatory serum fraction which came to be known as somatomedin B. Wasteson, Uthne and Westermark (1973) have used $^{35}SO_4$ incorporation into a culture of human foetal fibroblasts and have shown this to be stimulated by serum or somatomedin-rich plasma fractions.

Reasons for choosing the porcine costal cartilage bioassay for the present study:

The porcine costal cartilage assay of Van den Brande and Du Caju (1974) was chosen because it provided adequate sensitivity with good precision, there was no need for large numbers of hypophysectomised animals which were unobtainable locally and which are very expensive to import and travel extremely badly. In addition, the assay used cartilage discs of very uniform weight, individual weighing of cartilage fragments (a very tedious process) was not required and the Cape Town Municipal Abattoir was within 5 minutes drive of the laboratory and was known to be highly cooperative in assisting with medical research.

B Methodology

i) Source of cartilage

The costal cartilages (and sternum) from ribs 4 to 8 bilaterally were obtained from young pigs, weight 30-40 Kg, age 140-190 days. The pigs were slaughtered at the Cape Town Municipal Abattoir and due to the excellent cooperation of this institution, the cartilages could be excised within 20-30 minutes of the moment of death (i.e. as soon as the animals were bled and eviscerated).

ii) Transport of cartilage

The block of tissue including the costal cartilages was placed in a large sterile screw-cap jar of sterile saline kept at 4-6°C in an insulated container. The transit time from abattoir to laboratory was 5-7 minutes. Although transport in cold saline was used routinely it was found that transport at ambient temperature provided identical results.

iii) Preparation of cartilage

(a) On arrival at the laboratory the block of tissue was rinsed in 6 changes of sterile saline (at least 3 litres), placed in a sterile tray and with sterile instruments the muscle and connective tissue dissected from the costal cartilages. The perichondium was left intact, the 2 cm adjacent the costochondral junction and the 1 cm adjacent the sternocostal joint were excised and discarded. (b) The cleaned cartilage segments were sliced into 2mm thick slices (at right-angles to the long axis of the cartilage) with a razor blade guillotine (Fig. 3.1). (c) From the slices of cartilage uniform discs of cartilage were punched using a Trucut (Travenol Laboratories Inc., Deerfield, Illinois) biopsy needle, the tip of which was ground off square and sharpened. This needle was held in the chuck of a Minimat lathe/drillpress and punching was

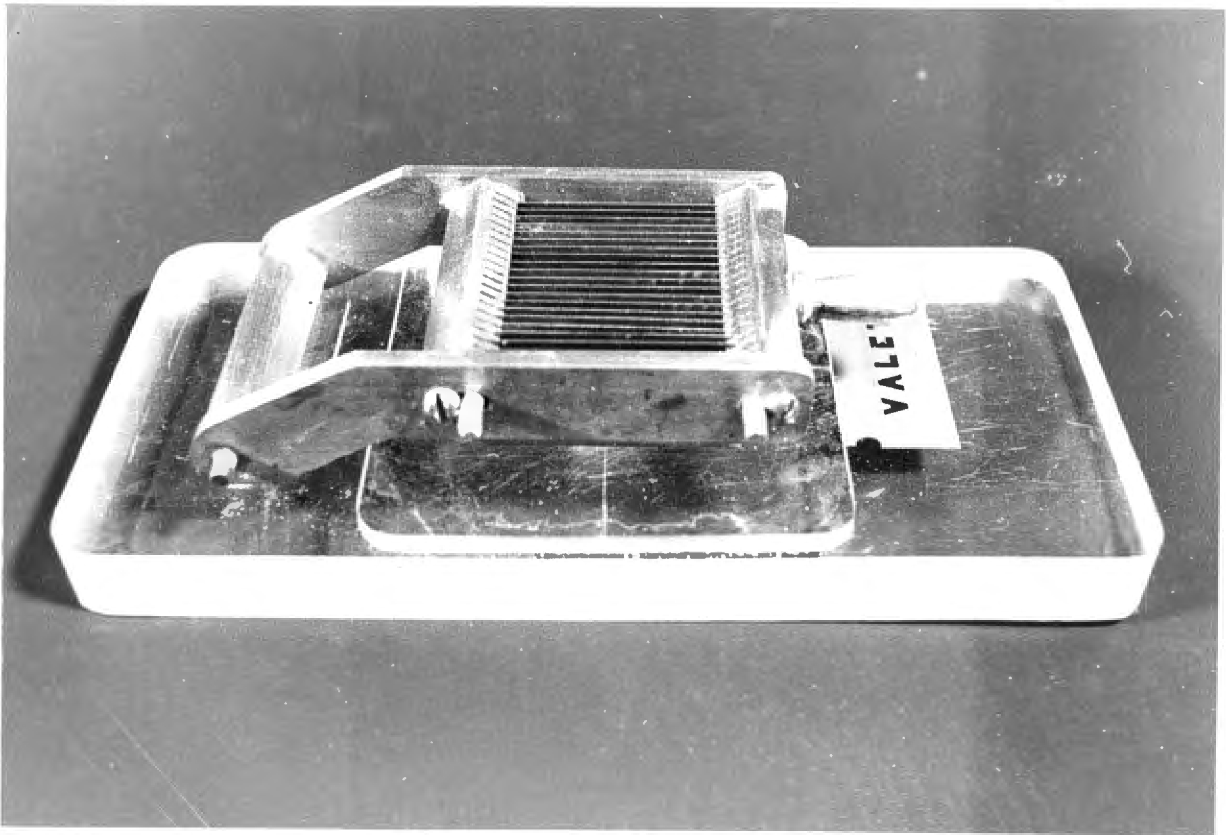


Fig. 3.1 Razor blade guillotine
Rigid single edge razor blades are held 2 mm apart in a hinged frame. This mechanism made it possible to cut uniform 2 mm slices of cartilage.

carried out under saline in a perspex chamber (Fig.3.2). From each slice of cartilage one to six discs of cartilage were punched, and expelled from the needle by pressure on the ground-down stillet (Fig. 3.3). Cartilage discs were punched out about 2 mm from the edge of the slice and from each other. (d) The saline was drained from the punching chamber and the cartilage discs ($\pm$ 300) placed in a sterile beaker with 50 ml of incubation medium. This was incubated (covered with sterile plastic film) for 20 hours at 37°C in a non-shaking incubator. The incubation medium was that described by Daughaday and Reeder (1966) and is essentially a Krebs phosphosaline buffer supplemented with amino acids, dextrose and antibiotics (Penicillin, Streptomycin and Kanamycin). Full details of the composition and method of preparation of this medium are provided in Appendix B.

iv) Incubation of cartilage with test substances (e.g. serum)

(a) The cartilage discs were incubated in a specially designed rack with a standard and unknown (Fig. 3.4 and Fig. 3.5). As can be seen from the figures, the cartilage discs were confined to the perforated teflon tubes which rest in pots containing the serum dilutions. The dilutions usually used were 40, 20, 10, 5 and 2.5% or 40, 20, 10 and 5%, the volume being 1 ml and each concentration being made up in triplicate. The rack could thus accommodate 6 unknowns and 1 reference standard as 10 point symmetrical assays, or 7 unknowns and 1 reference standard as 8 point symmetrical assays. In each case at least 3 tubes were filled with incubation medium only (0% serum). 30 to 50 discs were kept in the beaker in incubation medium for later determination of the weight of cartilage discs. (b) After 46 hours of incubation with dilutions of unknowns and standards the rack holding the perforated tubes was removed from

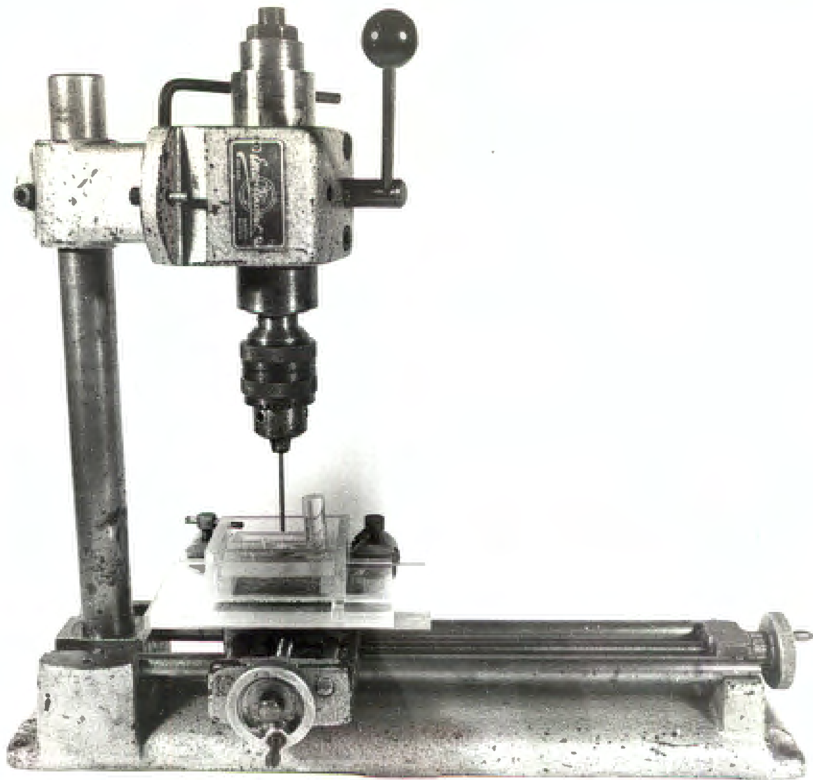


Fig. 3.2 Minimat lathe/drillpress with modified biopsy needle held in the chuck and perspex chamber clamped in place. The cartilage discs were punched out under saline in the perspex chamber.

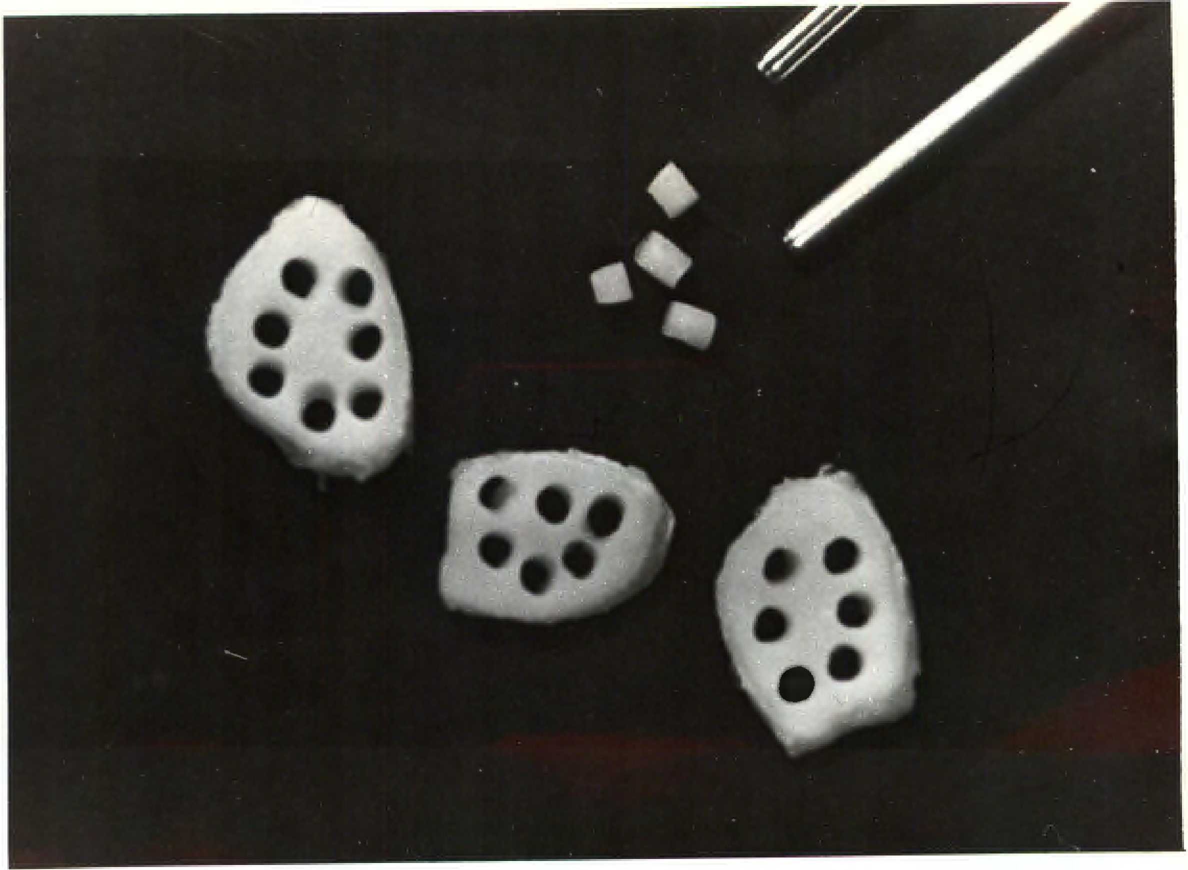


Fig. 3.3 Slices of cartilage with the cartilage discs punched from them by the modified biopsy needle (visible with its stillet at the upper right of the photograph).

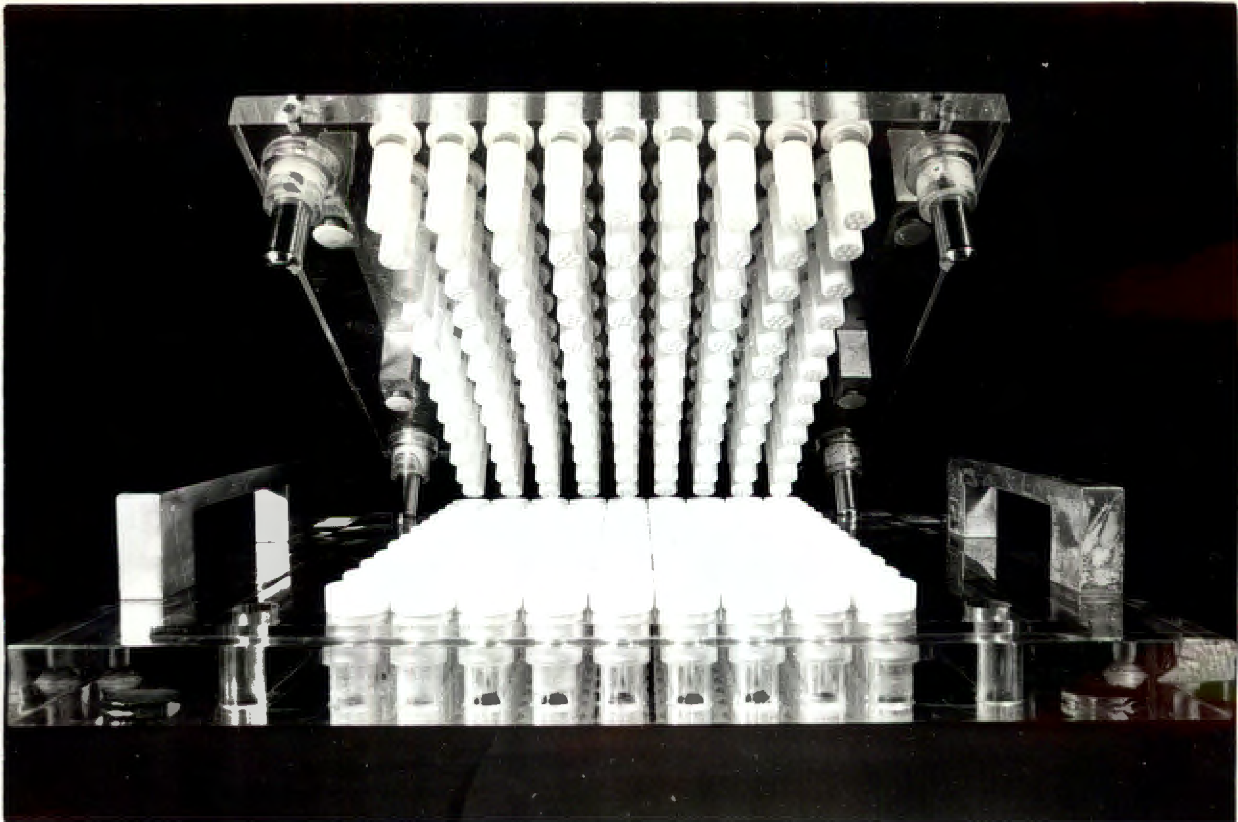


Fig. 3.4 Incubation rack: Perforated teflon tubes (above) fit into teflon pots (below). When in place the clearance between the tubes and the bottom of the pot is 1 mm.

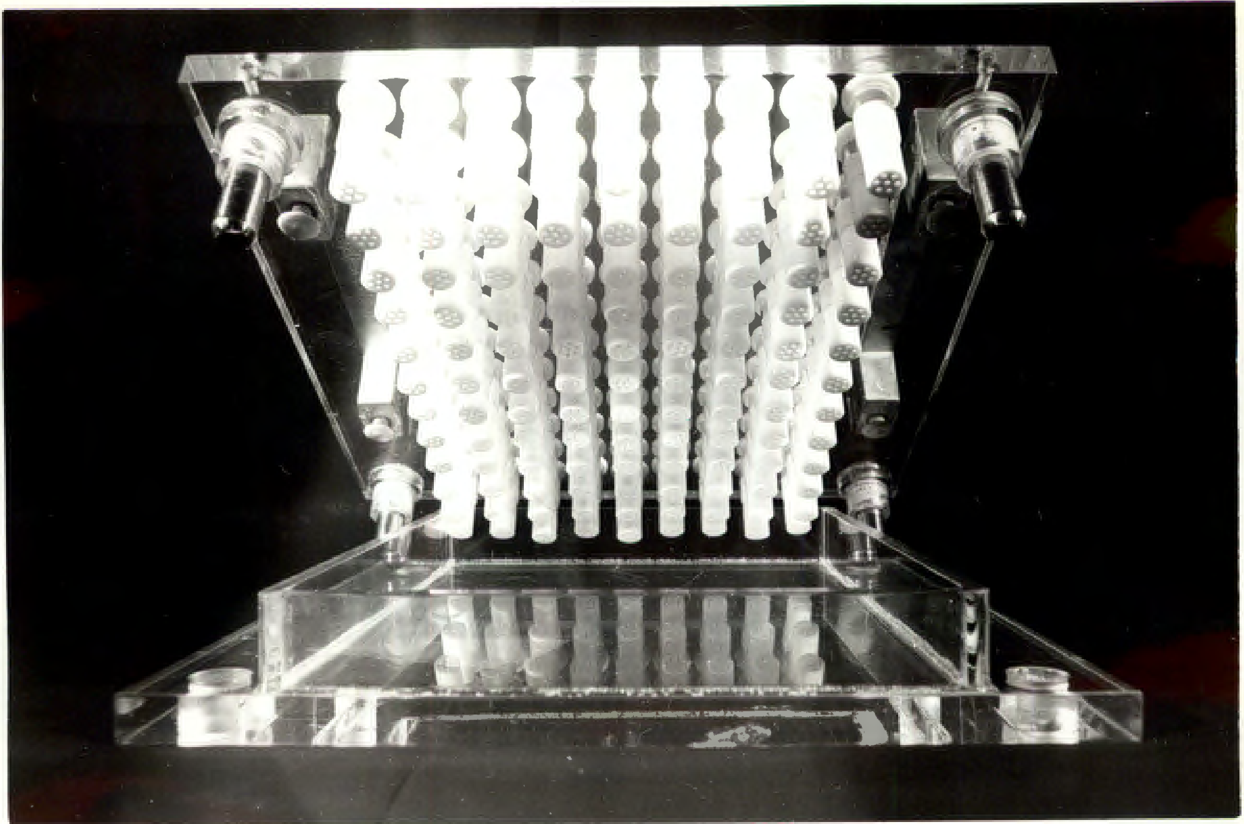


Fig. 3.5 Incubation rack and perspex bath for simultaneous incubation of all cartilage discs in the same $^{35}SO_4$ containing medium. The rack of perforated teflon tubes (above) is lowered into the perspex bath (below).

the rack holding the pots of samples. The lower ¼ to $^1/3$ of the perforated tubes were lowered into a perspex bath containing sterile normal saline, agitated for 30 seconds, removed and gently shaken free of residual saline. The perspex bath was then filled with incubation medium containing $Na_2{}^{35}SO_4$ 2 μCi/ml (carrier-free $Na_2{}^{35}SO_4$, The Radiochemical Centre, Amersham, U.K.) (Fig. 3.5). The perforated tubes were 1 mm from the bottom of the bath and the volume of medium was such as to completely submerge the cartilage discs. This incubation was maintained for 4 hours. (c) The rack containing the perforated tubes with their cartilage discs was transferred to a stainless steel bath fitted with a siphon which automatically filled and emptied the bath. After an initial rinse with tap water at room temperature the cartilage was killed by the addition of water at 80-90°C. This was retained for 10 minutes after which repeated filling and emptying of the bath was carried out over the next 30 minutes to rinse off all unincorporated $Na_2{}^{35}SO_4$. In 5 consecutive assays the radioactivity of the effluent wash was measured and found to be background after 7-9 exchanges. A period of 30 minutes provided at least 20 changes of wash water (Fig. 3.6).

v) Preparation of cartilage discs for counting

The 2 discs from each perforated tube were shaken out into 25 ml glass scintillation counting vials (Packard) each containing 0.5 ml 23M Formic Acid (Merck, Darmstadt). The vials were then loosely capped with polyethylene-lined caps (metal foil-lined caps were subject to corrosion) and heated to 80°C for 60-90 minutes in an oven, which resulted in complete dissolution. After cooling, Instagel (Packard) 10 ml was added to each tube, and after vigorous mixing the tubes placed in a cool dark place for 4 hours before $^{35}SO_4$ was determined by liquid scintillation counting. The 30-50 discs which were kept in assay buffer for the duration of the expe-

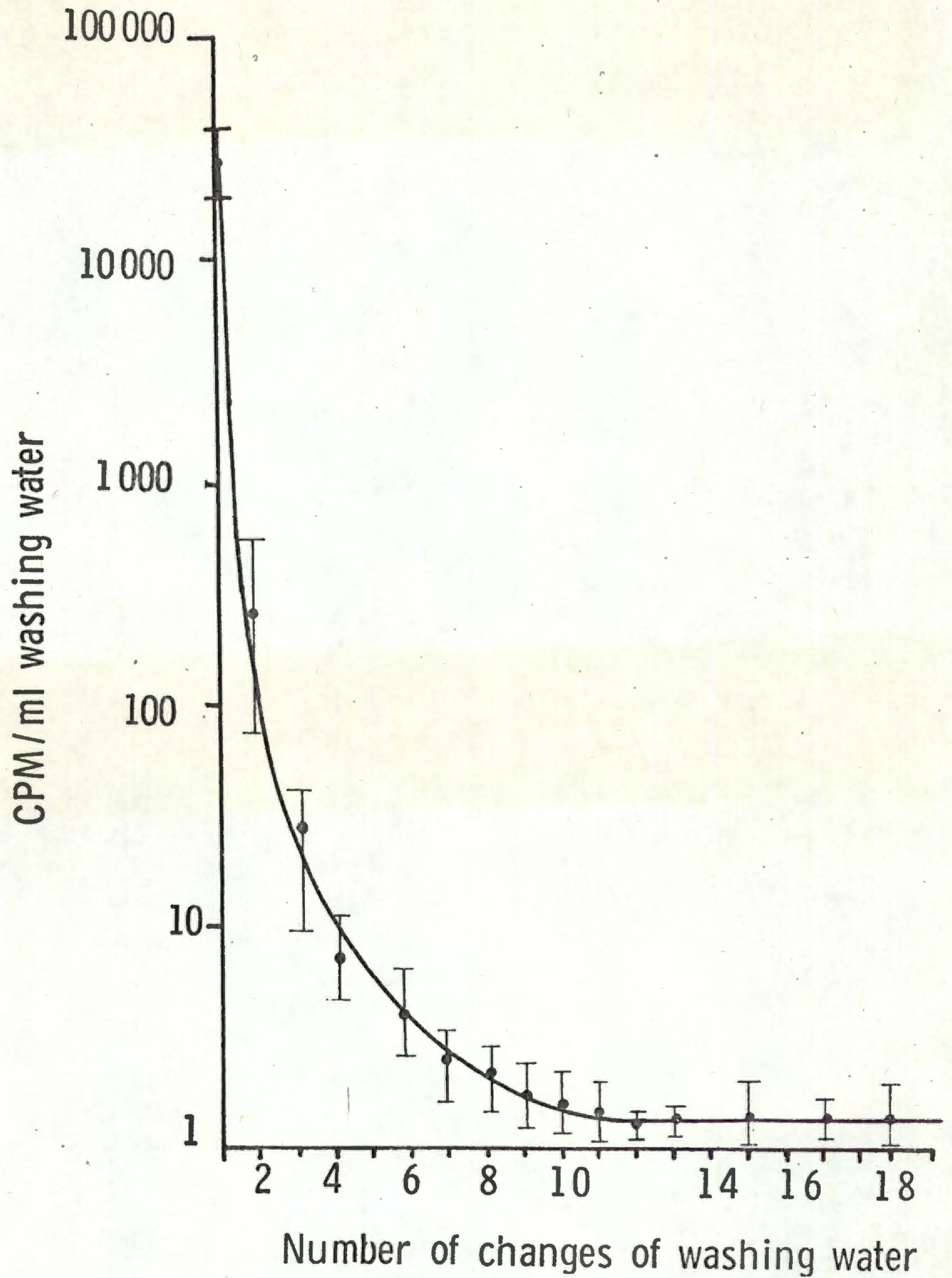


Fig. 3.6 Removal of unincorporated $Na_2\ {}^{35}SO_4$ from incubation rack containing cartilage fragments by washing in siphoning rinsing bath. Graph shows CPM ${}^{35}SO_4$/ml washing water with each change (mean ± SD, n = 5).

riment were washed in 3 changes of absolute alcohol over 12 hours and then 2 changes of ether (½ hour each) to dehydrate them. Individual discs were then weighed to the nearest 0.1 mg on a Metler 4 place digital balance. The mean dry weight of these representative samples of cartilage discs from each batch was 1.8-1.9 mg with a coefficient of variation from 5-7% on a sample of 8 consecutive assays. Later mean weight of cartilage discs was determined simply by weighing en masse and dividing by the number of discs weighed.

vi) The $^{35}SO_4$ in samples was determined by liquid scintillation counting with a Packard Tricarb Z650 appropriately set for ^{35}S (Appendix B).

vii) The somatomedin potency ratios were calculated using a Hewlett-Packard 9810 desk computer with plotter number and extended memory facilities. The programme listing was kindly provided by Dr. L. Phillips, Northwestern University College, U.S.A., and a full description of the programme is found in Appendix C. Essentially it provides a log-log transformation of the incorporation of $^{35}SO_4$ and serum concentration, with the linearised dose response line plotted by the computer, the linear portion is selected by eye and subjected to analysis. The programme provides for tests of linearity (95% confidence level), linear regression on the line and the index of precision (λ). The standard and unknown are then compared, and parallelism, heteroscedasticity, Finney's g and potency ratio with 95% confidence limits calculated. The statistics used were those for symmetrical bioassays (Finney, 1964) and are described in detail in Appendix C.

viii) Standard sera

In the absence of international reference preparations or even

international reference sera, use was made of a pool of serum made up of 30 ml of serum from 30 normal adult males (aged 20-48) who were clinically in good health and normal for height and weight. For rats, a pool of serum from 60 normal fed adult male rats was used. In both cases the blood was allowed to clot at 4°C, the serum separated by centrifugation (2000g for 30 min at 4°C), the sera pooled, aliquoted and frozen at -20°C until use.

C <u>Validation of the bioassay</u> (Methods and Results)

The method described in Section B above was used routinely to determine serum somatomedin activity. A number of experiments were carried out to validate and optimise the assay.

i) To prove the uptake of $^{35}SO_4$ was an active process

(a) Temperature dependence. Pairs of cartilage discs prepared as above were incubated with and without normal human serum at a concentration of 40% in triplicate (volume 1ml) in 6 ml plastic test tubes at 4, 17, 25, 30, 37, 40, 45, 55 and 80°C. Fig. 3.7 shows that both serum-free and 40% normal human serum stimulated $^{35}SO_4$ uptake were at their maximum at or near the physiological temperature of 37°C and that the stimulation by serum was clearly greatest at 37°C.

(b) Cartilage discs which were first killed by 5 minutes boiling in incubation medium failed to take up $^{35}SO_4$ above background, either with or without 40% normal serum.

(c) To prove that $^{35}SO_4$ uptake was energy dependent. Cartilage discs were incubated at 37°C with and without 4% normal human serum and dinitrophenol (Merck, Darmstadt) at a variety of concentrations. Dinitrophenol uncouples oxidative phosphorylation and

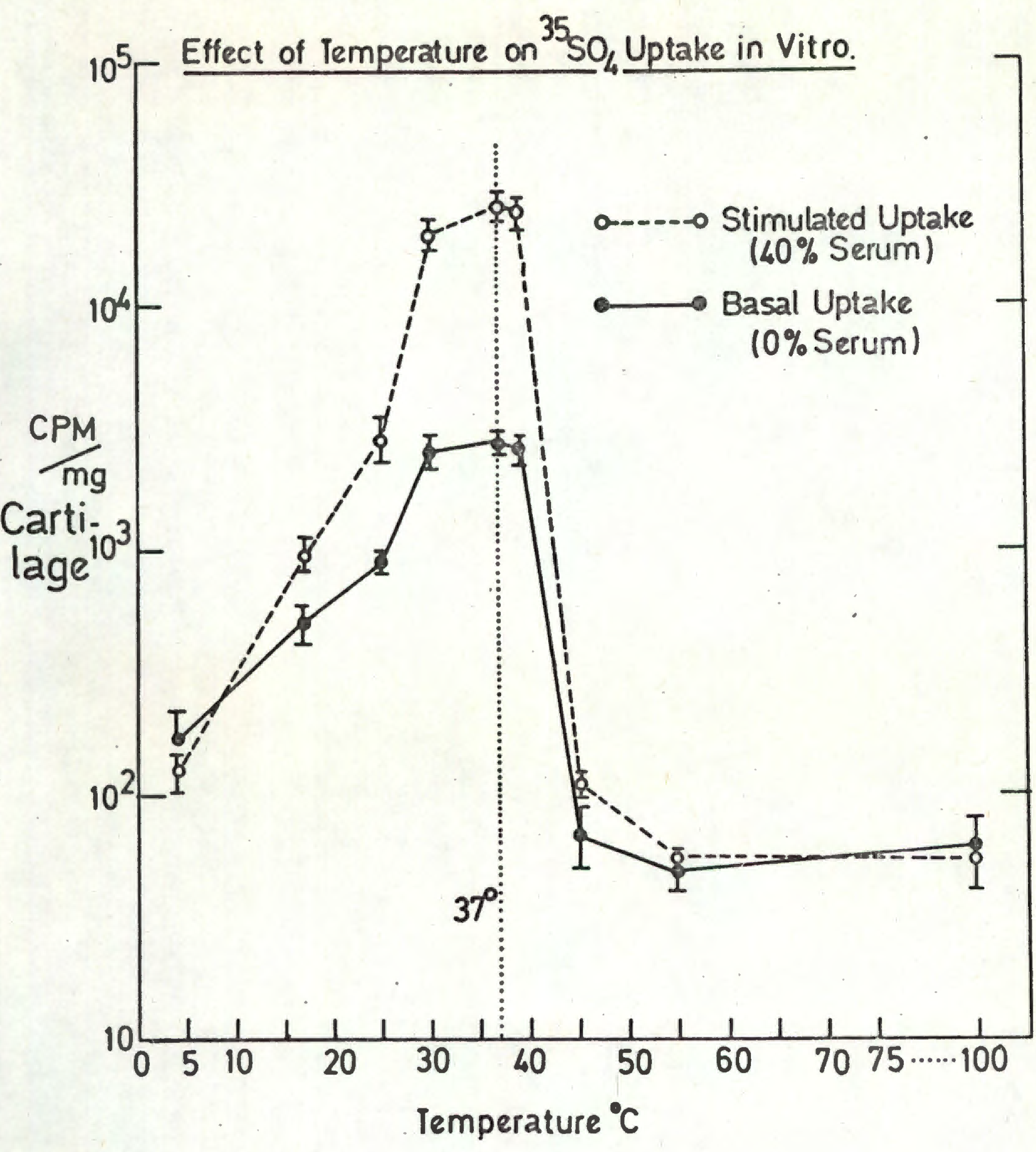


Fig. 3.7 The effect of temperature on basal (0% normal pooled human serum) and stimulated (40% normal pooled human serum) $^{35}SO4$ incorporation into porcine cartilage discs in vitro. Incorporation with or without serum is greatest at 37°C as is the stimulatory effect of serum. The point designated 100°C is cartilage discs boiled for 5 minutes and then subjected to incubation at 37°C. The graph shows mean ± SEM, n = 3).

Fig. 3.8 shows that both stimulated and unstimulated $^{35}SO_4$ incorporation was to a large extent dependent on oxidative phosphorylation.

(d) To show that $^{35}SO_4$ incorporated into cartilage was in the form of large molecular weight mucopolysaccharides. Cartilage discs were prepared as described above and incubated either in serum-free medium or with pooled normal rat serum or pooled normal human serum at a concentration of 40%. At the end of the assay procedure (after the washing step) instead of dissolving the cartilage in formic acid, a papain solution was used (0.5 mg/ml papain (Boehringer Mannheim) 2.0M cysteine and 2mM EDTA in 0.1M phosphate buffer at pH 6.5). Twenty cartilage discs from serum stimulated and serum-free incubations, were incubated with 1 ml papain solution at 50°C with agitation for 16 hours, which resulted in complete dissolution. An aliquot of the papain digest from stimulated and unstimulated cartilage was run on a Sephadex G100 gel filtration column (0.9 x 50 cm) in 0.1M phosphate buffer (pH 6.5). In both instances the $^{35}SO_4$ eluted as a high molecular weight form (see Fig. 3.9).

When aliquots of the papain digest were further incubated with testicular hyaluronidase (0.5 mg/ml) (Boehringer Mannheim) at 37°C for 8 hours, all the $^{35}SO_4$ eluted as small molecular weight form (see Fig. 3.9).

This method for the determination of the molecular weight of $^{35}SO_4$ mucopolysaccharides in cartilage has been used by Alford, Bellair, Burger and Lovett (1972) for rat cartilage, and the results for porcine cartilage are very similar.

ii) To prove that the age of the pigs providing the cartilage was compatible with adequate sensitivity

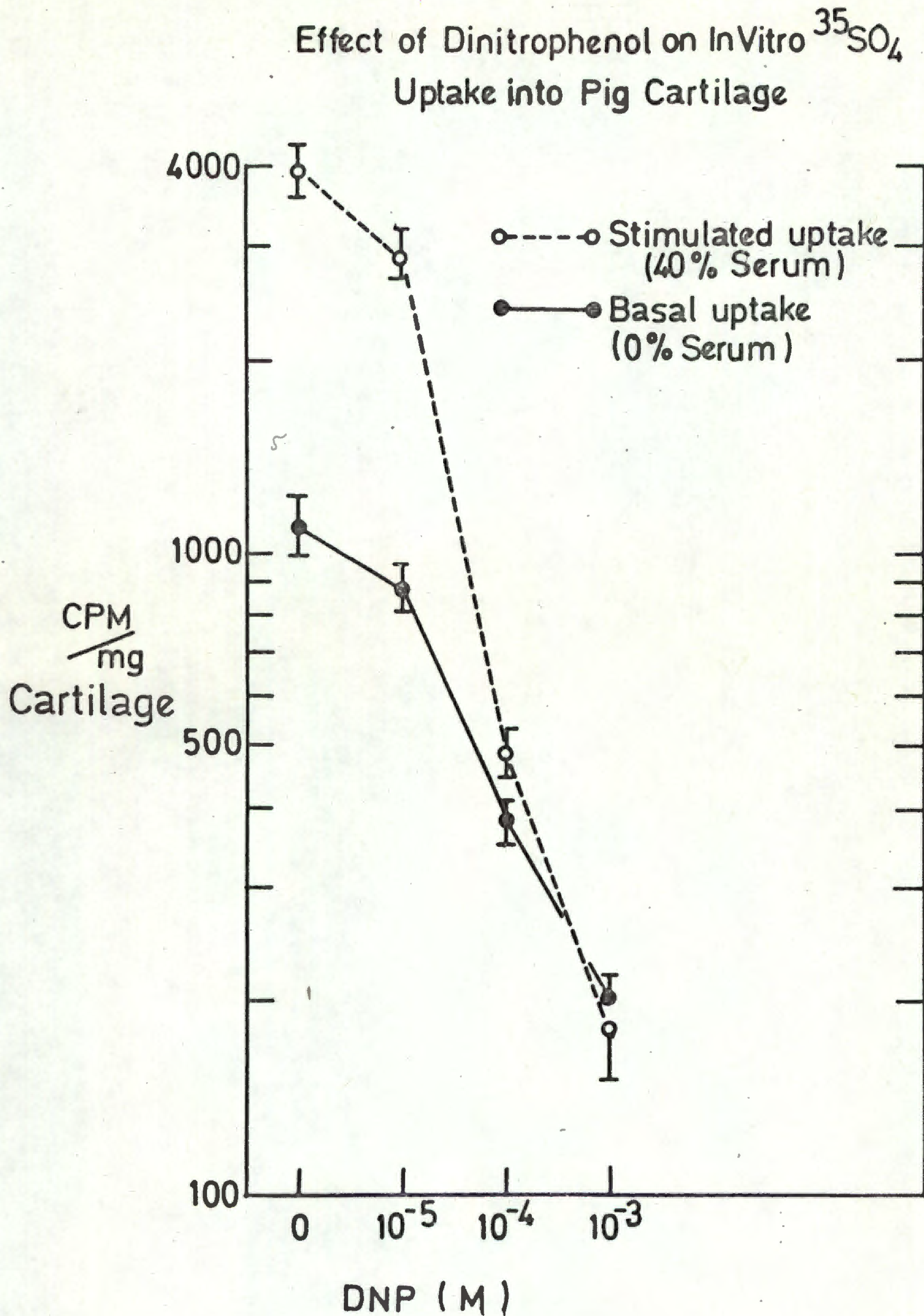


Fig. 3.8 The effect of dinitrophenol on $^{35}SO_4$ incorporation into porcine costal cartilage discs in vitro. There is a dose dependent inhibition of both basal and stimulated incorporation.

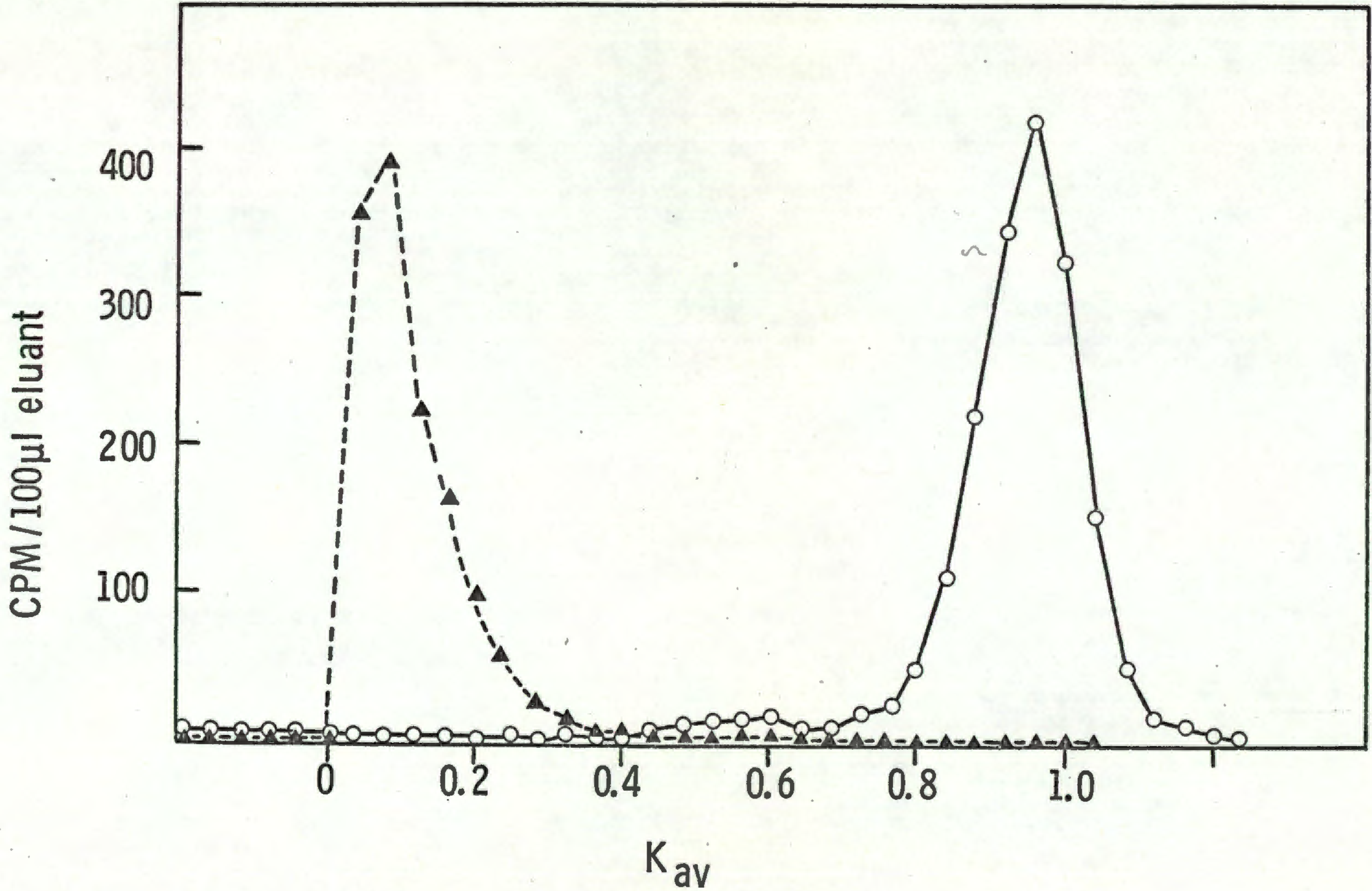


Fig. 3.9 Sephadex G-100f gel chromatography of papain digest of in vitro $^{35}SO_4$ labelled porcine costal cartilage discs (elution profile indicated by black triangles). After further digestion by hyaluronidase (elution profile indicated by open circles) there was conversion of $^{35}SO_4$ containing material from large to small molecular weight.

The stimulation of uptake of $^{35}SO_4$ by 30% normal human serum on cartilage discs from a number of pigs of ages from 6 weeks to 2 years is shown in Fig. 3.10. This confirms the findings of others that age is the most important factor in determining cartilage sensitivity to sulphation factor. Although the very young animals provided the most sensitive cartilage it was not practical to use them and pigs aged 6-9 months were used routinely.

iii) To prove that the area of cartilage chosen for the assay was uniformly responsive in $^{35}SO_4$ incorporation

Three blocks of tissue were obtained as described in Section B. (a) Samples of cartilage discs were obtained from the central portions of ribs 4 to 8. There was no significant difference at any rib level in the incorporation of $^{35}SO_4$, either unstimulated or stimulated with 40% serum. (b) Cartilage discs obtained from slices from the costochondral junction to the sternum were examined for basal uptake (serum free). Fig. 3.11 shows a far greater incorporation of $^{35}SO_4$ into discs prepared from the area within 20mm of the costochondral junction while discs from the rest of the cartilage had a uniform uptake. (c) Discs prepared from the mid-portions of costal cartilages 4 to 8 were punched from the periphery (2mm from the surface), intermediate (4mm from the surface) and centre of the cartilage slices. There was no statistical difference between the three areas, either basally or stimulated by 40% normal human serum.

iv) To prove that the sulphation factor measured with the porcine costal cartilage assay is growth hormone dependent

(a) Serum was obtained and assayed from patients with panhypopituitarism, isolated growth hormone deficiency (with and without

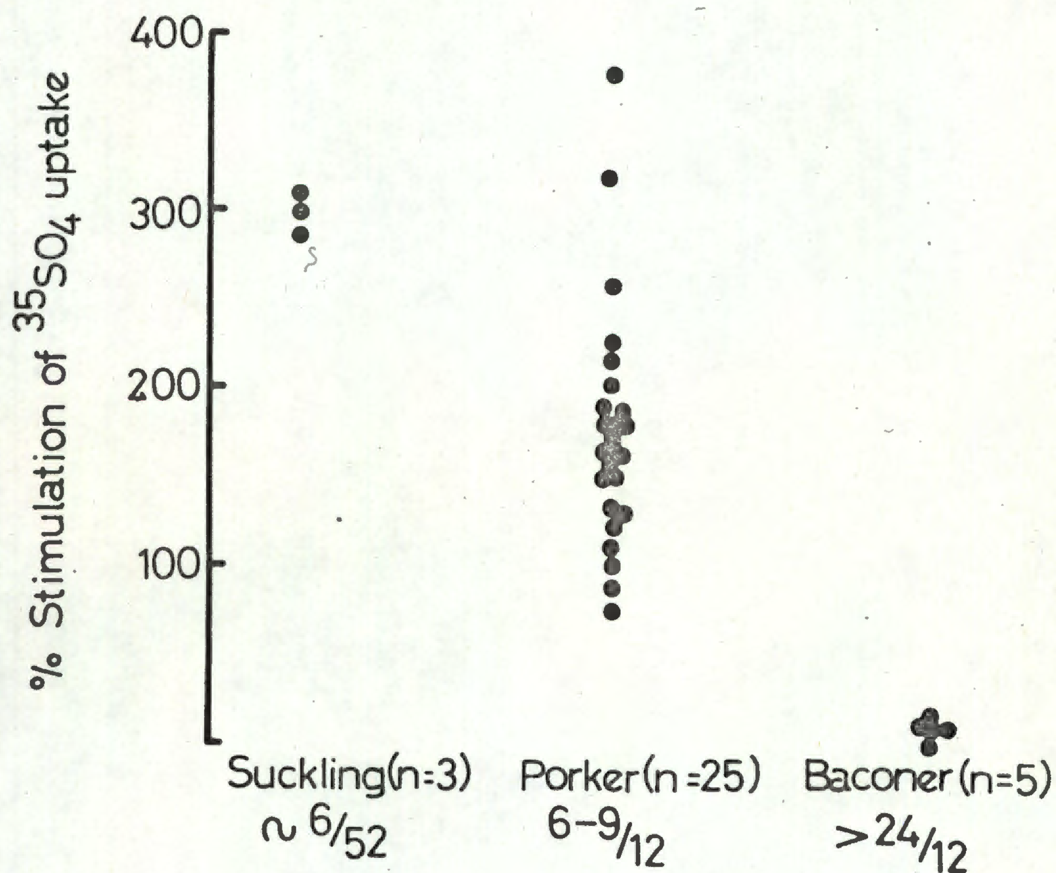


Fig. 3.10 The sensitivity to stimulation by 30% normal pooled human serum of porcine costal cartilage discs from pigs of different ages.

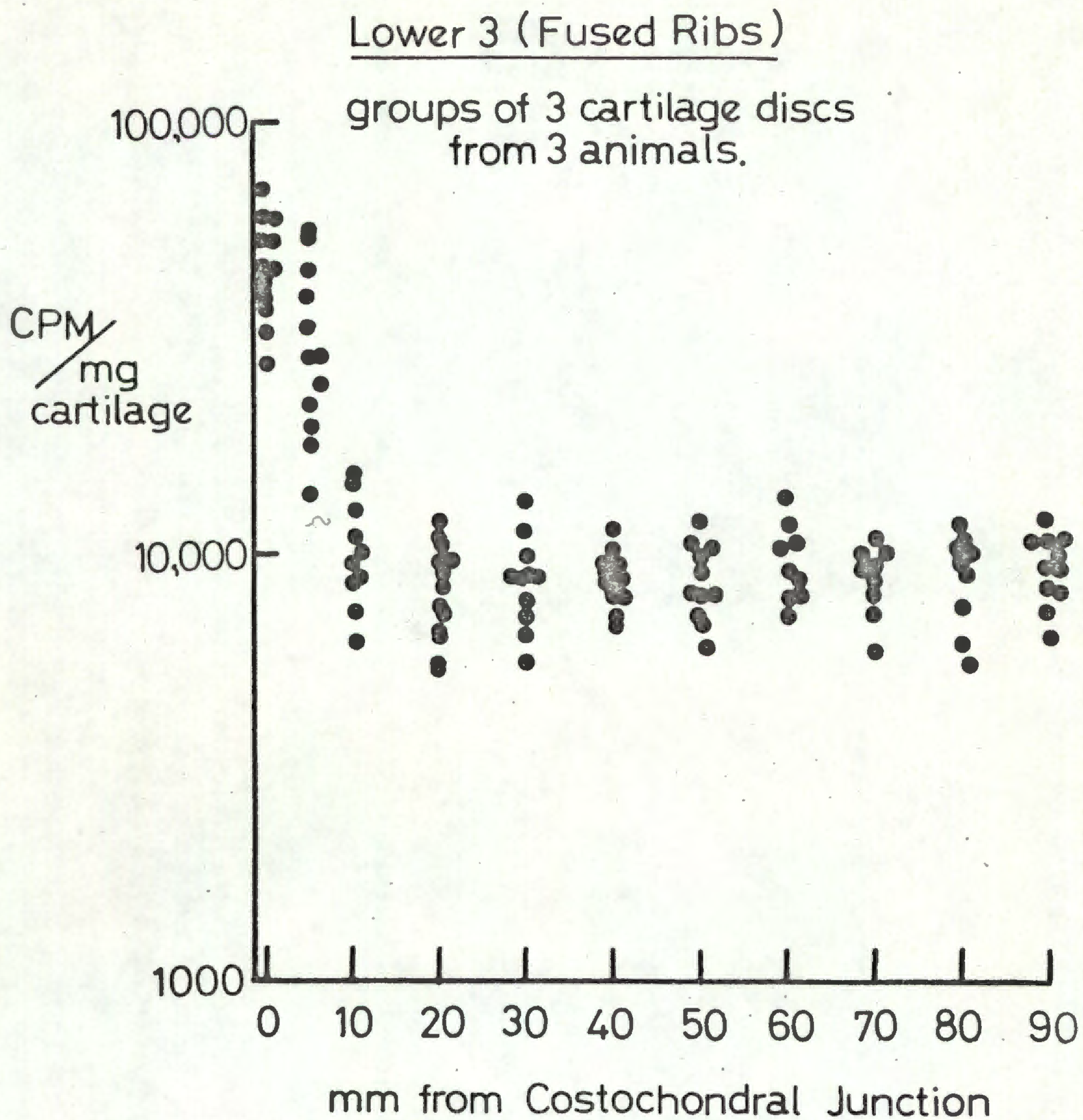


Fig. 3.11 Incorporation of $^{35}SO_4$ into porcine costal cartilage discs prepared from slices cut at intervals from the costochondral junction to the costosternal joint.

growth hormone treatment), untreated active acromegaly, treated acromegaly (treated with radiation, transsphenoidal hypophysectomy, transfrontal hypophysectomy or combined surgery and irradiation) and clinically normal persons. The findings of these assays is shown in Fig. 3.12 which shows that serum somatomedin activity is low in panhypopituitarism and isolated growth hormone deficiency, and responds to growth hormone treatment, that it is elevated in acromegaly and reduced in some treated cases of acromegaly.

(b) Great difficulty was experienced in obtaining hypophysectomised rats. A pool of hypophysectomised rat serum obtained from rats kindly hypophysectomised by Mr. D. de Wit of the State Vaccine Institute, Cape Town, was obtained and found to have a low somatomedin activity (0.19 U/ml, 95% confidence limits 0.09-0.30) and absent serum radioimmunoassayable rat growth hormone (no trace of pituitary tissue in the pituitary fossa). There were insufficient animals for growth hormone therapy to fully test growth hormone dependence in the rat but in view of the studies on man, the data available on the rat and the fact that the assay has been fully validated in the past on many occasions, growth hormone dependency was considered to be proven.

v) Reproducibility of somatomedin level results by the porcine costal cartilage bioassay

Two secondary pools of rat serum were prepared, each consisting of serum from 8 normal adult fed rats which were prepared in the same way as the primary rat standard, aliquoted and frozen at $-20^{o}C$. The secondary pools were measured against the primary rat standard on 6 occasions over a period of 15 months. Over the period studied there was a high degree of reproducibility in the somatomedin level assayed (see Table 3.2).

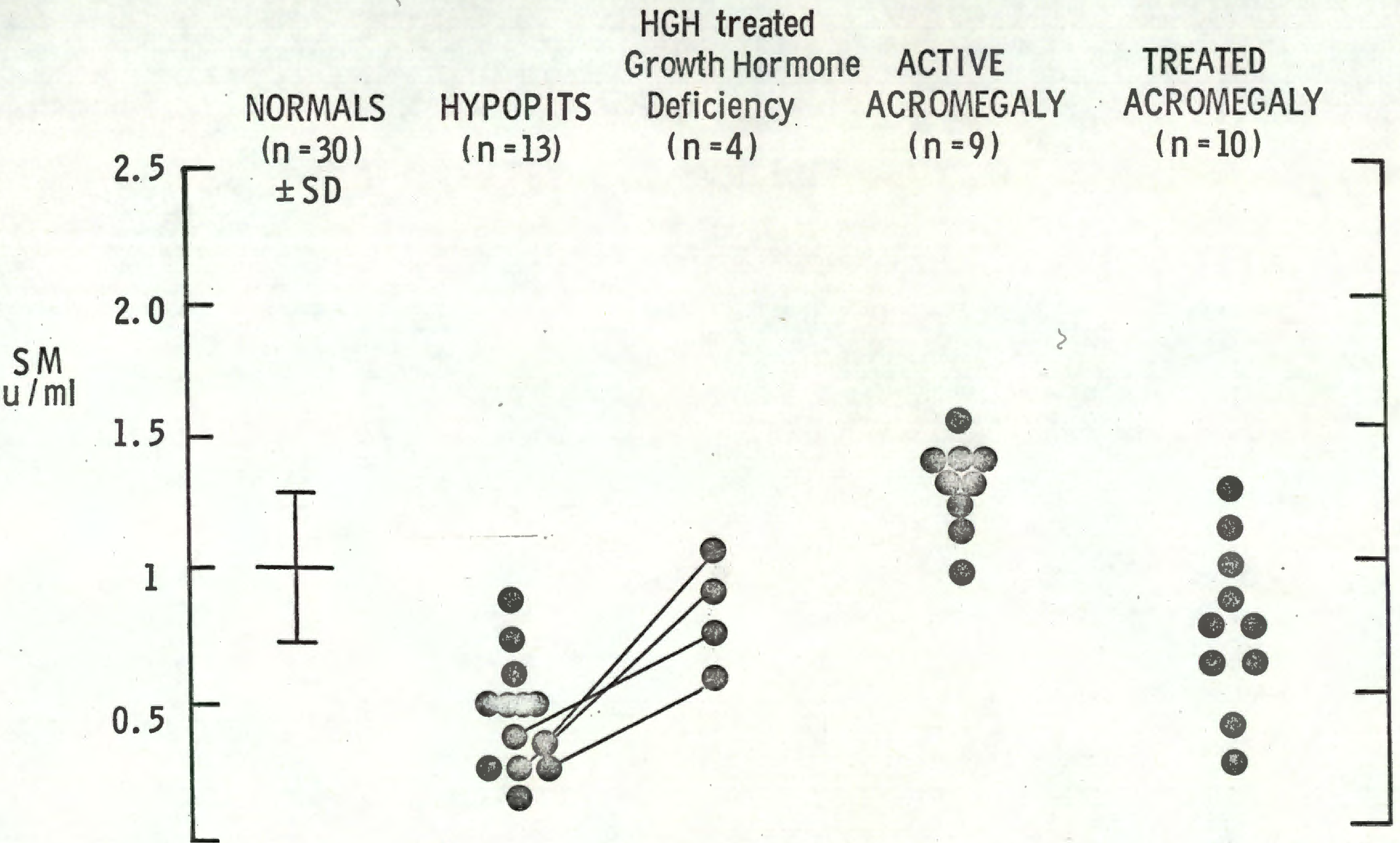


Fig. 3.12 Somatomedin levels in the normal subjects making up the standard normal human serum pool, 13 growth hormone deficient patients and the response of 4 to treatment with growth hormone, 9 acromegalics in the active phase of the disease and 10 acromegalics treated by various modalities. There was a significant correlation between the fasting growth hormone level and the somatomedin activity in these patients.

TABLE 3.2

REPRODUCIBILITY OF SOMATOMEDIN BIOASSAY

Date	Pool A Potency Ratio (± 95% Confidence limits)	Pool B Potency Ratio (± 95% Confidence limits)
Aug. 1976	1.11 (0.98 - 1.22)	0.84 (0.68 - 0.98)
Oct. 1976	1.23 (1.08 - 1.35)	0.81 (0.65 - 0.96)
Jan. 1977	1.14 (0.99 - 1.28)	0.80 (0.64 - 0.96)
May 1977	1.01 (0.90 - 1.12)	0.90 (0.77 - 1.02)
Sept. 1977	1.09 (0.94 - 1.25)	0.83 (0.68 - 0.97)
Nov. 1977	1.15 (1.01 - 1.30)	0.86 (0.71 - 1.01)

Serum somatomedin activity of aliquots deep frozen (-20°C) rat serum pools (8 normal adult male rats each) against the primary normal fed adult male rat standard (pool of 60) on 6 occasions over 15 months (the first within 1 week of bleeding).

vi) The precision of the porcine costal cartilage assay

The indices of precision obtained for sulphation factor activity are similar to those reported by others (Van den Brande and Du Caju, 1974; Beaton, 1976). (See Table 3.3).

TABLE 3.3

MEAN INDEX OF PRECISION FOR THE PORCINE COSTAL CARTILAGE BIOASSAY ($^{35}SO_4$ UPTAKE

		Mean λ	Range	No.
Assay of Human serum:	reference standard	0.18	(0.10-0.31)	8
	unknown samples	0.19	(0.11-0.31)	8
Assay of rat serum:	reference standard	0.15	(0.08-0.28)	25
	unknown samples	0.18	(0.09-0.30)	25

vii) The sensitivity of the porcine costal cartilage assay

The assay was found to be able to readily discriminate between serum-free medium and 5% normal pooled serum which represents by definition 0.05 U/ml somatomedin activity and was in keeping with that reported by others (Van den Brande and Du Caju, 1974; Phillips, Herington and Daughaday, 1974).

D Discussion

In validating this porcine costal cartilage bioassay for somatomedin, it has been shown to conform to the characteristics of porcine costal cartilage in the hands of other workers (Van den Brande and Du Caju, 1974; Phillips, Herington and Daughaday, 1974; Beaton, 1976).

Sulphate uptake alone was used because of its greater precision, the lower cost, and the fact that there is an excellent correlation between somatomedin activity when measured either as sulphation or thymidine factor. There is no evidence that somatomedin activity of serum has dissociated sulphation factor and thymidine factor activities.

The uniformity of basal and stimulated sulphate uptake by the cartilage discs from areas of tissue used has been proven. The uptake of sulphate has been shown to be an active temperature and energy dependent process in which the ^{35}S sulphate is incorporated into a high molecular weight mucopolysaccharide macromolecule.

The growth hormone dependence of the factor bioassayed has been shown. This is a prerequisite for all somatomedin bioassays (Van Wyk, Underwood, Hintz, Clemmons, Voina and Weaver, 1974; Daughaday, Hall, Raben, Salmon, Van den Brande and Van Wyk, 1972).

An adequate degree of reproducibility and an acceptable index of precision and sensitivity were obtained with this assay and it was considered suitable for further use in investigating somatomedin levels in experimental protein energy malnutrition.

CHAPTER FOUR

EXPERIMENTAL PROTEIN MALNUTRITION

"All flesh is Grass". The prophet Isaiah

A Introduction

i) Human clinical protein-energy malnutrition:

This presents a highly heterogenous spectrum, varying in age of onset, duration and severity. Concomitant vitamin and mineral deficiencies and water and electrolyte imbalance frequently occur, while presentation to medical attention is all too often precipitated by intercurrent infections and infestations. Protein deficiency of energy remains however the central core of the syndrome of protein-energy malnutrition, ranging from kwashiorkor to marasmus (Trowell, Davies and Dean, 1954). The diversity of the syndrome and the fact that withholding adequate food intake while non-therapeutic investigations and experiments are conducted is ethically unjustifiable, making an animal model of protein malnutrition highly desirable (Kirsch, Saunders and Brock, 1968).

ii) Animal models of protein malnutrition:

The perfectly valid animal model would simulate all the clinical and biochemical parameters of human malnutrition, avoid vitamin, mineral, water and electrolyte imbalances, infections and infestations, while being uniform in severity and age of onset. No such model exists. The problems have been reviewed by Kirsch, Saunders and Brock (1968).

Larger animals such as the pig (McCance and Widdowson, 1966; Platt, Heard and Stewart, 1964), the dog (Platt, Heard and Stewart, 1964) and the monkey (Deo, Sood and Ramalingaswami, 1965; Follis, 1957) have the disadvantages of high cost, slow breeding and the need for large scale animal housing which limits

the numbers of animals that can be studied. Rat models (McCance and Widdowson, 1966; Kirsch, Brock and Saunders, 1968; Edozien, 1968; Sidransky and Verney, 1970; Enwonwu and Sreebny, 1970; Stead and Brock, 1972) have the advantages of low cost, ease of breeding and housing and the availability of large numbers of animals for adequate statistical analysis of data.

Rats on low protein diets reduce food intake (Harper, 1959) which may be overcome by intragastric force feeding at the expense of high mortality (Sidransky and Verney, 1970) or "meal" feeding to reduce the anorexic effect (Stead and Brock, 1972; Padwaldesai, Ninjoor, Ramakrishnan, Sawant, Kumta and Sreenivasan, 1969). Alternatively the reduced intake can be accepted, in that although reduced, it remains appropriate for body weight and weight loss does not occur (Stead and Brock, 1972).

iii) The choice of an animal model:

The model of Stead and Brock (1972) was chosen because it is produced in early weaned rats fed a low protein diet ad lib, a situation not dissimilar to that in human protein energy malnutrition. A variety of endocrine aspects of this model had been previously studied in this laboratory and it was thus familiar, highly characterised and much detailed endocrine and metabolic data was was available. Le Roith and Pimstone (1973) using a meal fed variation, demonstrated a lowered exchangeable calcium pool and bone calcium accretion rate associated with poor calcium absorption and undermineralised bone. Kalk and Pimstone (1974) showed a low intestinal calcium binding protein but normal vitamin D metabolism. Abnormal glucose tolerance and poor insulin secretion was found (Weinkove, Weinkove and Pimstone, 1976) similar to that in human protein-energy malnutrition (Becker, Pimstone, Hansen and Hendricks, 1971).

The isolated in situ perfusion of livers from these animals has been used to investigate insulin degradation (Sacks, Pimstone, Waligora, Peires, Weinkove and Saunders, 1977).

B Methodology

i) Breeding colony:

Albino rats of the Wistar strain were used. This strain, inbred over many years is extremely homogeneous. Fertile females were introduced into a large (50 x 27 x 20 cm) plastic cage with several adult males and remained there until obviously pregnant. Each female was then placed in a separate plastic cage (40 x 25 x 23 cm) until delivery, only litters of 6-9 were used. All adults had continuous access to tap water and standard rat cubes (Epol Feeds, Cape Town), fresh shredded paper bedding was provided twice weekly.

ii) Dietary regime:

Unsexed weanling rats (i.e. fed maternal milk and just beginning to eat scraps of standard rat cubes) aged 21 days, weighing 28-38g were housed in mesh-bottomed (to prevent coprophagia) stainless steel cages (30 x 15 x 15 cm) at a density of 2-3 per cage (225-150 cm^2 floor surface/animal). All animals were kept in an air-conditioned room at 18-20°C, 40-60% humidity and a 12-hour light/dark cycle was provided by overhead fluorescent lighting and an automatic time switch. The rats were allowed free access to tap water and one of two diets: 20% protein in the form of cow's milk casein or 4% casein supplemented with 0.2% DL Methionine (this prevents specific methionine deficiency; methionine being the first limiting amino acid in casein (Harper, 1959)). Both diets are isocaloric and provide known and adequate amounts of vitamins and minerals (Harper, 1959) (See Tables 4.1 and 4.2).

TABLE 4.1

DIETARY COMPOSITION FOR THE EXPERIMENTAL MODEL OF PROTEIN MALNUTRITION (Stead and Brock, 1972)

	4% Casein Diet (g/Kg diet)	20% Casein Diet (g/Kg Diet)
Casein	40.0	200.0
Dextrin	855.0	697.0
Mineral Mixture	40.0	40.0
Vitamin Mixture	10.0	10.0
Maize Oil	40.0	40.0
Cod Liver Oil and 1% Vitamin E	10.0	10.0
Choline Chloride	3.0	3.0
DL Methionine	2.0	-

Casein : 90 mesh (South African Milk Board

Dextrin : (Glucose and Starch Products, Cape Town)

Maize Oil : Vereeniging Consolidated Mills, Vereeniging)

Cod Liver Oil : (Marine Oil Refineries, Hout Bay) provides 4000 IU Vitamin A and 2000 IU Vitamin D per Kg diet

Vitamin E : Provided as all α tocopheryl acetate (Merck, Darmstadt)

Choline Chloride : (BDH, Poole, England)

DL Methionine : (BDH, Poole, England)

TABLE 4.2

VITAMIN MIXTURE (Harper, 1959)

	mg/100g mixture
Inositol	1000.0
Ascorbic Acid	500.0
Vitamin B12 (as Cyanocobalamine)	400.0 (200μg B12)
Nicotinic Acid	250.0
Ca Pantothenate	200.0
Riboflavine	50.0
Thiamine HCl	50.0
Pyridoxine	25.0
Vitamin K_3 (Heterozeen)	2.5
Folic Acid	2.0
Biotin	1.0
Dextrin	97520.0

MINERAL MIXTURE (Harper, 1959)

	g/100g mixture
KH_2PO_4	34.31
$CaCO_3$	29.29
NaCl	25.06
$MgSO_4 \cdot 7H_2O$	9.98
$Fe(C_6H_5OH) \cdot 6H_2O$	0.623
$CaHPO_4 \cdot 2H_2O$	0.430
$MnSO_4 \cdot 4H_2O$	0.160
$CuSO_4 \cdot 5H_2O$	0.153
2nCl	0.020
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0025
KI	0.0005

All constituents were reagent grade and were ground to a very fine uniform powder and stored in airtight glass containers at $4^{o}C$.

Animals from each litter were allocated at random to the dietary regimes, those fed the 20% protein diet acted as "age matched" adequately protein nourished controls for the protein malnourished rats fed the 4% protein diet, while untreated weanlings acted as "weight matched" controls. Animals were marked by staining the fur on crown, back, belly or tail with picric acid. All blood sampling, tissue analyses, histology and liver perfusions were carried out on animals which had been fasted overnight (free access to water continued) for 14 to 16 hours in clean cages.

C Validation of nutritional status of the rat model

I The following parameters were determined for confirmation of a protein malnourished state:

i) Body weight: Animals were weighed to the nearest 0.5g twice weekly between 09.00 and 11.00 hours.

ii) Body length: Animals were measured under light ether anaesthesia at the beginning and end of the period on the experimental diet. (a) Total length (nose to tail-tip and (b) tail length (vent to tail-tip) were measured.

iii) Total serum protein was determined on serum obtained at the end of the experimental period by aortic puncture under light ether anaesthesia by the method of Lowry, Rosebrough, Farr and Randall (1951) (See Appendix B).

iv) Serum albumin was determined by the method of Doumas, Watson and Biggs (1971) (See Appendix B).

v) Liver fat content was measured by the decrease in weight of the dried liver after extraction with petroleum ether (b.p. 40°-60°C) (Hazlewood and Nichols, 1969) (See Appendix B).

vi) Liver histology was carried out on 10 µm frozen sections of tissue fixed in 10% buffered formalin and stained with

oil red O in iso-propanol (Lillie and Ashburn, 1943) and Mayer's Haemalum.

vii) Wet and dry weights of livers. Livers were weighed immediately after sacrifice and again after drying at 100°C for 24 hours and the wet:dry weight ratio calculated.

II Results

i) Body weight: Animals fed the 4% protein diet failed to gain weight during the experimental period while those fed the 20% protein diet gained weight steadily (see Fig. 4.1).

ii) Body length: There was no significant growth in total or tail length in the animals fed the 4% protein diet while those fed the 20% protein diet increased by over 50%. (See Table 4.3 and Fig. 4.2).

iii) Total serum protein and serum albumin were significantly lower in the 4% protein fed rats than those fed 20% protein or in weanlings (see Table 4.3).

iv) Liver lipid was greatest in the 4% protein fed rats and liver histology confirmed the characteristic periportal fatty infiltration only in these animals (see Fig. 4.3 and Table 4.3).

v) Liver wet:dry weight ratio was significantly greater in the 4% protein fed rats than those fed 20% protein or in weanlings (see Table 4.3).

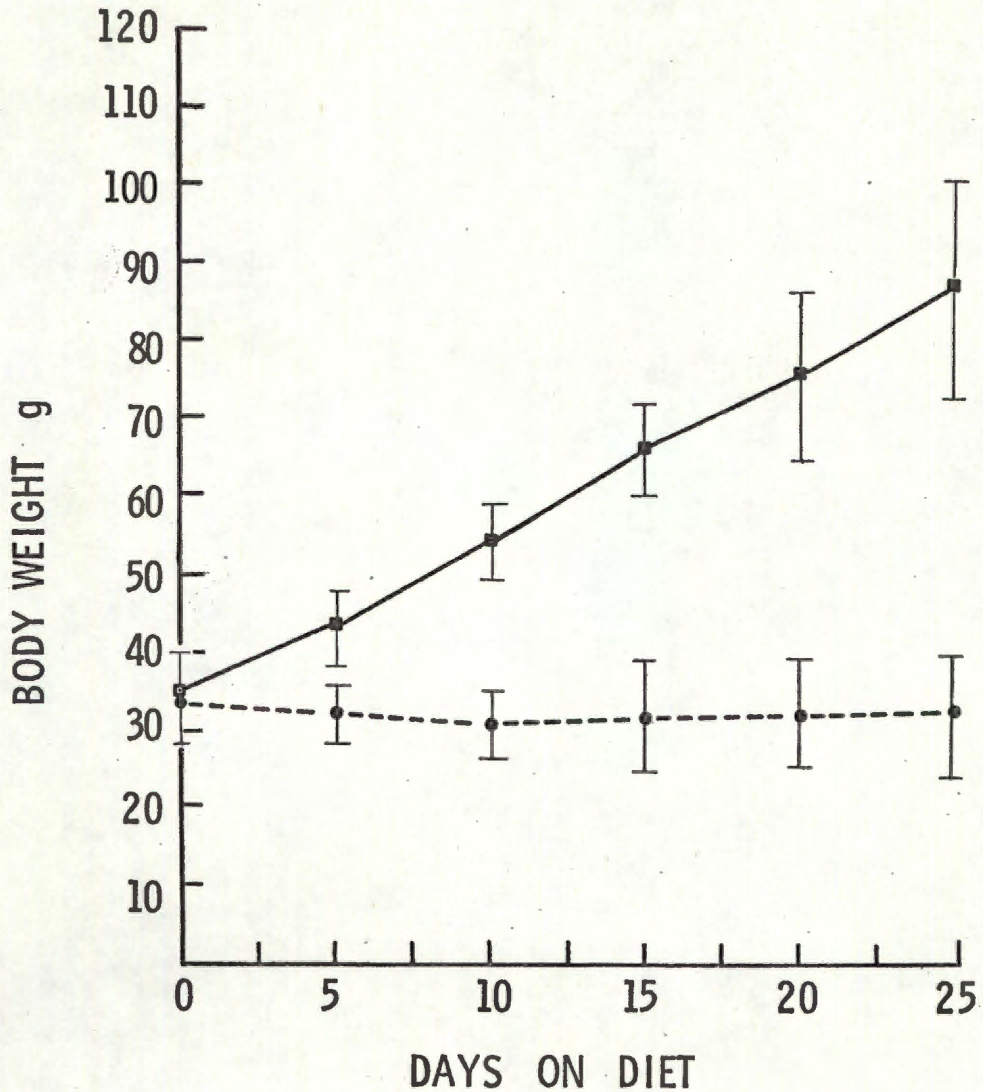


Fig. 4.1 The effect of 20% and 4% protein diets on weight gain

Rats on 20% protein diet (solid line) gain weight steadily while those on 4% protein diet (dashed line) fail to gain weight. Mean ± SD, n = 20.

TABLE 4.3

MORPHOLOGICAL AND BIOCHEMICAL FEATURES OF PROTEIN MALNOURISHED AND CONTROL RATS

	(a) 4% Protein Diet (n = 20)	(b) 20% Protein Diet (n = 20)	(c) Weanlings (n = 20)
Weight (g)	32.2 ± 7.9 *o	87.1 ± 14.1	33.7 ± 4.2
Total length (cm)	20.4 ± 1.3 *o	28.4 ± 2.3	18.9 ± 2.0
Tail length (cm)	9.5 ± 0.7 *o	13.8 ± 1.5	8.8 ± 1.1
Total serum protein (g/100 ml)	3.8 ± 0.2 *†	5.8 ± 0.2	5.4 ± 0.2
Serum albumin (g/100 ml)	2.1 ± 0.1 *†	3.4 ± 0.1	3.1 ± 0.1
Fatty liver (Oil Red O staining)	Positive	Negative	Negative
Extractable liver lipid (% Dry liver weight)	9.9 ± 0.9 *†	4.2 ± 0.5	3.4 ± 0.4
Liver wet:dry weight ratio	4.12 ± 0.12 *†	3.43 ± 0.16	3.51 ± 0.21

Mean and Standard Deviations

Student's t test: a vs b; a vs c

* $p < 0.001$ (a vs b)

† $p < 0.001$ (a vs c)

o Not significant

Fig. 4.2 General appearance of the three types of experimental animals. Animals fed a 4% protein diet for 25 days from weaning fail to grow. Yellow marks are picric acid stains to identify individual animals.

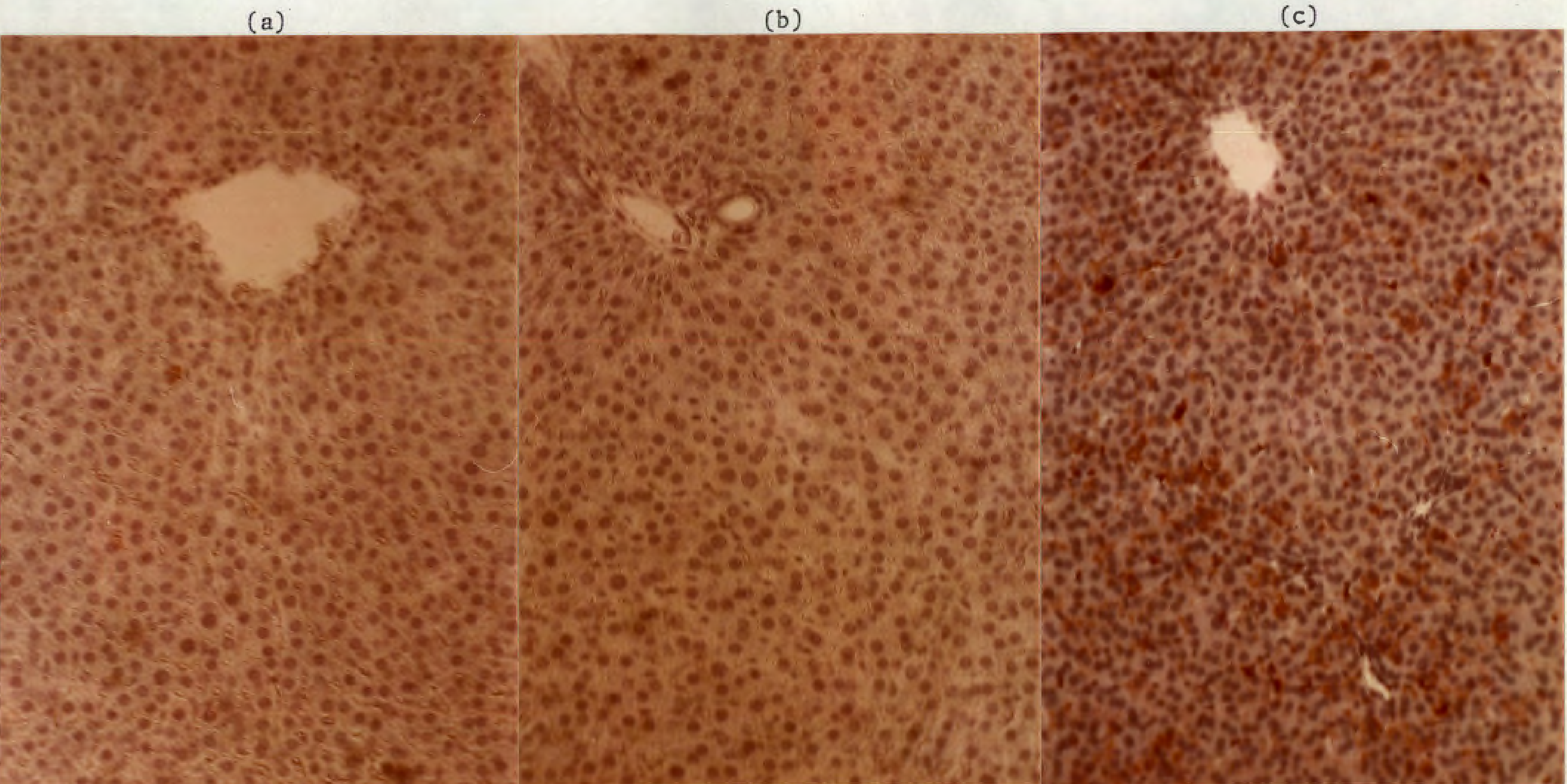


Fig. 4.3 Photomicrographs of frozen sections stained with oil red O from the livers of (a) Weanling rat (b) 20% protein fed rat (c) 4% protein fed rat. Only section (c) shows the characteristic red staining for fat.

TABLE 4.4

PARAMETERS OF THIS MODEL OF PROTEIN MALNUTRITION DETERMINED BY OTHERS AND TO WHICH REFERENCE IS LATER MADE

	(a) 4% Protein Diet	(b) 20% Protein Diet	(c) Weanlings
Total body water[1] (% body weight)	77.7 ± 1.1 (7) o∅	77.6 ± 0.7 (8)	75.6 ± 0.7 (5)
Liver glycogen[1] (mg/g liver weight)	19.7 ± 3.1 (6) *††	6.5 ± 2.3 (6)	2.2 ± 0.3 (5)
Fasting plasma insulin[1] (ng/ml)	0.3 (10) *†	1.0 (12)	0.8 (11)
Liver Protein[2] (mg/g wet liver weight)	162 ± 4 (10) **	193 ± 5 (11)	-
Liver DNA[2] (mg/g wet liver weight)	6.1 ± 0.2 (11) **††	5.0 ± 0.1 (13)	6.1 ± 0.2 (11)
Food intake[3] (Kcal/Kg $^{0.73}$/day)	166.81 ± 5.45 (6)	244.38 ± 16.05 (6)	

Means ± SEM, number of experiments in parenthesis

Student's t test	a vs b	a vs c
	** p < 0.001	†† p < 0.001
	* p < 0.01	† p < 0.05
	o not significant	∅ not significant

(1) Weinkove, Weinkove and Pimstone (1976)

(2) Sacks (1977)

(3) Stead and Brock (1972)

D Discussion and Conclusions

i) After 25 days of free access to the diet containing 4% protein the rats developed a syndrome characterised by many of the features of protein-energy malnutrition in humans. The features were: failure of weight gain and linear growth, hypoproteinaemia and albuminaemia and fatty infiltration of the liver. These findings were in keeping with others using this model (Stead and Brock, 1972; le Roith and Pimstone, 1973; Weinkove, Weinkove and Pimstone, 1976; Sacks, Pimstone, Waligora, Peires, Weinkove and Saunders, 1977) and is a reflection of its high degree of reproducibility.

ii) Oedema was not a feature of the syndrome and occult accumulation of water has been excluded by dilution studies with tritiated water (Weinkove, Weinkove and Pimstone, 1976). Oedema has also not been found in other rat models of malnutrition (Ramalingaswami and Deo, 1968; Widdowson and McCance, 1957). The short period of protein deprivation (25 days) may be a factor, in that after 15 weeks on a pure cassava diet (Schnieden, Hendrickse and Haigh, 1958) rats developed weight loss, a fall in serum albumin from 4.2 to 1.2 g/100 ml and a rise in total body water from 69.9 to 77.4%.

iii) Food intake was not measured but is known to be reduced in animals fed 4% protein diet, the intake is however sufficient to maintain constant body weight which excludes simple starvation (Stead and Brock, 1972). Equal food intake can only be achieved by highly unphysiological force feeding with resultant high mortality (Sidransky and Verney, 1970).

iv) In spite of the problems of producing pure protein deprivation (Weinkove, 1974; Kirsch, Saunders and Brock, 1968) and the difficulties of extrapolating any animal pathological states to humans, it was considered that the syndrome produced by feeding a

4% protein diet was one of protein malnutrition; not too dissimilar to that observed in humans, and warranted use in this study.

CHAPTER FIVE

IN VIVO $^{35}SO_4$ INCORPORATION INTO CARTILAGE AND CARTILAGE HISTOLOGY AND HISTOCHEMISTRY

"As thou knowest not what is the way of the spirit nor how the bones do grow.....". Ecclesiastes 11.5.

A Introduction

i) A failure of linear growth is a striking feature of protein energy malnutrition (Berridge and Prior, 1954; Trowell, Davies and Dean, 1954). The linear growth of long bones, which is the main contributor to height in man, occurs at the epiphyses (Davies and Coupland, 1967) and abnormal, narrow epiphyses have been noted in protein-energy malnutrition (Higginson, 1954).

ii) Much of the emphasis on the study of the skeleton in protein malnutrition has been directed towards calcium and bone metabolism rather than to that part of the skeleton especially relevant to growth, namely the epiphyseal cartilage (Higginson, 1954; Platt and Stewart, 1962; Deo, Sood and Ramalingaswami, 1965; Jha, Deo and Ramalingaswami, 1968; Adams and Berridge, 1969; Dickerson and John, 1969; le Roith and Pimstone, 1973). When cartilage has been studied it has frequently been that of the costochondral junction rather than that of the long bones and only in the case of Deo et al (1965) have special cartilage stains been used (PAS-Alcian blue).

iii) The incorporation of $^{35}SO_4$ into the mucopolysaccharides of cartilage reflects a functional balance between three factors: the intrinsic growth activity of the cartilage (and other tissues), its modification by humoral factors and the dilution of the radioactive tracer in the freely exchangeable sulphate pool of the body. Thus the in vivo incorporation of $^{35}SO_4$ into cartilage was chosen

as an overall functional indicator of cartilagenous activity in the state of protein malnutrition in the rat. It has been shown that in growing rats within 24 hours of an intraperitoneally administered dose of $Na_2{}^{35}SO_4$, most of the $^{35}SO_4$ present in cartilage is incorporated in the mucopolysaccharides of the matrix and very little is free (Dziewiatkowski, 1954; Dziewiatkowski, Di Ferrante, Bronner and Okinaka, 1957; Boström, 1952). The in vivo incorporation of $^{35}SO_4$ into cartilage matrix is influenced by changes in hormonal milieu, especially hypophysectomy (Dziewiatkowski, 1964).

iv) A detailed morphometric, histological and histochemical study of the epiphyses and costal cartilages was made to correlate functional activity, as reflected by $^{35}SO_4$ incorporation, epiphyseal plate width, histological parameters of epiphyseal activity and the nature of the matrix. Much of the previous work on cartilage in malnutrition concentrated on the costochondral junction and confirmation of the finding in long bone epiphyseal plates was required. The choice of the upper tibial epiphyseal plate for detailed study was because of its uniformity which in the rat has made this the site of choice for the study of epiphyseal growth, as in the bioassays of growth hormone (Evans, Simpson, Marx and Kibrick, 1943; Greenspan, Li, Simpson and Evans, 1949).

B Methodology

i) Administration of $^{35}SO_4$: Saline control (250 µl) or carrier free $Na_2{}^{35}SO_4$ (The Radiochemical Centre, Amersham, U.K.) was administered by intraperitoneal injection in doses of 0.1, 1.0, 2, 5, 10 µCi/g body weight to 5 animals of each type (20% protein fed, 4% protein fed and weanlings) in 250 µl of sterile saline. All animals were housed in mesh-bottomed stainless steel cages and all contaminated excreta collected and disposed of.

After 24 hours, animals were sacrificed by exsanguination under light ether anaesthesia.

ii) Serum sulphate was determined on 8 animals of each type by the method of Kleeman, Taborsky and Epstein (1956), in which the serum was deproteinized with uranylacetate, which simultaneously removed phosphate. The sulphate in the protein-free supernatant was precipitated as benzidine sulphate and the benzidine in the precipitate quantitated by photometric measurement after reaction with B Naphthoquinone 4 sulphonate. (For detailed methodology see Appendix B).

iii) Quantitative measurement of $^{35}SO_4$ incorporation into costal cartilage: The rib cages of the rats were dissected by the method of Herbai (1970) (Fig. 5.1) in which the whole rib cage was removed, dipped into boiling water and the ribs stripped of muscle and divided into lateral, middle and medial segments. The segments from left and right sides were pooled (except for the left seventh rib which was reserved for sectioning). After drying at 80°C for 12 hours the cartilage segments were weighed on a microtortion balance, dissolved in 500 µl 23M formic acid at 80°C for 1 hour and counted by liquid scintillation counting in 10 ml of Insta-gel (Packard, USA).

iv) Preparation of bony tissue for histology, histochemistry and autoradiography: The tibiae with intact knee and ankle joints were dissected free of most attached muscle. In the case of some animals the humerus with intact shoulder and elbow joints and the 7th rib including the costochondral junction were similarly dissected. All tissues were immediately placed in 10% formalin with 0.5% cetylpyridinium chloride (Engfeldt and Hjertquist, 1967) for 24 hour fixation. (This has been shown to elute the smallest amount

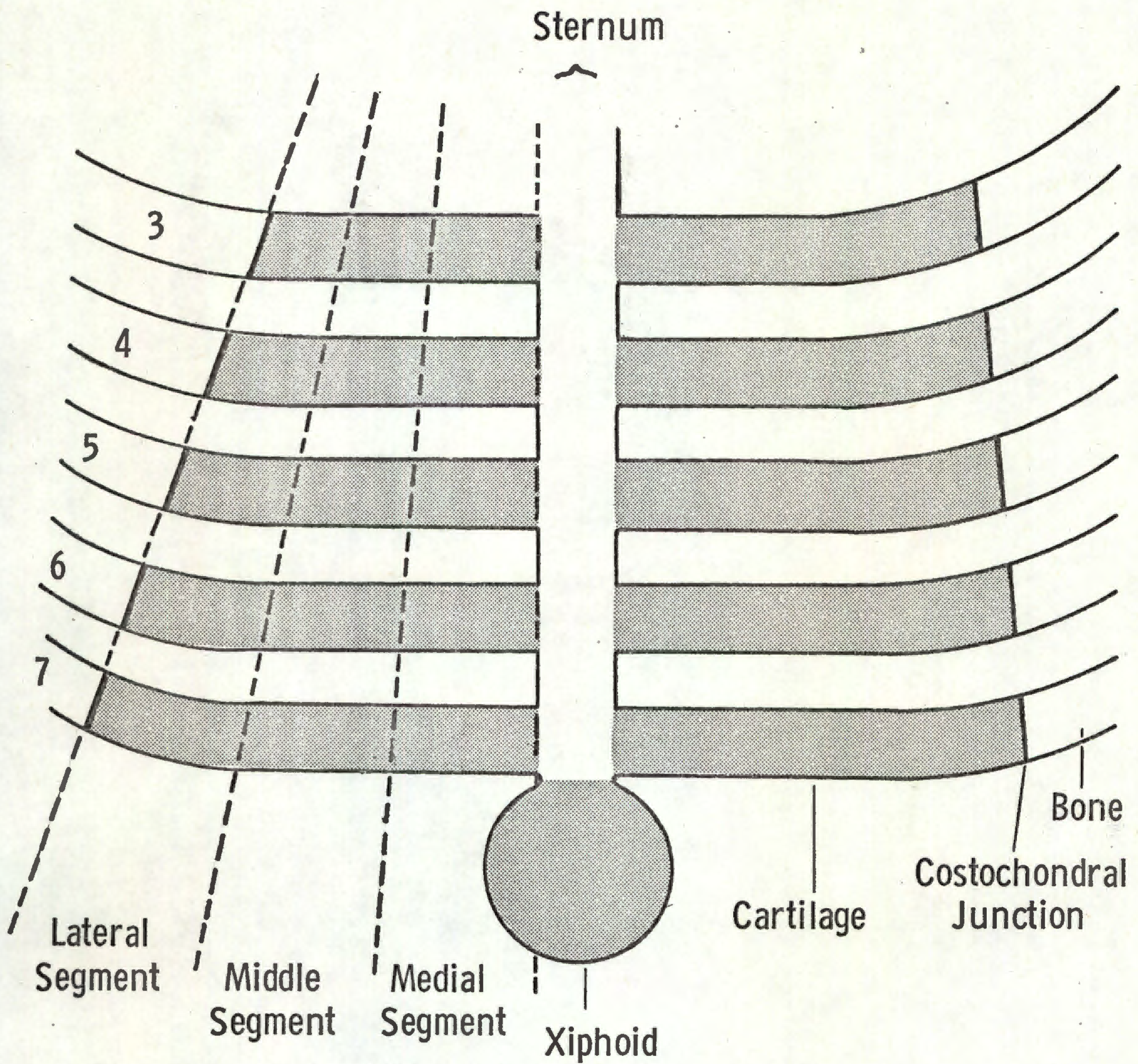


Fig. 5.1 Dissection of the rat anterior thoracic wall (based on that of Herbai (1970) for the mouse).

Tissue was decalcified in Gooding and Stewart's Medium (Culling, 1974) for 72 hours. The decalcified long bones were bisected (equal numbers in the sagittal and coronal planes), dehydrated in graded alcohols, cleared in xylol and embedded in paraffin wax. Multiple sections (where possible serial sections) 8 μm thick were cut with a Jung sliding microtome and dewaxed in xylol and graded alcohols.

v) Semiquantitative macroautoradiography (a modification of the method of Dziewiatkowski, 1951). Sections of long bones and ribs 8 μm thick were air dried for 24 hours after dewaxing and then placed in direct contact with the emulsion of Kodak Ortho Process Film 4154, thick, cut into strips 20 x 60 mm. The film was held in place by means of a clean microscopic slide and a rubber band at each end. After periods ranging from 5 to 40 days the film was developed for 5 minutes in Kodak D19 developer, washed for 30 seconds in 1% acetic acid and fixed in Amphix (Maybaker, Cape Town) diluted 1:4 for 20 minutes, rinsed in tap water for 30 minutes and distilled water for 1 minute, then dried and mounted, emulsion uppermost, on clean microscopic slides. All work with film while sensitive was carried out under low intensity light filtered with a Kodak No. 1 Safelight filter. After autoradiography, the tissue sections could be used for histological staining. The importance of unlabelled controls to exclude spurious images on the film due to direct chemical action has been stressed (Dziewiatkowski, 1951).

vi) Histological and histochemical staining (see Appendix A for detailed methodology): The following techniques were used: Mayer's haemalum and eosin for routine cellular morphology; the resorcin-crystal violet and picro-fuchsin method of Sweet (1968) stains chondroitin sulphate pale blue and keratosulphate pink;

Toluidine blue 0.25% in Michaelis' veronal acetate-HCl buffer for metachromasia (Culling, 1974), Alcian blue 8GX 1% in 0.1M HCl (pH 1.0) for sulphated mucopolysaccharides; Alcian blue 8GX 1% in 0.5M acetic acid (pH 2.5) for carboxyl and sulphate groups of mucopolysaccharides (Culling, 1974); Alcian blue 8GX 1% in 0.1M acetate buffer (pH 5.7) with $MgCl_2$ at 0.1, 0.7 and 1.0M according to the critical electrolyte concentration technique of Quintarelli, Scott and Dellovo (1964) for differentiating acid from sulphated mucopolysaccharides. Some sections were subjected to ovine testicular hyaluronidase digestion prior to staining with Alcian blue 8GX 1% in 0.1M HCl (Culling, 1974). All sections were dehydrated in graded alcohols, cleared in xylol and mounted in Depex (GT Gurr, U.K.).

vii) Measurement of tibial length and epiphyseal width: Tibial lengths were measured to the nearest mm with a transparent rule on stained mounted sections, from the centre of the tibial plateau to the upper articular surface of the ankle mortice. The width of the upper tibial epiphysis in its central half was measured at 5 equidistant points by an eyepiece micrometer on at least 5 sections from the tibia of each animal (see Fig. 5.2). All sections and autoradiographs were assigned a random number and examined and measured without the observer having any knowledge of which group of animals they came from.

C Results

i) Serum inorganic sulphate:

There was no significant difference in the sulphate content of the sera from the three groups of experimental animals. (See Table 5.1).

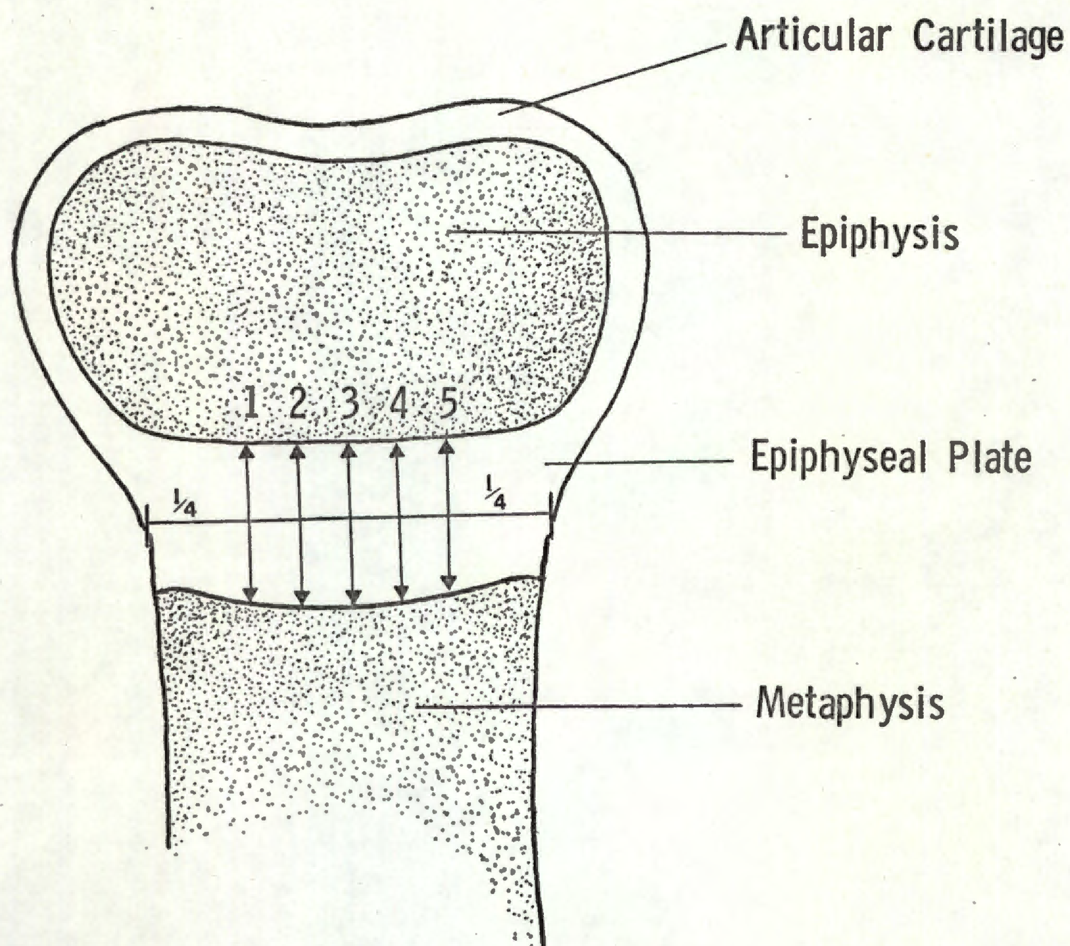


Fig. 5.2 Points of measurement of the tibial epiphyseal width. Width was measured by eyepiece micrometer at 5 equidistant points in the middle half of the epiphysis.

TABLE 5.1

SERUM SULPHATE LEVELS

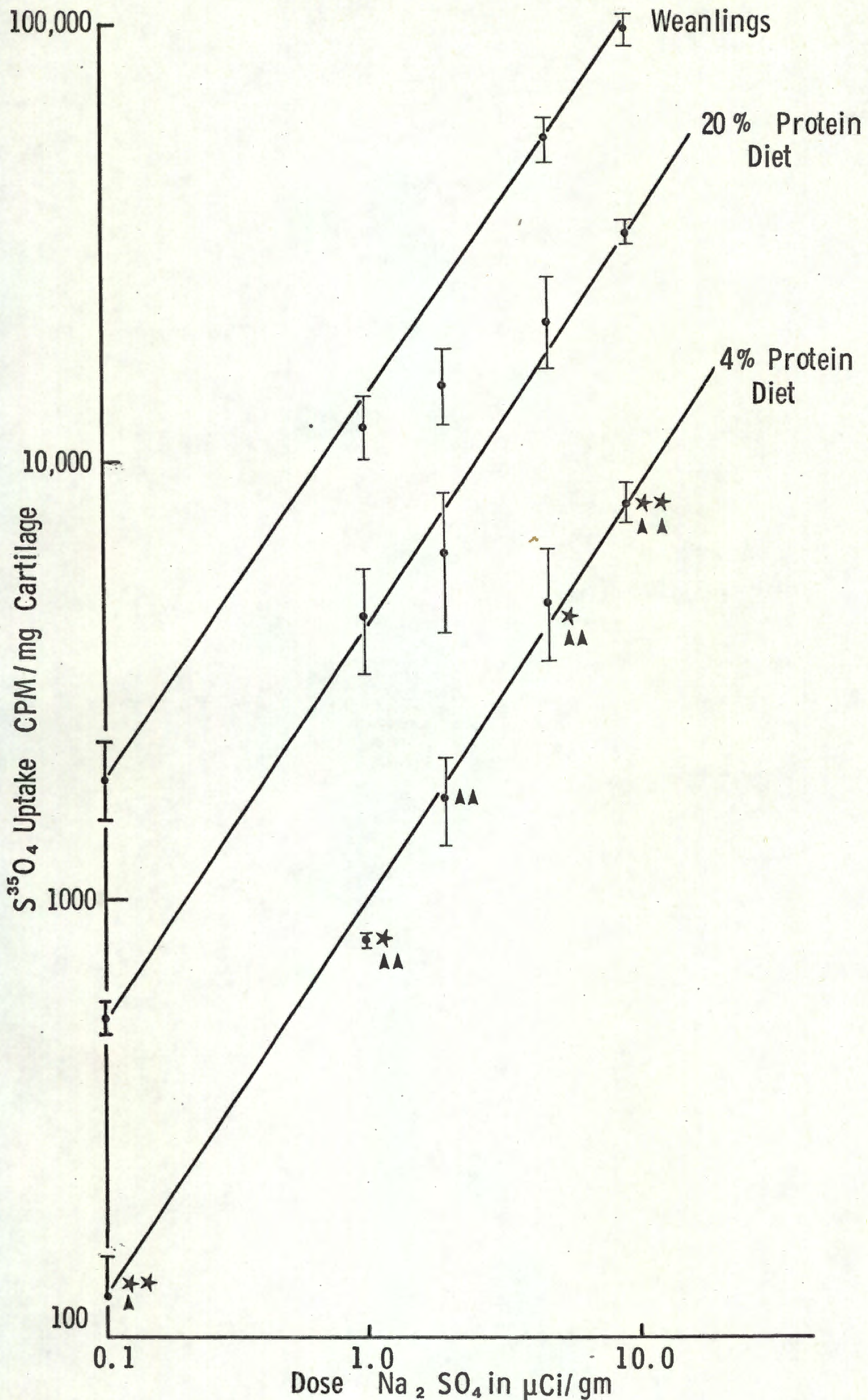
	Mean Serum Sulphate (µg/ml) ± SEM
4% Protein diet (n = 8)	63.0 ± 3.5
20% Protein diet (n = 8)	60.4 ± 2.7
Weanlings (n = 8)	62.7 ± 3.1

Unpaired Student's t test

$p > 0.5$ for all groups

ii) The incorporation of $^{35}SO_4$ into costal cartilage: The following features were demonstrated: Incorporation of $^{35}SO_4$ was lowest at all doses in the 4% protein fed rats, the 20% protein fed rats (age-matched, wellnourished controls) had an intermediate $^{35}SO_4$ incorporation, while the rapidly growing younger weanlings (weight-matched controls) had the greatest incorporation. These differences were significant (see Fig. 5.3).

iii) The regional distribution of $^{35}SO_4$ incorporation into costal cartilages: This confirms the findings of Herbai (1970). For all three groups of animals the highest incorporation occurred in the lateral segments which includes the costochondral junction and was lowest in the medial segment. The total incorporation per unit cartilage weight was slightly higher in the upper than the lower ribs (probably because the costochondral junction area made up a larger fraction of the total) (Table 5.3). The ratio between the lateral and middle and medial segments was significantly lowest in the 4% protein fed rats relative to age and weight-matched controls (see Tables 5.2 and 5.3).



100,000
10,000
1000
100
Weanlings
20 % Protein Diet
4% Protein Diet
$S^{35}O_4$ Uptake CPM/mg Cartilage
0.1
1.0
10.0
Dose Na_2SO_4 in µCi/gm

Fig. 5.3 Incorporation of $^{35}SO_4$ into costal cartilage in vivo of weanling, 20% protein fed and 4% protein fed rats, 24 hours after intraperitoneal injection of $Na_2\,^{35}SO_4$ 0.1 to 10 μCi/gm body weight.

Each bar represents mean ± SEM for 5 animals.

** $p < 0.001$ (4% vs 20%)

* $p < 0.05$ (4% vs 20%)

▲▲ $p < 0.001$ (4% vs Weanling)

▲ $p < 0.05$ (4% vs Weanling)

(Comparisons by Student's t test)

TABLE 5.2

INCORPORATION OF $^{35}SO_4$ INTO COSTAL CARTILAGE

AT DOSE 1 μCi/g BODY WEIGHT

(Similar results were obtained at other dose levels)

	Lateral	Middle	Medial	Ratio (Lateral: $\frac{\text{Middle + Medial}}{2}$)
	(Mean CPM/mg dry cartilage)			
4% Protein diet (n = 5)	1,130	674	643	1.716
20% Protein diet (n = 5)	7,620	3,840	3,010	2.225
Weanling (n = 5)	20,830	8,470	7,490	2.610

TABLE 5.3

INCORPORATION OF $^{35}SO_4$ INTO COSTAL CARTILAGE
AT DOSE 1 μCi/g BODY WEIGHT (n = 5)

(Similar results were obtained at other dose levels)

	Mean CPM/mg dry cartilage (whole cartilage)		
Rib No.	4% Protein Diet	20% Protein Diet	Weanling
3	1,180	5,840	16,270
4	934	5,430	14,380
5	814	4,550	12,140
6	762	3,810	11,720
7	701	3,210	11,010

iv) Semiquantitative macroautoradiography of long bone epiphyses and costochondral junctions: The incorporation of $^{35}SO_4$ into epiphyses of long bones as demonstrated by this method confirmed the quantitative findings in costal cartilages. There was a striking decrease of $^{35}SO_4$ in the sections of tissue from 4% protein fed rats, this applied especially to the epiphyseal but also to the articular cartilage, bone periosteum and joint capsule. In the case of costal cartilages the maximum incorporation was in cartilage adjacent to the costochondral junction in all groups of animals but again was least in the malnourished animals. Unlabelled control sections produced no spurious images on the film (see Fig. 5.4).

v) Micrometry of tibial epiphyseal width: The upper tibial epiphyses of the 4% protein fed rats were significantly narrower than age or weight-matched controls, both absolutely, or if expressed as μm/mm total tibial length (see Table 5.4).

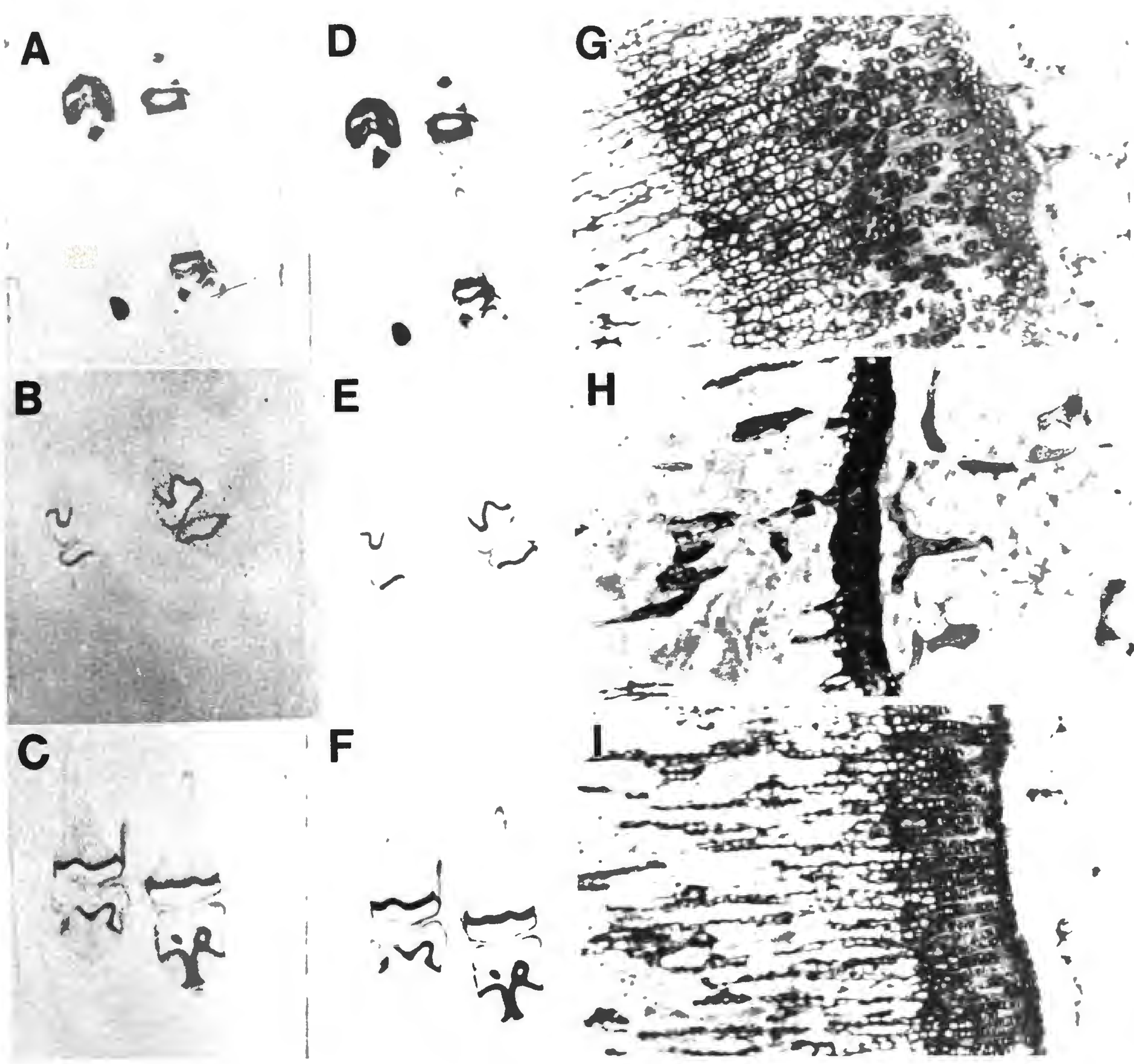

A
D
G
B
E
H
C
F
I

Fig. 5.4

Panels A-C	Autoradiographs of sections after administration of $Na_2{}^{35}SO_4$ (1 μCi/g body weight) 24 hours before sacrifice.
Panel A	Weanling (weight-matched control) marked uptake of $^{35}SO_4$ into epiphyses.
Panel B	4% Protein fed (protein deficient) poor uptake of $^{35}SO_4$ into epiphyses.
Panel C	20% Protein fed (age-matched control) good uptake of $^{35}SO_4$ into epiphyses.
Panels D-F	The identical sections from which the autoradiographs were made stained with Alcian Blue at pH 1.0.
Panels G-I	Photomicrographs of the upper tibial epipysis of the stained sections.
Panels D & G	Weanling (weight-matched control) with very wide active epiphysis.
Panels E & H	4% Protein fed (protein deficient) with inactive epiphysis.
Panels F & I	20% Protein fed (age-matched control) with active epiphysis.

TABLE 5.4

UPPER TIBIAL EPIPHYSEAL WIDTH

	4% Protein Diet	20% Protein Diet	Weanlings
Tibial epiphyseal width (μm)	202 ± 46	367 ± 52	578 ± 40
Ratio of epiphyseal width to tibial length (μm/mm)	9.4 ± 1.9	14.2 ± 2.4	28.2 ± 2.3

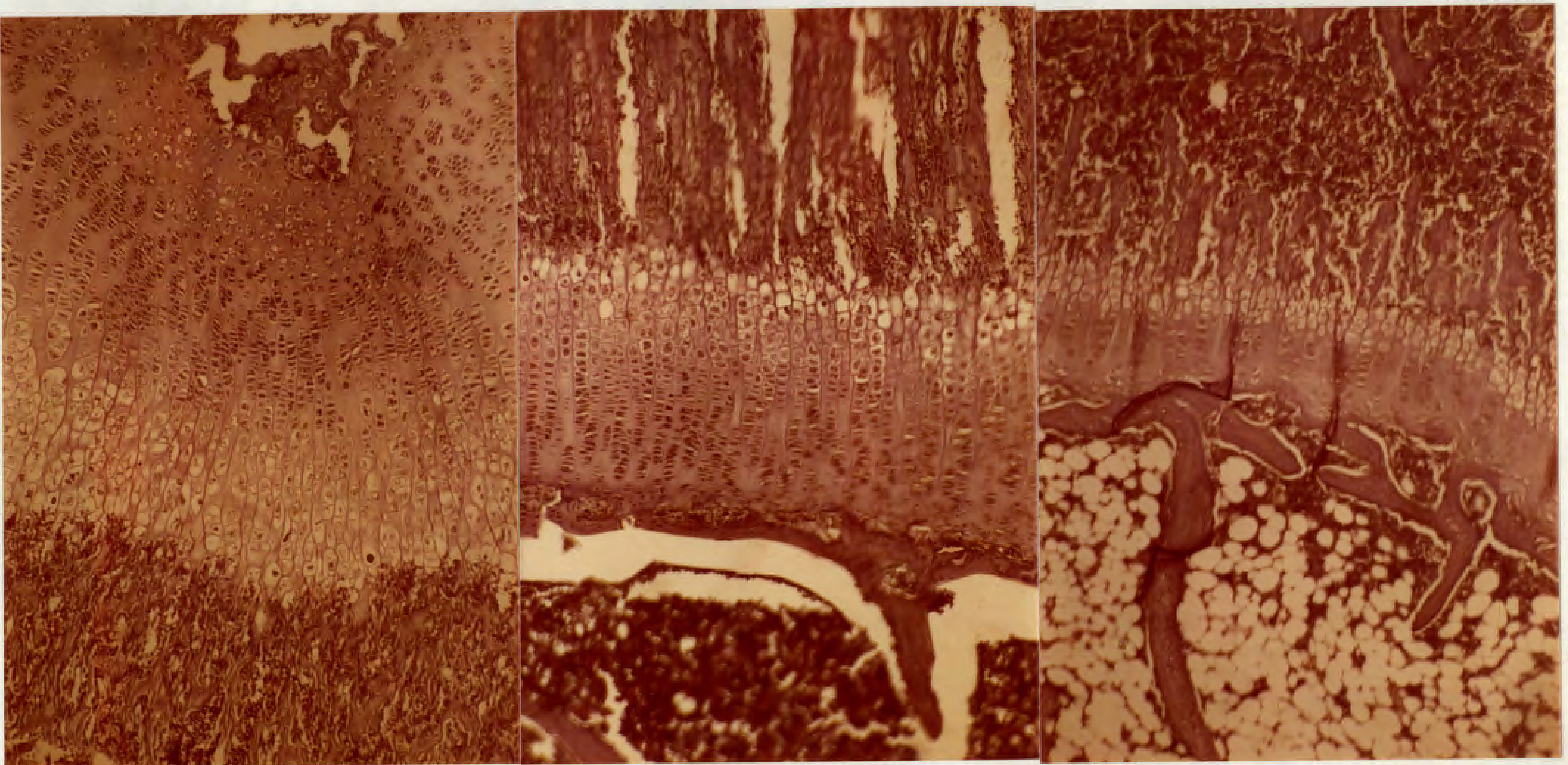
(Mean ± SEM) (n = 20)

Similar results were found for lower tibial, lower femoral, upper humeral and lower humeral epiphyses but the variance of micrometric measurements were far greater, confirming the uniformity of the upper tibial epiphyses as an area for study.

vi) Histological features: The narrowing of the epiphyseal plate in the 4% protein fed animals was due to a diminution in the thickness of all zones, resting, proliferating, maturing and degenerating cartilage. The proliferating zone was most severely affected. The osteoid tissue and osteoblasts were atrophic. The cartilage cells were small, flattened with frequent pyknotic nuclei. The metaphyseal trabeculae were narrow and poorly branched. There was poor vascular invasion of the metaphyseal-epiphyseal junction (see Fig. 5.5).

vii) Histochemistry of cartilage matrix: There was no significant difference in the staining properties of the matrix of the cartilage from the three groups of animals, when studied by the methods detailed above suggesting that the acid and sulphated mucopolysaccharides are qualitatively similar but quantitatively less in the narrow epiphyses of the 4% protein fed rats (see Figs. 5.5 to 5.9).

Fig. 5.5 Sections of tibial epiphyses stained with Mayer's haemalum and eosin



(a) Weanling (weight-matched control) (b) 20% Protein fed (age-matched control) (c) 4% Protein fed.

Fig. 5.6 Sections of tibial epiphyses stained with Sweet's resorcin-crystal violet and picro-fuchsin method.

(a) Weanling (weight-matched control)

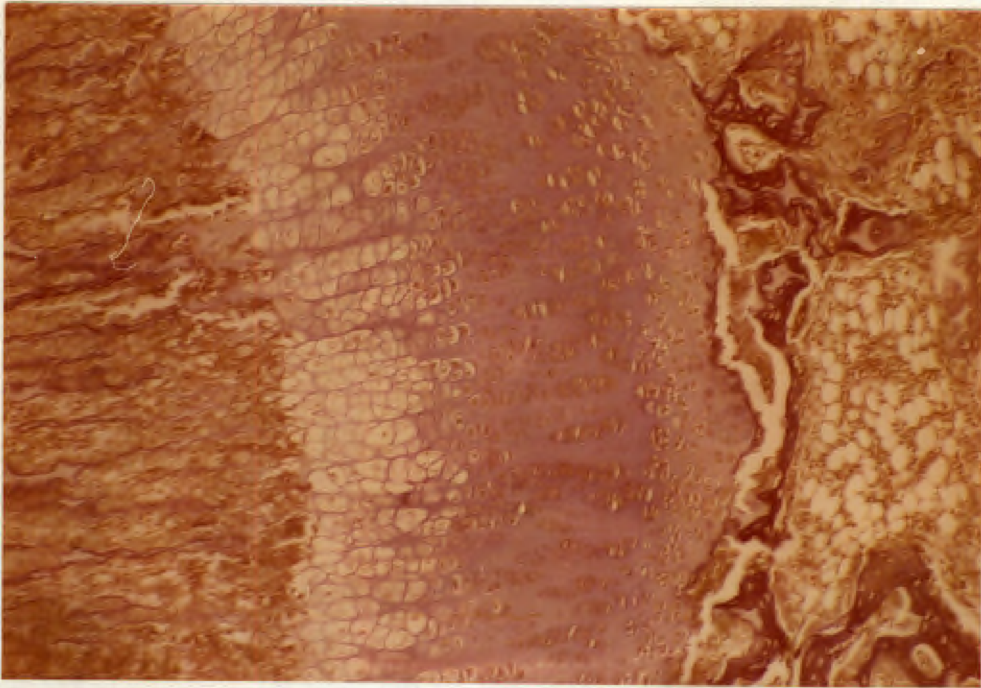
(b) Epiphysis of 20% protein fed (age-matched control)

(c) Articular cartilage from same 20% protein fed rat tibial section to show contrast in staining of epiphyseal and articular cartilage

(d) 4% Protein fed.

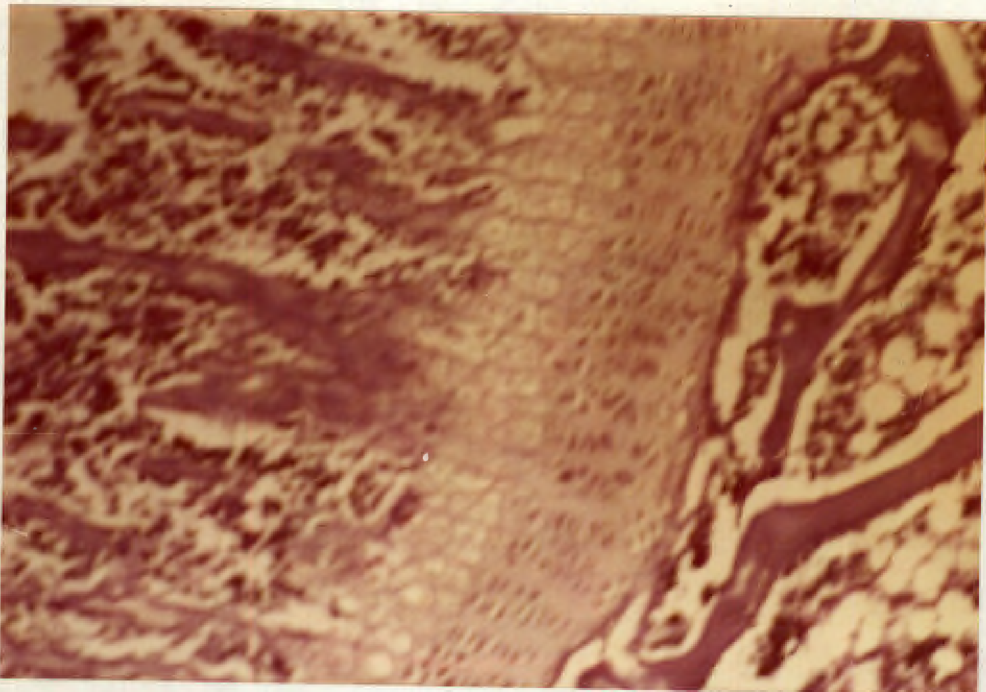
Fig. 5.6 Sweet's stain

(a)



Weanling (weight-matched control)

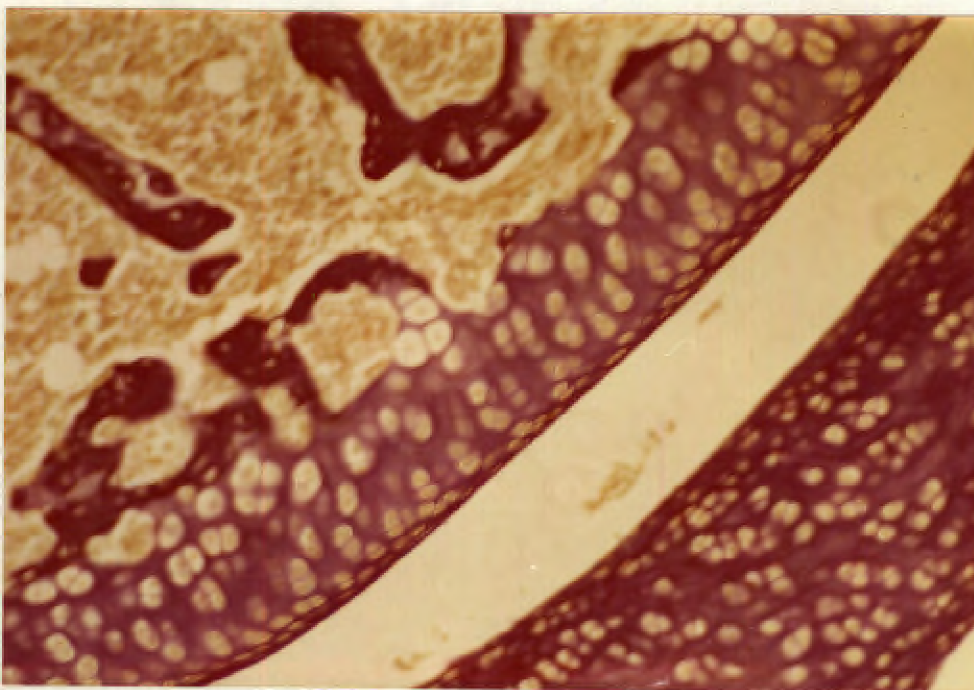
(b)



Epiphysis of 20% protein fed (age-matched control)

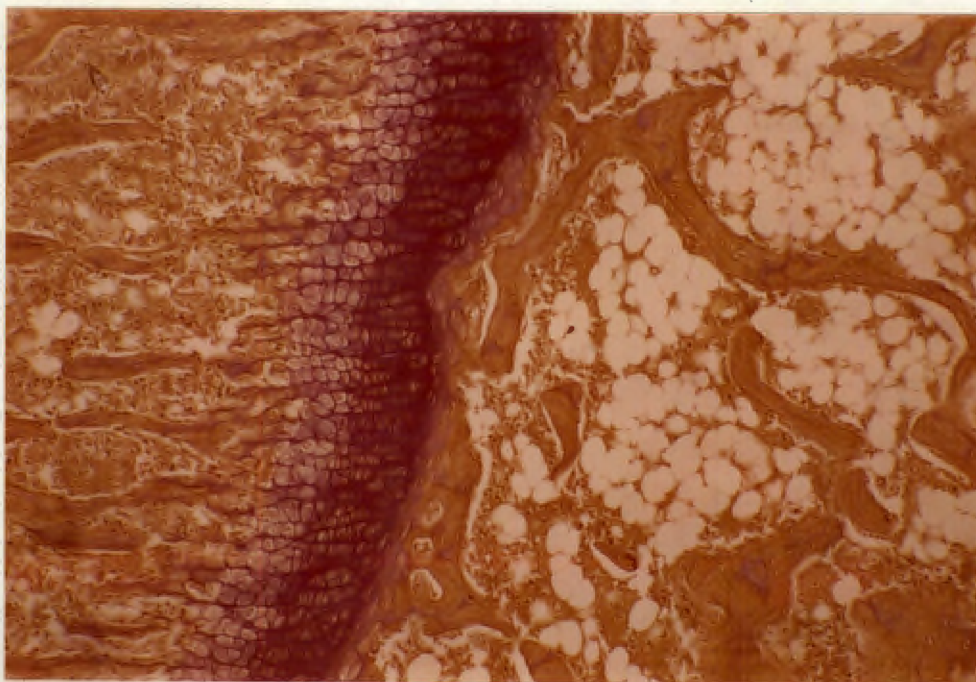
Fig. 5.6 Sweet's stain (continued)

(c)



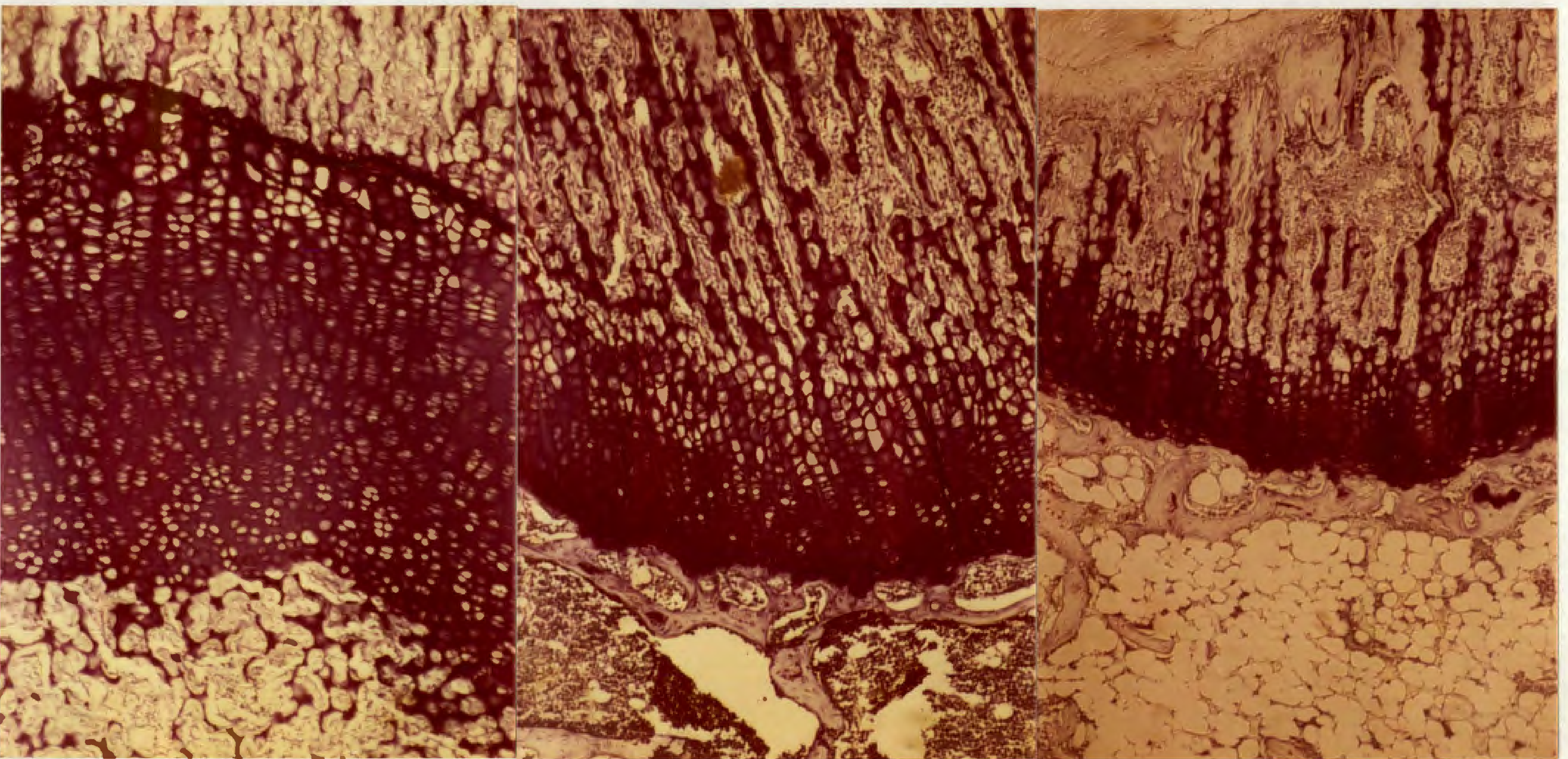
Articular cartilage from same 20% protein fed rat tibial section to show contract in staining of epiphyseal and articular cartilage

(d)



4% Protein fed

Fig. 5.7 Metachromatic staining with toluidine blue of sections of tibial epiphyses



(a) Weanling (weight-matched control) (b) 20% Protein fed (age-matched control) (c) 4% Protein fed

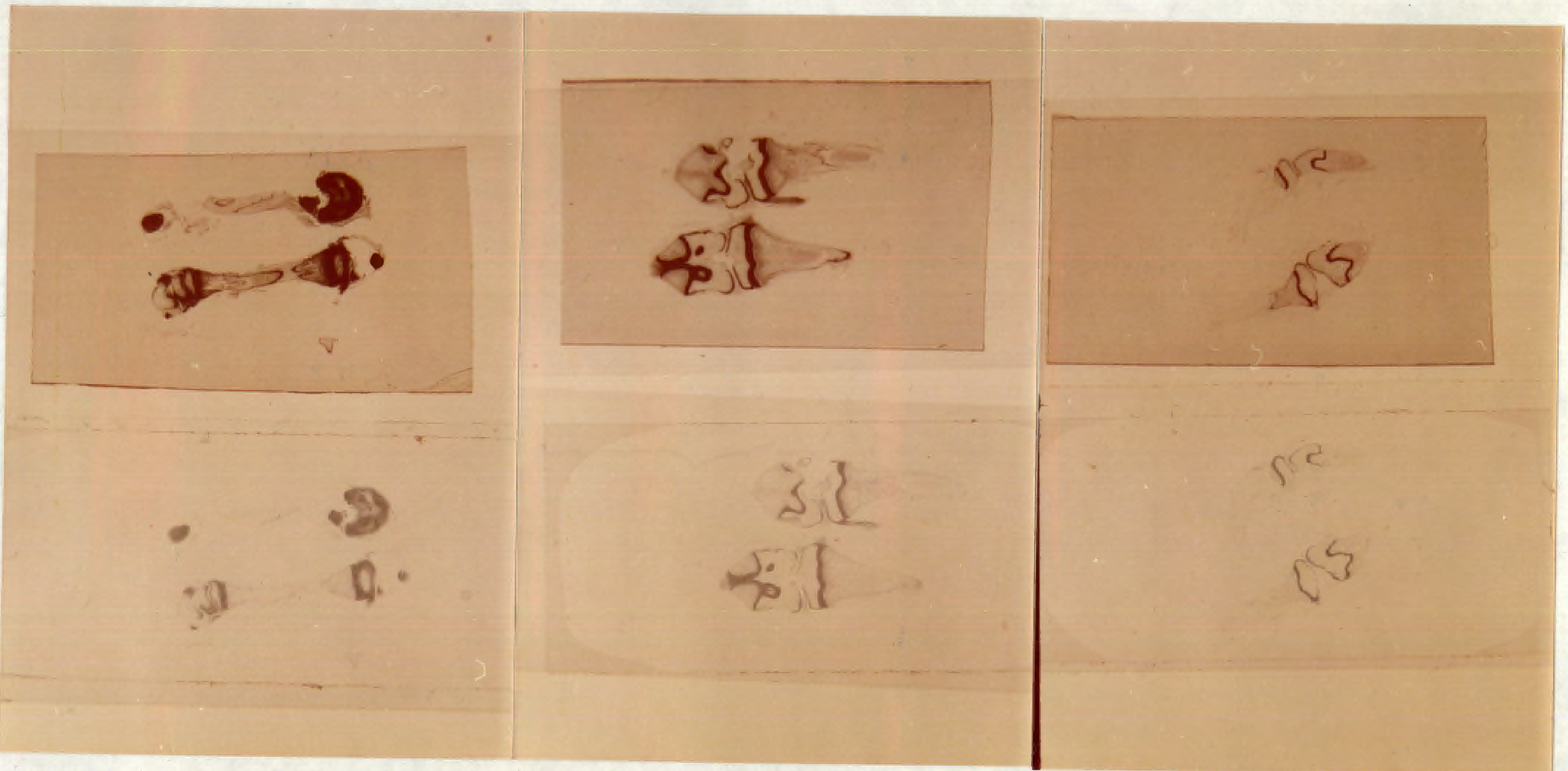
Fig. 5.8 Autoradiographs of sections of tibial epiphyses from rats administered $Na_2\,^{35}SO_4$ 1.0 μCi/g body weight 24 hours before sacrifice. Sections were subsequently stained with Alcian blue 8GX at pH 1.0 for sulphated mucopolysaccharides. Note the concordance between the labelling with $^{35}SO_4$ and the staining with Alcian blue 8GX.

(a) Weanling (weight-matched control)

(b) 20% Protein fed (age-matched control)

(c) 4% Protein fed.

Fig. 5.8 Autoradiographs/Alcian blue 8GX (ph 1.0)



(a) Weanling (weight-matched control) (b) 20% Protein fed (age-matched control) (c) Protein fed

Fig. 5.9 Section of tibial epiphyses stained by the critical electrolyte technique using Alcian blue 8GX at pH 5.7 with $MgCl_2$ at 0.1 and 1.0M.

(a) Weanling (weight-matched control) at 0.1M $MgCl_2$

(b) Weanling (weight-matched control) at 1.0M MgC12

(c) 20% Protein fed (age-matched control) at 0.1M $MgCl_2$

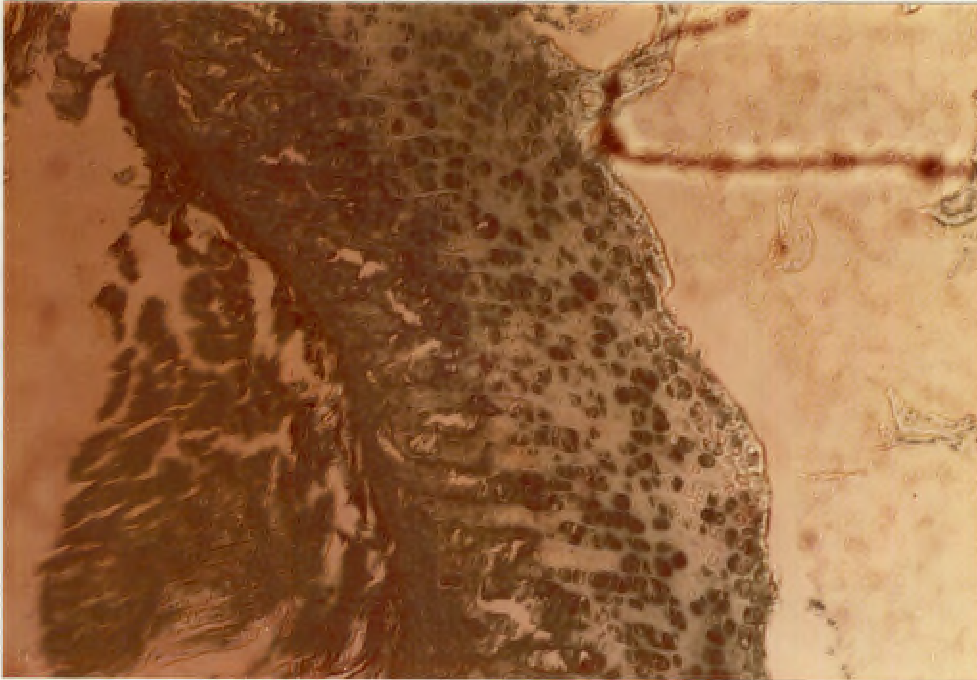
(d) 20% Protein fed (age-matched control) at 1.0M MgC12

(e) 4% Protein fed at 0.1M $MgCl_2$

(f) 4% Protein fed at 1.0M $MgCl_2$.

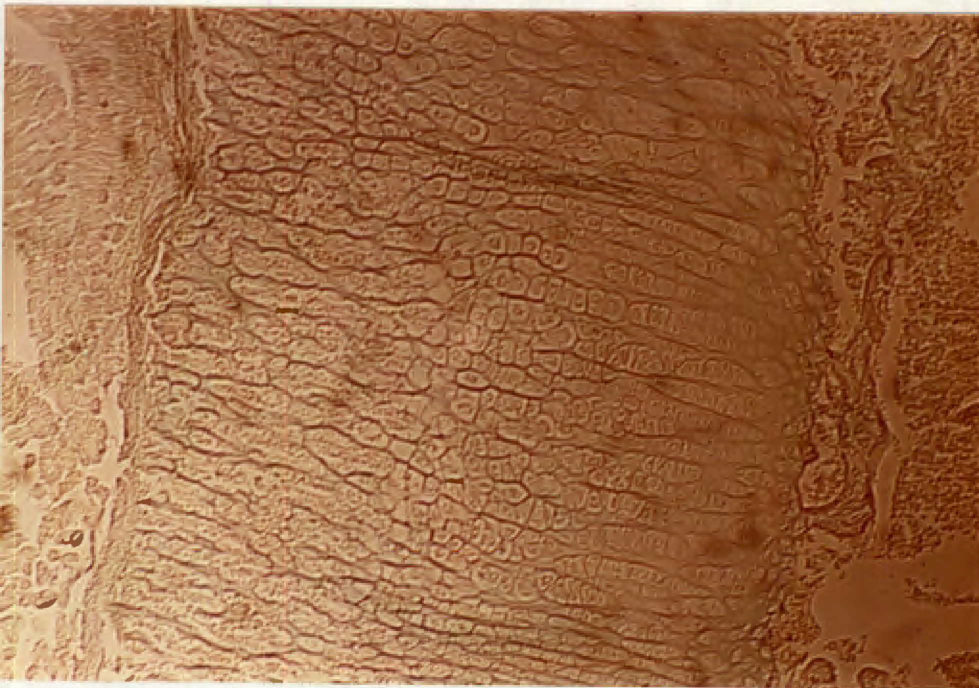
Fig. 5.9 Critical electrolyte technique

(a)



Weanling (weight-matched control) at 0.1M $MgCl_2$

(b)



Weanling (weight-matched control) at 1.0M $MgCl_2$

Fig. 5.9 Critical electrolyte technique (continued)

(c)

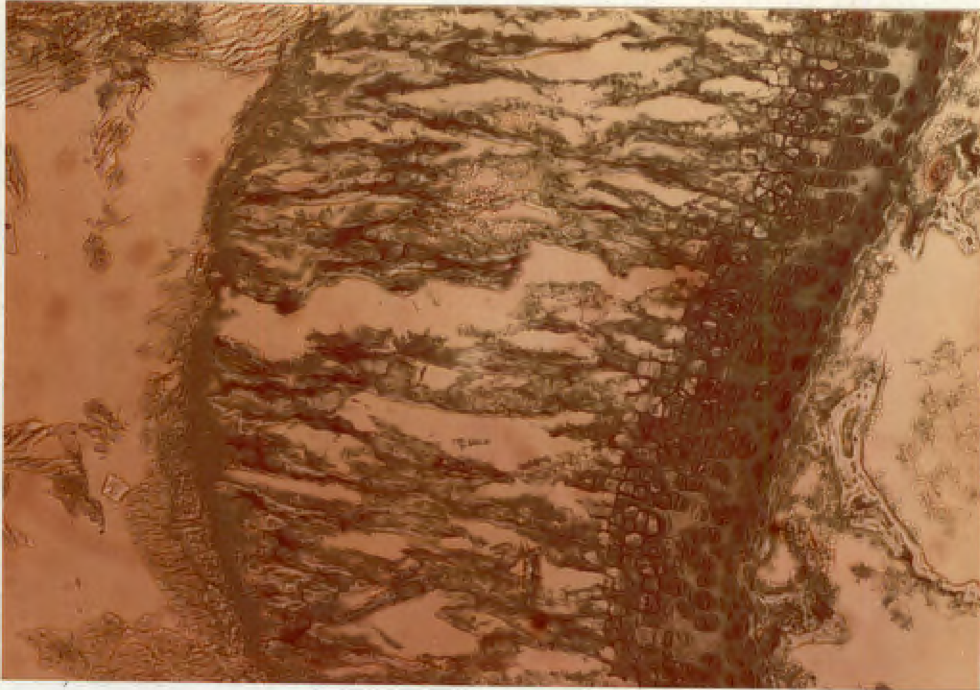
20% Protein fed (age-matched control) at 0.1M $MgCl_2$

(d)

20% Protein fed (age-matched control) at 1.0M $MgCl_2$

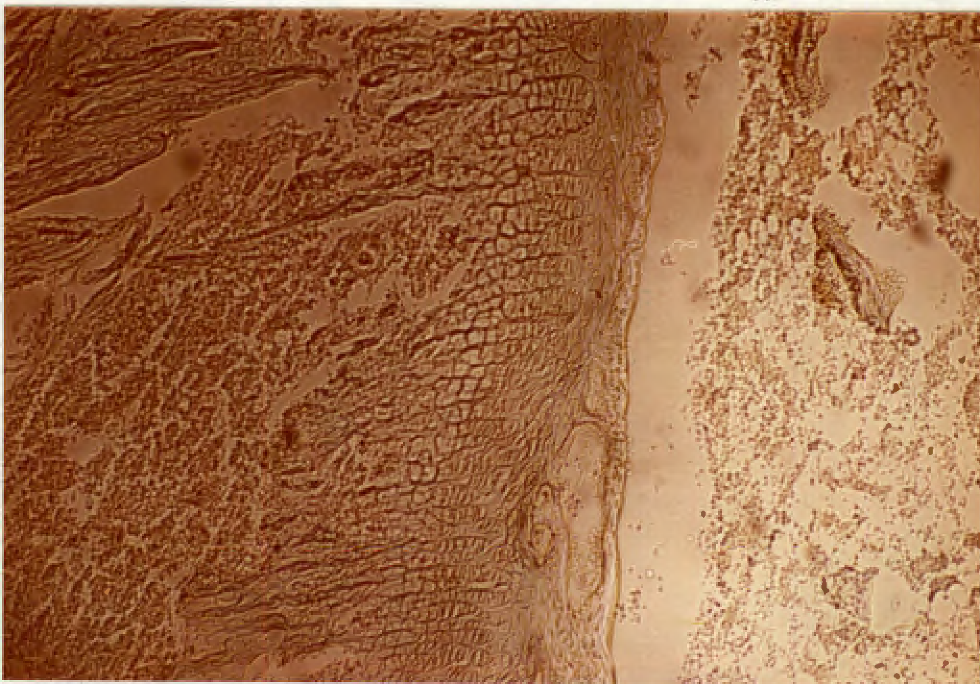
Fig. 5.9 Critical electrolyte technique (continued)

(e)



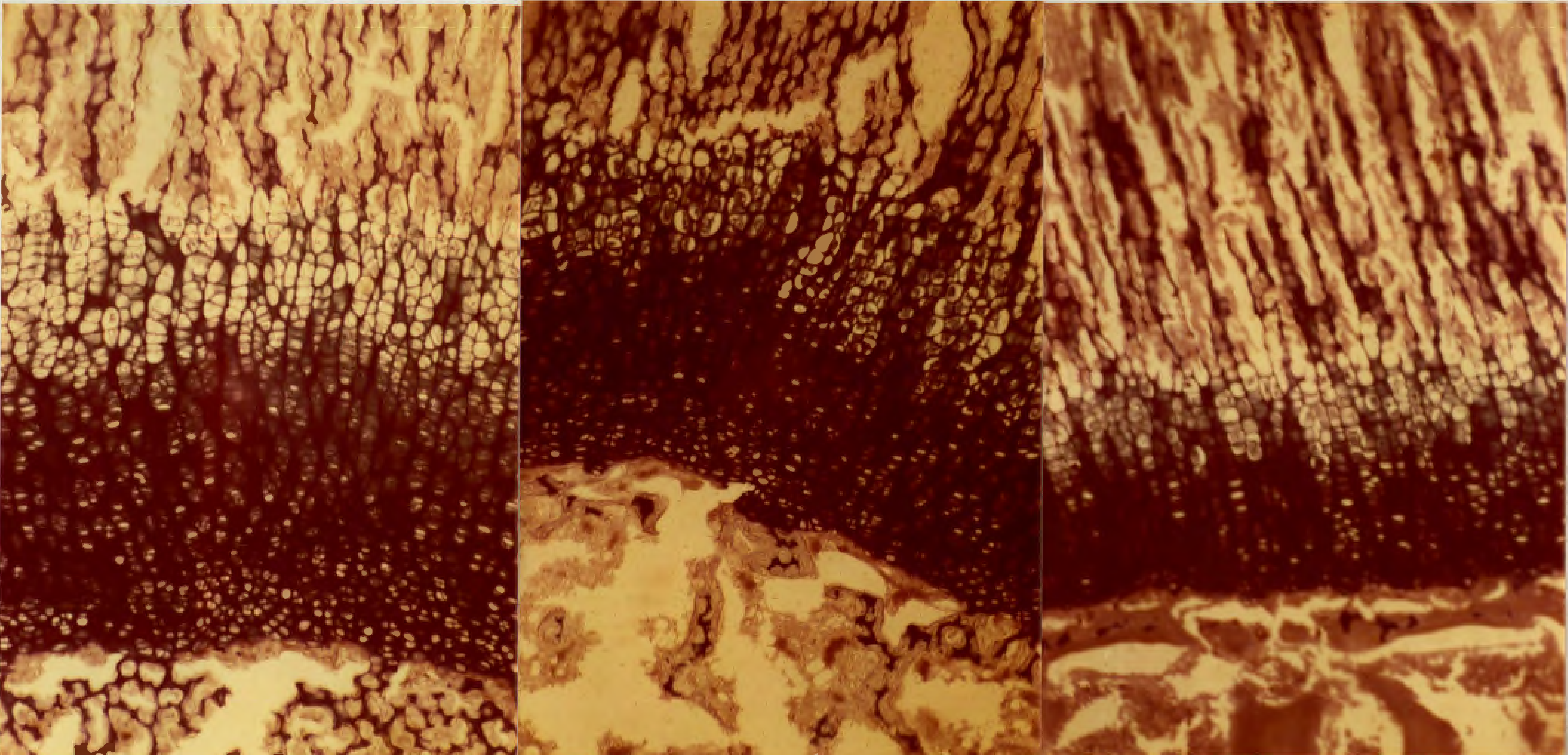
4% Protein fed at 0.1M $MgCl_2$

(f)



4% Protein fed at 1.0M $MgCl_2$

Fig.5.10 Alcian blue 8GX (pH2.5)



(a) Weanling (weight-matched control)

(b) 20% Protein fed (age-matched control)

(c) 4% Protein fed

D Conclusions

i) The similar serum sulphate contents of the 3 groups of animals and the fact that the malnourished animals have been shown to have the same total body water content as controls (Weinkove, Weinkove and Pimstone, 1976) suggests that when the same dose of $Na_2{}^{35}SO_4$ is administered per gram body weight, the cartilage in the different groups of animals is able to compete equally for the radioactive label. Differences in $^{35}SO_4$ uptake under these conditions would be determined by intrinsic cartilage avidity and humoral factors which may influence this. The use of costal cartilage for quantitative work was necessitated by the difficulty in dissecting out epiphyseal growth plates free of bony contamination in an animal as small as the rat. The autoradiographic and histological findings however support the premise that costal and epiphyseal cartilage share many features.

ii) The narrow epiphyseal plates confirmed the work in human malnutrition and the radiographic findings in this animal model (le Roith and Pimstone, 1973). The histological findings in general confirmed the narrowing, inactivity and cellular changes previously described. They also showed no significant differences in the staining reactions but a decrease in the amount of the mucopolysaccharides in the narrowed epiphyses of the malnourished animals. The oedema and hyalinisation described by Silberberg and Silberberg (1940) in female guinea pigs subjected to overall food restriction were not observed.

iii) The growth, histology and $^{35}SO_4$ incorporation in vivo of cartilage are subject to hormonal influences. The histological features (Freud, Levie and Kroon, 1939; Ingalls and Hayes, 1941;

Ray, Evans and Becks, 1941) and autoradiographic features (Dziewiatkowski, 1964) of epiphyseal cartilage in hypophysectomised rats are not dissimilar to the findings in 4% protein fed rats and thus the study was extended to include some of the hormonal influences on cartilage and the $^{35}SO4$ incorporation by cartilage in vitro in the absence of hormonal influences.

CHAPTER SIX

SERUM SOMATOMEDIN ACTIVITY (SULPHATION FACTOR) IN EXPERIMENTAL PROTEIN MALNUTRITION IN THE RAT

"Blood is a very special juice" *Johann Wolfgang von Goethe*

A Introduction

i) The growth failure and abnormal skeletal and cartilage morphology discussed in Chapters 4 and 5 have long suggested an abnormality of growth hormone in protein energy malnutrition. Bioassay of growth hormone in the pituitary glands of protein deprived or starved rats shows reduction; this has been interpreted as being due to diminished production (Srebnik and Nelson, 1962) or increased secretion (Friedman and Reichlin, 1965) and popularised the use of the term pseudohypophysectomy, first used to describe the pituitary atrophy produced by malnutrition in animals (Mulinos and Pomerantz, 1940). Mönckeberg, Donoso, Oxman, Pak and Meneghello (1963) have claimed that malnourished children failed to respond to refeeding without simultaneous growth hormone administration, suggesting a deficiency of growth hormone. However the general experience is that providing refeeding supplies adequate protein and energy, catchup growth occurs (Ashworth, Bell, James and Waterlow, 1968) and that in untreated cases exogenous growth hormone is without effect (Hadden and Rutishauser, 1967). The similarity of metabolic responses in starvation and after growth hormone administration (Russell, 1957) and the abnormal glucose tolerance in protein deprived animals (Heard and Turner, 1967) led these investigators to predict that growth hormone might be elevated in these circumstances.

ii) The accurate measurement of circulating growth hormone in man was made possible by radioimmunoassay and in children with

kwashiorkor and marasmic kwashiorkor, growth hormone was found to be elevated (Pimstone, Wittmann, Hansen and Murray, 1966; Pimstone, Barbezat, Hansen and Murray, 1967; Hadden, 1967; Graham, Cordano, Blizzard and Cheek, 1969; Milner, 1971a). In general, the elevation of growth hormone in marasmus is lower than that occuring in kwashiorkor (Pimstone, Barbezat, Hansen and Murray, 1968). That elevated growth hormone resulted from a hypersecretion rather than reduced clearance was proven by the administration of somatostatin which directly inhibits pituitary growth hormone secretion. The half life of growth hormone disappearance after somatostatin in protein energy malnutrition is similar to that of exogenously administered growth hormone in normal subjects (Pimstone, Becker, and Kronheim, 1975). In addition to being elevated, growth hormone is not suppressed by oral glucose (Pimstone, Barbezat, Hansen and Murray, 1967) or by carbohydrate feeding (Pimstone, Barbezat, Hansen and Murray, 1968). Oral mixed amino acids (Milner, 1971b) and milk protein feeding (Becker, Pimstone, Hansen and Hendricks, 1971) rapidly drop the elevated growth hormone. The level of growth hormone elevation in protein energy malnutrition correlates best with the degree of protein deficiency, as reflected by the reduction in serum albumin. Below a plasma albumin of 25 to 30 g/l growth hormone tends to be elevated (Lunn, Whitehead, Hay and Barker, 1973). In spite of the inverse correlation between growth hormone and albumin and certain amino acids (especially alanine) in untreated protein energy malnutrition (Pimstone, Becker and Hansen, 1972), the growth hormone elevation does not appear to be causally related to the reduced albumin or amino acids in that intravenous infusions of albumin (Becker, Pimstone, Hansen and Hendricks, 1971), mixed amino acids (Becker, Pimstone and Hansen, 1975) or alanine (Becker, Pimstone, Kronheim and Weinkove, 1975)

failed to reduce the elevated growth hormone. Thus the hypoalbuminaemia is a reflection of the the severity of protein deficit which results in elevation of growth hormone by an as yet unexplained mechanism. That energy intake is not causally related is suggested by the rapid return of growth hormone to normal (with normal glucose suppressibility) after protein rather than carbohydrate feeding (Pimstone, Barbezat, Hansen and Murray, 1968; Becker, Pimstone, Hansen and Hendricks, 1971).

iii) The apparent paradox of grossly elevated growth hormone in a condition associated with growth failure and narrow inactive growth cartilages was resolved by the observation that bioassayable somatomedin (the putative mediator of growth hormone on skeletal tissue) was greatly reduced in protein-energy malnutrition, returning progressively towards normal on refeeding as the growth hormone fell and serum albumin rose (Grant, Hambley, Becker and Pimstone, 1973; Van den Brande and Du Caju, 1973; Hintz, Suskind, Amatayakul and Olsen, 1976). Thus impaired feedback of somatomedin may result in elevation of growth hormone, as has been suggested by Laron, Pertzelan, Karp, Kowadlo-Silbergeld and Daughaday (1971) in a form of familial dwarfism characterised by elevated growth hormone and reduced somatomedin.

Not only was the bioassayable somatomedin reduced but frequently diluted in a dose response non-parallel to standard pooled normal serum (Grant, Hambley, Becker and Pimstone, 1973; Van den Brande and Du Caju, 1973). In the studies by Grant et al (1973) with an embryonic chick cartilage assay, mixing experiments failed to demonstrate an inhibitor, while Van den Brande and Du Caju (1973) found a heat labile inhibitor. With refeeding, increasing serum albumin and normalisation of growth hormone, the non-parallel somatomedin dose response returned to normal. Non-parallel dose

responses have been reported in the very different nutritional condition of acute starvation in the rat which resulted in the development of an inhibitor which was not dialysable, antagonised the action of sulphation factor activity from normal fed rat serum, inhibited basal $^{35}SO_4$ incorporation into cartilage in vitro and was inactivated by trypsin (Salmon, 1975).

iv) At the time this study was initiated no data on somatomedin activity in protein malnourished experimental animals was available, in spite of the advantages, both practical and ethical, of animal models of protein-energy malnutrition outlined in Chapter 4. The obvious growth failure and abnormal epiphyseal morphology, histochemistry and in vivo $^{35}SO_4$ incorporation strongly suggested that an abnormality of somatomedin activity was present in the Stead and Brock (1972) model.

B <u>Methodology</u>

i) Protein malnourished 4% protein fed rats, their well-nourished, aged-matched 20% protein fed controls, and wellnourished, weight-matched controls (weanlings) were prepared as outlined in Chapter 4.

ii) Blood samples were obtained by aortic puncture under light ether anaesthesia, allowed to clot at 4°C and the serum separated by centrifugation (3000g for 10 min at 4°C) and stored at -20°C until assayed.

iii) Pooled serum from 60 rats fed a 20% protein diet, with a nominal potency ratio of 1.0 U/ml served as a reference standard.

iv) Somatomedin activity in the serum was measured by a porcine costal cartilage bioassay modified from that of Van den Brande and Cu Caju (1974) as described in Chapter 3. Assays were performed at

four different dilutions of serum (5, 10, 20 and 40%). Potency ratios and parallelism were calculated using the computer programme of Phillips and Herington (1975).

v) Mixing experiments based on those of Phillips and Young (1976a) were performed; standard pooled rat serum was mixed in a 1:1 ratio with pooled serum from 4 and 20% protein-fed rats or weanlings, and the stimulation of $^{35}SO_4$ incorporation into porcine cartilage discs determined at 10 and 40% dilutions. The stimulation of the standard, 4 and 20% protein-fed and weanling serum alone at the same concentrations was measured in the same assay.

vi) The concentration of rat growth hormone was determined by radioimmunoassay (Birge, Peake, Mariz and Daughaday, 1967) with NIAMDD anti-rat GH serum 3, ^{125}I-labelled (by the Chloramine-T method), NIAMDD rat GH 122 as tracer, NIAMDD rat GH RP1 as standard and a second antibody separation (rabbit antimonkey α globulin produced in our laboratory). The assay was performed in strict adherence to the method recommended by the NIAMDD and is presented in detail in Appendix B.

vii) Rat insulin was determined by radioimmunoassay (Weinkove, Weinkove, and Pimstone, 1974) using guinea pig anti-porcine insulin antibody (Burroughs Wellcome Ltd., U.K.) ^{125}I labelled (by the Chloramine-T method), porcine monocomponent insulin (Novo) as tracer, crystalline rat insulin as standard (Novo) and a second antibody separation (rabbit anti guinea pig antiserum, Burroughs Wellcome Ltd., U.K.). The detailed methodology of the assay appears in Appendix B.

viii) Administration of exogenous hormone. Human growth hormone (Kabi, Stockholm; Crescormon) or bovine (NIAMDD BG-18) was

administered daily at a dose of 4 μg/g body weight by intraperitoneal injection for 7 days (days 19-25 of diet) to six 4% protein-fed rats.

C Results

i) The results of bioassay of serum somatomedin activity are shown in Fig. 6.1. In summary they show that serum somatomedin activity in rats fed a standard rat cube diet, a 20% protein diet and 21 day old weanlings did not differ significantly from each other, but that after 4 days on the 4% protein diet there was a striking fall. The low level of serum somatomedin activity present after 25 days of 4% protein diet did not alter significantly after 7 days of treatment with bovine or human growth hormone (days 19-25 of special diet).

Fig. 6.1 Bioassayable somatomedin activity in serum from:

(a) Normal adult rats fed standard rat cubes (n = 20)

(b) Rats fed a 20% protein diet for 25 days (n = 20)

(c) Weanling rats (n = 20)

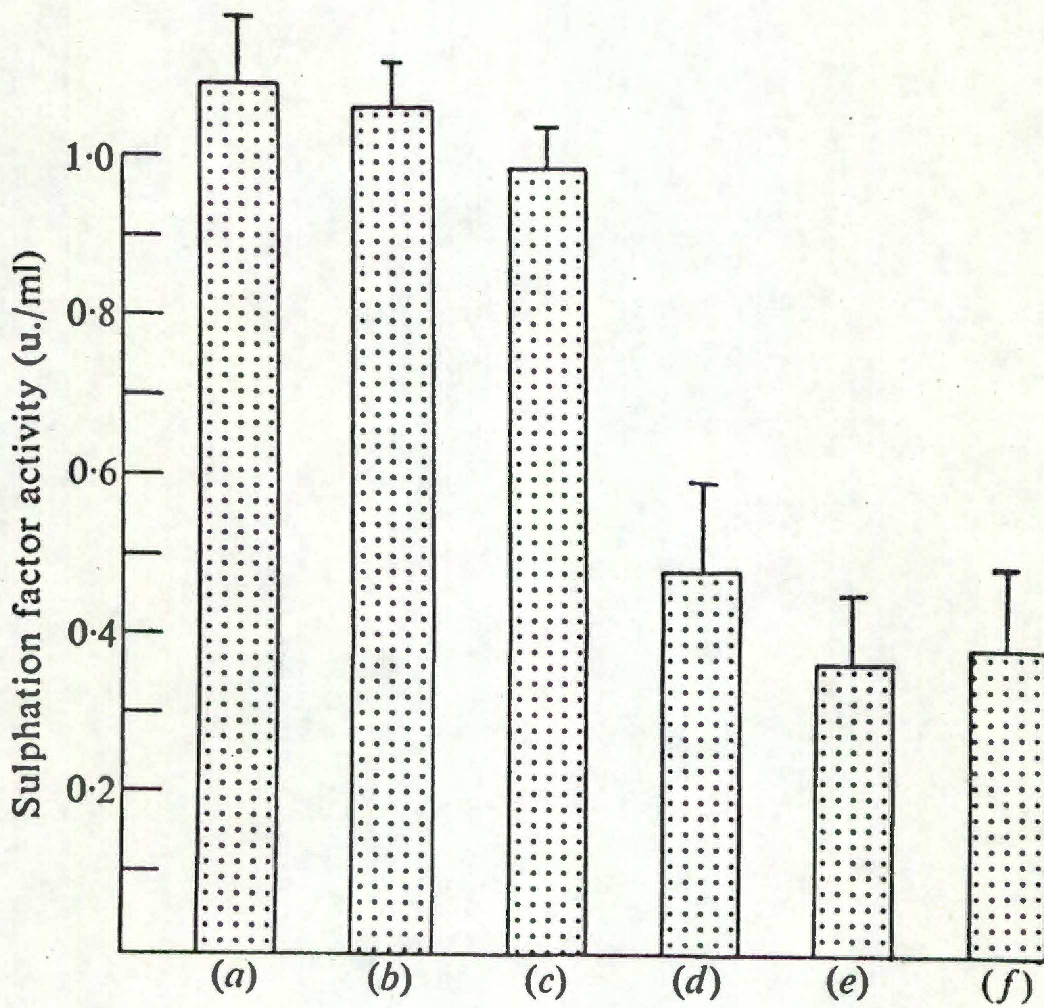
(d) Rats fed a 4% protein diet for 4 days (n = 6)

(e) Rats fed a 4% protein diet for 25 days (n = 20)

(f) Rats fed a 4% protein diet for 25 days and treated with human or bovine growth hormone (4 μg/g/day) for the last 7 days (n = 12).

Bars represent means and the vertical lines SEM.

Fig. 6.1 Bioassayable somatomedin activity



ii) Mixing experiments failed to demonstrate an obvious inhibiting factor in the serum from protein malnourished rats (see Table 6.1).

TABLE 6.1

MIXING EXPERIMENTS TO INVESTIGATE THE POSSIBLE INHIBITORY ACTION OF SERUM FROM PROTEIN MALNOURISHED RATS ON THE PORCINE COSTAL ASSAY FOR SOMATOMEDIN (Means ± SEM)

Serum	Predicted Potency (U/ml)*	Actual Potency	P
Standard	-	1.00	-
Standard + serum from rats fed 20% protein	1.03	0.94 ± 0.21	> 0.50
Standard + serum from rats fed 4% protein	0.68	0.61 ± 0.26	> 0.50
Standard + weanling rat serum	0.99	0.92 ± 0.31	> 0.50

* Half (activity of standard + previously assayed activity of test serum). Previously assayed pooled serum was mixed 1:1 with standard (potency defined as 1.00 U/ml) and the potency of the mixture compared with that calculated from the components of the mixture assayed separately.

iii) The failure of growth hormone to affect the morphological and biochemical features of protein energy malnutrition is shown in Table 6.2.

TABLE 6.2

MORPHOLOGICAL AND BIOCHEMICAL FEATURES OF PROTEIN-DEFICIENT RATS WITH AND WITHOUT GROWTH HORMONE TREATMENT

	4% Protein Diet (n = 20)	4% Protein diet + 4 μg hGH or bGH g^{-1} day^{-1} for 7 days* (n = 6 each)
Weight (g)	32.2 ± 7.9	34.0 ± 4.8
Total length (cm)	20.4 ± 1.3	20.8 ± 1.8
Fatty liver (Oil red O)	Positive	Positive
Serum protein (g/100 ml)	3.8 ± 0.2	3.9 ± 0.2
Serum albumin (g/100 ml)	2.1 ± 0.1	2.2 ± 0.1
Tibial epiphyseal width (μm)	202 ± 46	216 ± 50

* As neither human nor bovine growth hormone had a significant effect, the results are combined.

iv) The hypoinsulinaemia previously observed in this model of protein malnutrition was confirmed (in the fasting state) and the rat growth hormone levels in the three groups of rats did not differ significantly from each other (see Table 6.3).

TABLE 6.3

THE LEVELS OF rGH AND INSULIN (ng/ml) IN THE PLASMA OF PROTEIN-DEFICIENT AND CONTROL RATS AFTER AN OVERNIGHT FAST (Means + SEM)

	A Weanlings (n = 8)	B 20% Protein Diet (n = 8)	C 4% Protein Diet (n = 8)
Insulin	0.84 ± 0.09	1.03 ± 0.11	1.31 ± 0.08*†
rGH	78 ± 26	89 ± 22	86 ± 18

Student's T (unpaired)

*† $p<0.001$: Group A vs Group C and Group B vs Group C resp.

$p>0.500$: Group A vs Group C and Group B vs Group C.

D Discussion

i) Somatomedin activity in experimental protein malnutrition.

We have shown in the Stead and Brock (1972) model of protein malnutrition that there is reduced serum somatomedin activity similar to that occurring in human kwashiorkor except that non-parallel dose response lines are infrequent (Shapiro and Pimstone, 1978).

At the time this work was initiated there was no data available on serum somatomedin activity in protein malnourished animals. Recently it has been reported in abstract that a diet low in protein is associated with low serum somatomedin activity and that there is a positive correlation between serum somatomedin activity (and growth) and dietary protein content (Reeves, Lee, Dickinson, Kilgore, Branham and Elders, 1977; Reeves, Dickinson, Lee, Kilgore, Branham and Elders, 1977). These workers also report a negative correlation between serum somatomedin activity and dietary fat con-

tent. Somatomedin A measured by radioreceptor assay has been found to be reduced in animals fed a low protein diet (Takano, Hizuka and Shizume, 1977, and Takano, personal communication, 1977). There is thus a relationship between dietary composition and somatomedin activity which may be at least one of the mediators of growth disturbance in protein malnutrition.

ii) Somatomedin in acute starvation.

The relationships between the changes in somatomedin (and other hormones) in protein malnutrition and those occurring in acute starvation of previously wellnourished animals, are not yet clear. Decreased somatomedin activity was reported after a 48 hour fast (Daughaday and Kipnis, 1966). Phillips and Young (1976a) have shown that a significant fall in serum somatomedin activity occurred within 24 hours of the onset of an acute fast and reached hypopituitary levels after 72 hours. The fall was not prevented by exogenously administered bovine growth hormone but refeeding resulted in a significant increase in serum somatomedin activity within 6 hours which returned to normal within 24 hours. Mixing experiments failed to reveal an obvious inhibitor. Work in man and rats (Takano, Hizuka and Shizume, 1977) in which radioreceptor assayable rather than bioassayable somatomedin was found to be reduced in acute fasting confirms that at least part of the reduced bioassayable somatomedin activity is due to a true reduction in at least one component of total somatomedin activity (somatomedin A). The return to normal of serum somatomedin activity after a 72 hour fast was directly proportional to the calorie intake provided and protein was more effective than carbohydrate in stimulating this recovery (fat was still more stimulatory!) (Phillips and Orawski, 1976). In contrast to the findings of Phillips and Young (1976a), Salmon (1975) found evidence for an inhibitor of somatomedin activity. It is possible

that an inhibitor present in low concentrations may not be detected by mixing experiments (Phillips and Young, 1976a) and thus the two groups of findings may not be irreconcilable.

iii) The response of somatomedin activity to growth hormone in protein malnutrition.

The failure of pharmacological doses of growth hormone (known to be biologically active in the rat) to have any effect on growth or somatomedin in the rat model of protein malnutrition studied is in keeping with its failure to increase somatomedin in acutely starved animals (Phillips and Young, 1976a) and to stimulate lipolysis in human protein malnutrition (Hadden and Rutishauser, 1967).

iv) The relationship of somatomedin activity and growth hormone in experimental protein malnutrition in the rat.

That the rat growth hormone in the protein malnourished rats was not elevated was somewhat different from the human syndrome, but there remained the dichotomy of low somatomedin activity in the face of normal growth hormone levels. The control and dynamics of growth hormone in the rat differs radically from that in man, stress, anaesthesia and hypoglycaemia suppressing growth hormone secretion (Martin, Durand, Gurd, Faille, Audet and Brazeau, 1978). However growth hormone might be elevated in protein energy malnutrition rats if sampling were done frequently and basal and peak patterns assessed. To avoid such sampling error, chronic cannulation with very frequent sampling of fully conscious freely moving animals would be required (Dr. J. Martin, personal communication, 1977), a procedure found to be quite impractical in malnourished rats weighing only 30-40g. The plasma rat growth hormone values in our experiments were all from samples obtained between 09.30 and 10.30 (the usual time of the mid-morning growth hormone peak in the rat) under rapidly induced light anaesthesia and demonstrate that the protein

malnourished rat has at least growth hormone levels in the normal range and that the low serum somatomedin activity cannot be ascribed to very low or absent growth hormone concentrations (a situation analogous to hypophysectomy).

v) The relationship of somatomedin activity and insulin in experimental protein malnutrition in the rat.

The hypoinsulinaemia occurring in protein malnutrition was confirmed in the overnight fasted state and is also known to be blunted in response to such stimuli as glucose (Weinkove, Weinkove and Pimstone, 1976). The role of insulin in somatomedin generation may be important in that insulin has been shown to increase body weight, length and epiphyseal cartilage width (Salter and Best, 1953) in hypophysectomised rats. Furthermore hypoinsulinaemic streptozotocin induced diabetes resulted in reduced somatomedin activity normalised by insulin administration (Phillips and Young, 1976b) and somatomedin is generated from isolated rat livers perfused with insulin-containing medium (Daughaday, Phillips and Mueller, 1976). Insulin may also be a factor in some of the bizarre growth patterns reported post-operatively in patients with craniopharyngioma who may grow in spite of low or undetectable growth hormone and who have normal somatomedin levels (Finkelstein, Kream, Ludan and Hellman, 1972; Costin, Kogut, Phillips and Daughaday, 1976), those children who demonstrate increased appetite (occasionally to the point of pathological hyperphagia) with increased insulin levels being the ones who show "catch-up growth".

The hypoinsulinaemia of protein malnutrition may thus be at least one factor for reduced somatomedin activity in serum in spite of normal (in rats) or elevated (in man) growth hormone.

CHAPTER SEVEN

IN VITRO $^{35}SO_4$ INCORPORATION INTO CARTILAGE

"It is too bad that we cannot cut the patient in half in order to compare two regimens of treatment" *Béla Schick*

A Introduction

Having demonstrated in the protein malnourished rat, growth failure, narrow epiphyses, reduced $^{35}SO_4$ incorporation into cartilage in vivo and reduced serum somatomedin activity, an in vitro study of the $^{35}SO_4$ incorporation into the costal cartilages of malnourished and wellnourished control rats was undertaken to study the role of the end-organ. The $^{35}SO_4$ incorporation in vitro in the absence of serum was taken as a measure of intrinsic cartilage activity, while the stimulation of $^{35}SO_4$ incorporation by serum was taken as a measure of responsiveness to somatomedin. As the response to sera from normally nourished and malnourished animals was studied this provided, in effect, another bioassay system for the somatomedin activity of malnourished serum.

B Methodology

i) Dissection of tissue

Under light ether anaesthesia malnourished and wellnourished (age and weight matched controls) rats were positioned on their backs on a dissecting board, the fur on the ventral surface was trimmed with an electric hair clipper and the whole ventral surface was vigorously swabbed with 95% ethanol. The skin was incised from jaw to groin with sterile scissors and the skin displaced laterally by blunt dissection. Using a new set of sterile instruments the abdomen was opened, the abdominal aorta exposed and the animal killed by exsanguination via a 21 gauge needle and sterile syringe.

The blood was allowed to clot at 4°C and the serum separated by centrifugation at 4°C and the pooled serum (equal volumes from each animal within each group) stored at -20°C for future use.

The entire anterior chest wall, including all the sternally attached costal cartilages, was removed as a single block and placed between two sterile gauze swabs moistened with 0.9% sterile saline in a sterile stainless steel dissecting tray. Using yet another set of sterile instruments and gloves the costal cartilages from the 3rd to the 7th ribs were excised, with the exception of the most lateral 2 mm including the costochondral junction, stripped free of muscle and perichondrium and diced into pieces 2-3 mm long. The lateral 2 mm of the costal cartilages 3 to 7 including the costochondral junctions were dissected out, pooled separately, preincubated and incubated as for the cartilage fragments. These cartilage fragments were then placed in an Erlenmeyer flask with 75 ml of supplemented phosphosaline buffer (composition as described in Appendix B). In all, cartilage fragments from 5 animals of each type were dissected and pooled on four occasions.

ii) Preincubation

The three flasks containing the pooled cartilage fragments or costochondral junctions from the three different groups of animals were "preincubated" at 37°C in air with occasional gentle agitation for 18 hours.

iii) Unstimulated and serum stimulated $^{35}SO_4$ uptake

Pooled cartilage fragments from 4% protein-fed malnourished rats and their 20% protein-fed age matched and weanling weight matched controls were incubated with two concentrations of pooled serum from each type of animal.

Under sterile conditions groups of cartilage fragments (8-10/

tube) were placed in plastic test tubes (12 x 75 mm) containing 1 ml of supplemented phosphaline buffer, a 10% or 40% dilution of pooled serum from each type of animal, each in triplicate. Sterile carrier-free $Na_2{}^{35}SO_4$ (Radiochemical Centre, Amersham, U.K.) was added in a volume of 20 µl by "Repette" (Hemel Hempstead, U.K.) to give a final concentration of 2 µCi/ml.

After 20 hours incubation in air at 37°C the incubation medium was aspirated and the cartilage killed by boiling water. The tops of the tubes were covered with nylon mesh held in place with rubber bands and repeatedly rinsed with cold tap water. After 10 rinses $^{35}SO_4$ was no longer detectable in the wash water, but an additional 20 rinses were carried out.

iv) $^{35}SO_4$ determination

After rinsing, the cartilage fragments in their tubes were dried at 80°C for 24 hours and after cooling weighed by microtortion balance, dissolved in 23M formic acid in Packard Scintillation vials (80°C for 1 hr) and after cooling, $^{35}SO_4$ determined by liquid scintillation counting in 10 ml of Instagel (Packard).

C Results

1) This experiment was performed 4 times (i.e. 20 animals of each type) (The results are shown in Table 7.1, Table 7.2, Fig. 7.1 and Table 7.3).

ii) In summary the results were:-

(a) Cartilage from 4% protein-fed rats in the absence of serum incorporated $^{35}SO_4$ identically to that from 20% protein-fed rats of the same age. Cartilage from the younger weanlings, under the same conditions had a higher incorporation of $^{35}SO_4$.

TABLE 7.1

INCORPORATION OF $^{35}SO_4$ INTO COSTOCHONDRAL JUNCTIONS IN VITRO WITH AND WITHOUT SERUM

	4% Protein Diet Cartilage (n=20)	20% Protein Diet Cartilage (n=20)	Weanling Cartilage (n=20)
Buffer (0% serum)	2170 ± 190	2400 ± 240	7225 ± 355†
4% Protein diet Pooled serum 40%	2620 ± 240‡	2875 ± 255‡	8880 ± 360‡†
20% Protein diet Pooled serum 40%	5425 ± 275*	5560 ± 380*	23140 ± 515*†
Weanling Pooled serum 40%	5020 ± 370*	5345 ± 385*	20475 ± 470*†

CPM/Mg cartilage Mean ± SEM, number of animals in parenthesis is made up of 4 groups of 5 animals

Student's t test for unpaired groups

1 Serum stimulated vs Buffer $p < 0.01^{*}$

Serum stimulated vs Buffer $p > 0.50^{\ddagger}$

2 Weanling vs 4% or 20% cartilage $p < 0.01^{\dagger}$

TABLE 7.2

INCORPORATION OF $^{35}SO_4$ INTO COSTAL CARTILAGE IN VITRO (EXCLUDING COSTOCHONDRAL JUNCTIONS) WITH AND WITHOUT SERUM

	4% Protein Diet Cartilage (n=20)	20% Protein Diet Cartilage (n=20)	Weanling Cartilage (n=20)
Buffer (0% serum)	1240 ± 160	1420 ± 220	3210 ± 205†
4% Protein diet) Pooled serum) 40%	1540 ± 210 ‡	1650 ± 195 ‡	3290 ± 275‡†
20% Protein diet) Pooled serum) 40%	3100 ± 230 *	2910 ± 215 *	8900 ± 290*†
Weanling) Pooled serum) 40%	2970 ± 185 *	3170 ± 225 *	9100 ± 310*†

CPM/mg cartilage Mean ± SEM, number of animals in parenthesis is made up of 4 groups of 5 animals

Student's t test for unpaired groups

1 Serum stimulated vs Buffer $p < 0.01^{*}$

Serum stimulated vs Buffer $p > 0.50^{‡}$

2 Weanling vs 4% or 20% cartilage $p < 0.01^{†}$

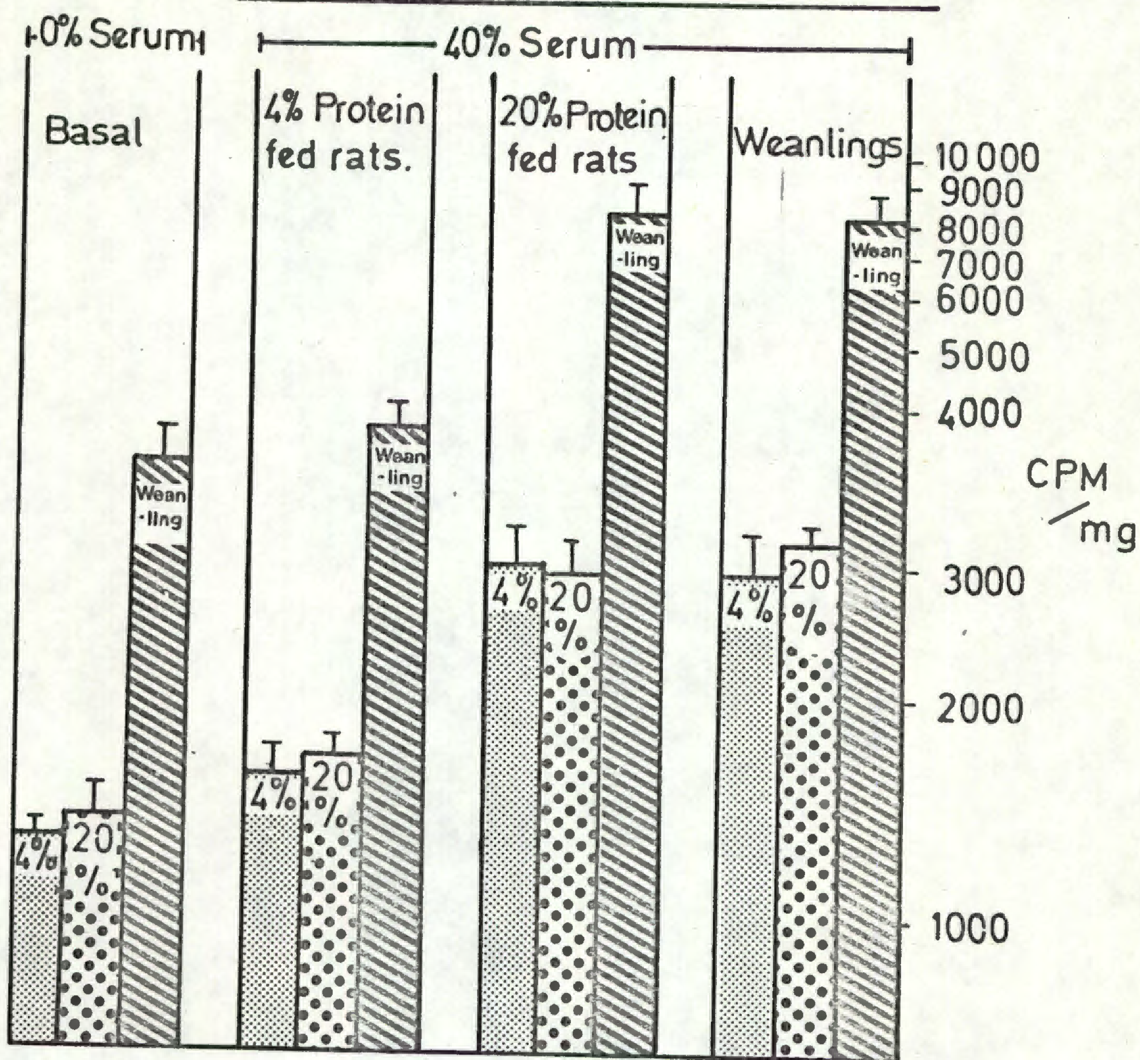


Fig. 7.1 In vitro uptake of $^{35}SO_4$ by pooled costal cartilage fragments from rats fed a 4% protein diet (hatched bars), 20% protein diet (small stippled bars) or weanlings (large stippled bars), incubated in the absence of serum or with 40% serum from controls (20% protein fed age-matched controls or weanling weight-matched controls) or protein malnourished rats (4% protein fed). Results are means ± SEM of three experiments and each pool of cartilage was derived from five rats.

TABLE 7.3

INCORPORATION OF $^{35}SO_4$ INTO COSTAL CARTILAGE IN VITRO (EXCLUDING COSTOCHONDRAL JUNCTIONS) WITH AND WITHOUT NORMAL POOLED HUMAN SERUM OR SERUM FROM PATIENTS WITH PROVEN PANHYPOPITUITARISM AND ABSENT CIRCULATING GROWTH HORMONE

	4% Protein Diet Cartilage (n=5)	20% Protein Diet Cartilage (n=5)	Weanling Cartilage (n=5)
Buffer (0% serum)	1140 $\pm$ 190	1280 $\pm$ 210	3540 $\pm$ 215
Normal human Pooled serum 40%	3320 $\pm$ 310*	3290 $\pm$ 290*	10270 $\pm$ 325*
Hypopituitary human serum 40%	1680 $\pm$ 230	1720 $\pm$ 215	4770 $\pm$ 270

CPM/mg cartilage Mean $\pm$ SEM, number of animals in parenthesis

Student's t test for unpaired groups

Normal serum vs hypopituitary serum $p < 0.05$*

(b) Cartilage from 4% protein-fed rats and 20% protein-fed rats was equally stimulated to incorporate $^{35}SO_4$ by pooled normal human serum while weanling cartilage was greater and the dose response steeper. Serum from hypopituitary humans was only about $^1/5$ as stimulating as normal serum (Table 7.3).

(c) Pooled serum from 20% protein-fed or weanling rats was equally stimulatory to the incorporation of $^{35}SO_4$ by both 20% protein-fed rat cartilage and 4% protein-fed rat cartilage, with parallel dose response curves when studied at two dilutions (10 and 40%). The stimulation of $^{35}SO_4$ by weanling cartilage was even greater and the dose response steeper.

(d) In contrast pooled serum from 4% protein-fed rats was only poorly stimulatory to all three types of cartilage but dose responses were still parallel to those of 20% protein-fed and weanling rat serum (Fig. 7.1).

(e) The behaviour of the costochondral junctions was essentially like that of the rest of the costal cartilage but the incorporation was greater under all circumstances. As there were fewer costochondral junction fragments only 3-5 could be placed in each tube for incubation in triplicate. (Table 7.2).

D Discussion

i) Although it is a highly artificial system, the in vitro incubation of cartilage fragments has been widely used to study the behaviour of cartilage in a variety of conditions in the rat, namely starvation and refeeding (Phillips and Young, 1976a), streptozotocin diabetes (Phillips and Young, 1976b), cortisone-induced growth

arrest (Mosier, Jansons, Hill and Dearden, 1976), propylthiouracil-induced hypothyroidism (Mosier, Dearden, Jansons and Hill, 1977) and the effect of ageing (Heins, Garland and Daughaday, 1970; Beaton, 1976). Such systems have the advantage of permitting the manipulation of variables acting on cartilage in a manner impossible in vivo. As with the above studies, costal cartilages were chosen because of the ease of dissection and the adequate amount of tissue obtained and $^{35}SO_4$ incorporation used as an indication of cartilage growth activity. The dissection of epiphyseal growth plate proved impractical in terms of the tissue required, the amount of cartilage obtained and the inaccuracy of the dissection. Costal cartilage has been widely used as being representative of growth cartilage in general and the results of in vivo $^{35}SO_4$ labelling experiments (Chapter 4) showed costal and epiphyseal cartilage to have behaved in a qualitatively similar way.

(iia) The system utilized in this study differed from those previously described in that all cartilage was subjected to an initial serum-free preincubation in an attempt to minimize the "carryover" of the in vivo somatomedin and other humoral effects. That such carryover occurs is exemplified in the porcine costal cartilage bioassay for somatomedin (Van den Brande and Du Caju, 1974) in which the cartilage fragments are subjected to a preincubation step and in which the incubation with $^{35}SO_4$ follows the incubation with serum, but $^{35}SO_4$ incorporation is related to the somatomedin stimulus of the preceeding serum incubation. There is some evidence for the "carryover" of somatomedin effect in vivo to in vitro cartilage incubation with $^{35}SO_4$ (Phillips and Young, 1976a). The reduced serum somatomedin activity which occurs in acute starvation in the rat is paralleled by decreased $^{35}SO_4$ incorporation into cartilage from starved animals incubated in a serum-free in vitro system. Such

cartilage was however still sensitive to stimulation of $^{35}SO_4$ incorporation by normal rat serum.

(iib) The incorporation of $^{35}SO_4$ in this system was proven to be active in that boiled cartilage failed to incorporate $^{35}SO_4$.

(iic) The incorporation of $^{35}SO_4$ in this system was shown to be somatomedin dependent by the experiment in which stimulation of incorporation by panhypopituitary human serum was $\sim$ 20% that of normal serum. Human hypopituitary serum was used because of the non-availability of hypophysectomised rat serum and came from patients without detectable growth hormone and who were on full replacement therapy (thyroxine, cortisone, androgens or oestrogens).

(iii) Cartilage from 20% protein-fed and 4% protein-fed rats behaved similarly in that in the absence of serum they incorporated $^{35}SO_4$ and responded equally to pooled serum from 20% protein-fed and weanling rats. The response to pooled 4% protein-fed rat serum was less than that to normally nourished serum but was similar with both types of cartilage. Thus as in acute starvation (Phillips and Young, 1976a), in experimental protein malnutrition in the rat, the cartilagenous end-organ was normally responsive to serum somatomedin activity and the reduced serum somatomedin in experimental protein malnutrition is probably the major factor in the reduced $^{35}SO_4$ labelling in vivo and reduced skeletal growth.

(iv) The cartilage of weanling rats had a higher serum-free $^{35}SO_4$ incorporation and showed a steeper dose response to serum containing somatomedin than 20% protein-fed and 4% protein-fed rat cartilage (Fig. 7.1). This is in keeping with the fact that age appears to be the most important single factor determining cartilage responsiveness to somatomedin activity (Heins, Garland and Daughaday,

1970; Beaton, 1976). The response to 4% protein-fed rat serum was once again less than that to normally nourished serum.

(v) The heterogeneity of the costal cartilage is well recognised (Herbai, 1970), the costochondral junction area having the greatest $^{35}SO_4$ incorporation. In the protein malnourished rats and their age and weight matched wellnourished controls this also proved to be the case but the response to different sera showed the same pattern as the costal cartilage itself.

E <u>Conclusions</u>

i) Experimental protein malnutrition does not alter the intrinsic ability of the cartilage to incorporate $^{35}SO_4$, whether unstimulated or stimulated by serum.

ii) The age of the animal appears to be the major determinant of $^{35}SO_4$ incorporation into cartilage, in that unstimulated and stimulated incorporation is greatest in weanlings and the slope of the dose response to serum is also steeper.

iii) Serum from protein malnourished rats is poorly stimulatory to all types of cartilage, demonstrating the anticipated low somatomedin activity.

iv) Thus the poor $^{35}SO_4$ incorporation (and presumptively the growth) of cartilage in the protein malnourished rat in vivo is primarily due to a disturbance of the humoral factors acting on cartilage.

CHAPTER EIGHT

SOMATOMEDIN ACTIVITY GENERATION BY THE ISOLATED PERFUSED LIVER IN EXPERIMENTAL PROTEIN ENERGY MALNUTRITION

"He looked in the liver" *Ezekiel 21.21.*

In Section I of this chapter the evidence for the liver being a major site of somatomedin activity and the validation of a liver perfusion technique will be presented, while in Section II, the generation of somatomedin activity by protein malnourished and control livers will be considered.

SECTION I

A The role of the liver in somatomedin generation

Evidence for the liver as a major site of somatomedin generation is based on four groups of observations:

i) Uthne and Uthne (1972) noted that partial hepatectomy in the rat resulted in a rapid fall of serum somatomedin activity which rose with hepatic regeneration. The entire phenomenon may have been a non-specific response to a severe traumatic insult and biochemical derangement and the return to normal of somatomedin activity with liver regeneration the recovery from that insult.

ii) In chronic liver disease serum somatomedin activity is reduced in spite of normal or elevated growth hormone (Wu, Grant, Hambley and Levi, 1974; Giordano, Foppiani, Minuto, Perroni and DiCicco, 1975). Similarly NSILA is reduced in patients with cirrhosis (Samaan, Stillman and Fraser, 1962).

iii) A trans-hepatic gradient of somatomedin activity has been shown in the dog, the levels in the hepatic vein being significantly higher than the portal vein (or femoral vein or artery)

(Schimpff, Donnadieu, Glasinovic, Warnet and Girard, 1976).

iv) Generation of somatomedin activity has been demonstrated by a variety of in vitro liver preparations:

a) A crude microsomal fraction of rat liver in the presence of growth hormone and co-factors (Hall and Uthne, 1971).

b) Isolated perfused rat liver preparations in response to growth hormone (McConaghey and Sledge, 1970; McConaghey, 1972; Williams and Hughes, 1974; Ash and Francis, 1975; Francis and Hill, 1975; Phillips, Herington, Karl and Daughaday, 1976; Daughaday, Phillips and Mueller, 1976; Wondergem, Gaspard, Hamamdzic and Klitgaard, 1977).

c) Isolated perfused liver preparations in response to prolactin (Francis and Hill, 1975; Hill, 1977) and insulin (Daughaday, Phillips and Mueller, 1976). In addition NSILA has been generated in such systems in response to growth hormone (Schalch, Heinrich, Johnson, Koch and Miller, 1977; Heinrich, Schalch, Miller and Johnson, 1977).

d) Superfused rat liver slices in response to growth hormone (Hintz, Clemmons and Van Wyk, 1972).

e) Incubated rat liver slices in response to growth hormone (McConaghey, 1972).

B <u>Methodology of liver perfusion</u>

i) Choice of experimental animals: In the preceeding chapters, the protein malnourished rat was studied using a wellnouri-

shed (20% protein fed) age-matched control and a wellnourished weanling weight-matched control. The weanling controls were especially valuable in the study of cartilage behaviour (in vivo and in vitro) because of their known rapid growth and highly active cartilage. The weanlings were shown to have serum somatomedin activities similar to those of 20% protein fed and standard diet fed rats, thus in attempting to study liver somatomedin generation by the isolated perfused livers of protein malnourished rats, 20% protein fed rats and standard diet fed rats were used as controls as the small friable vasculature and livers of the weanlings made perfusion technically difficult. The standard diet fed rats had the additional advantage of being readily available and were especially valuable in establishing techniques and in pilot studies for which the rats fed a special diet could not be spared.

ii) The liver perfusion circuitry: The apparatus used permitted recycling perfusion by the method of Hems, Ross, Berry and Krebs (1966). The liver, left in situ in the animal, was perfused via the cannulated portal vein and the perfusate returned to the reservoir via a cannula from the inferior vena cava. The bile duct was cannulated where possible and the hepatic artery ligated (see section iv below for detailed operative technique).

The perfusate was pumped from the reservoir by a peristaltic pump (initially a SARNS model 5500, Ann Arbor, Michigan, U.S.A. and later a Gilson Minipuls, France) and flowed down the inside of the glass "lung", which had a large dimpled surface and a countercurrent gas flow of 95% O_2/5% CO_2 at 3 l/min. This provided pO_2 in the inflowing perfusate of 300-475 mmHg. An overflow from the lower end of the lung to the reservoir provided a constant pressure head. The reservoir was gently stirred by a magnetic stirrer and gassed with 95% O_2/5% CO_2. Some livers were perfused with a con-

stant set flow rate by means of a second peristaltic pump (Gilson Minipuls) interposed between the lung outflow and the liver. Flow rates were adjusted to provide sufficient oxygen for the maintenance of liver viability (as confirmed by the tests detailed below) for 2 hours by alteration of lung height above liver and adjustment of valve V (see Fig. 8.1) or by direct setting of flow in the second pump.

The entire circuitry except for the peristaltic pumps was housed in a perspex fronted, asbestos board cabinet provided with fluorescent lighting. The environment in which the experiments were conducted was maintained at 38°C by a thermostatically controlled heater, high humidity was maintained by a steam inlet from an electrically heated evaporator, the air in the cabinet was circulated by an electric fan. Liver temperatures of 37°-38°C were confirmed by a telethermometer probe (Simpson Electrical Co., Chicago, U.S.A.) placed between adjacent liver lobes. The liver itself was further protected against surface drying by covering with a gauze swab moistened with warm Plasmalyte B (Saphar Laboratories, South Africa).

iii) Preparation of perfusate: The perfusate was a semisynthetic medium containing aged human erythrocytes to increase oxygen carrying capacity.

a) Outdated human donor blood was obtained from the Western Province Blood Transfusion Service. This blood was 4-5 weeks old and had been anticoagulated in the standard way with acid-citrate-dextrose and stored at 4°C. After centrifugation at 2000g for 5 min at 4°C, plasma and buffy coat were removed by aspiration. The erythrocytes were then washed with three times their own volume of chilled Krebs

Fig. 8.1 Liver perfusion apparatus

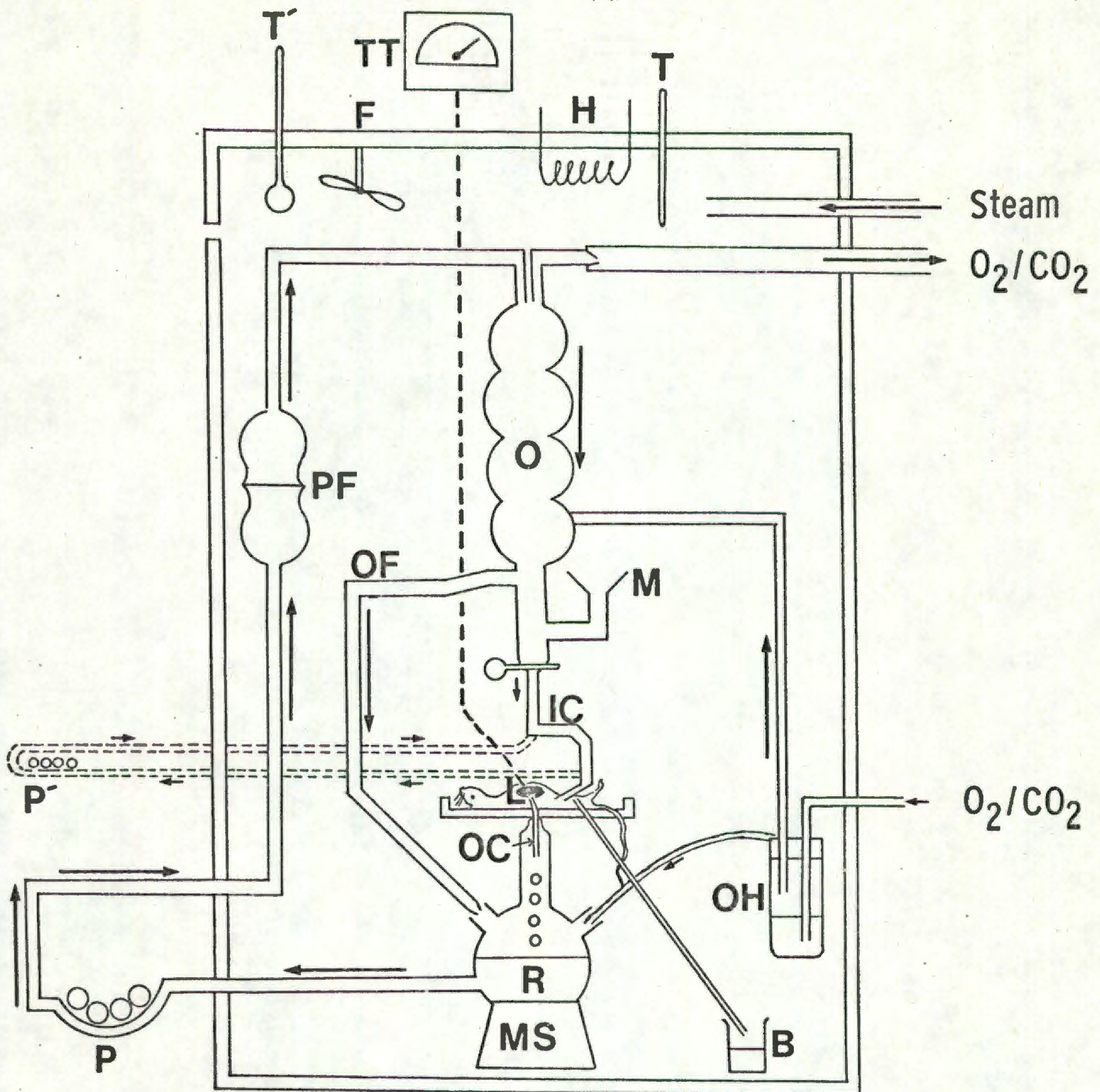


Fig. 8.1 Liver perfusion apparatus

B	-	Bile collection
F	-	Fan
H	-	Heater
IC	-	Inflow cannula (in portal vein)
M	-	Manometer
MS	-	Magnetic stirrer
O	-	Oxygenator
OC	-	Outflow cannula (in inferior vena cava)
OF	-	Overflow
OH	-	Humidifier for oxygen/carbon dioxide
P	-	Primary pump (reservoir to oxygenator)
P′	-	Secondary pump (oxygenator to liver - not used in all experiments)
PF	-	Perfusate filter
T	-	Thermostat
T′	-	Thermometer (for cabinet temperature)
TT	-	Telethermometer (connected to thermo-sistor under lobe of liver)

Henseleit bicarbonate buffer (KHB). The wash was removed by centrifugation and aspiration as before. In all, three washes were performed. The final erythrocyte pellet was reserved and kept at $4^{o}C$ until use (within 1 hour).

b) Krebs Henseleit bicarbonate buffer (KHB), gassed with 95% O_2:5% CO_2 for 10 minutes at room temperature ($20-22^{o}C$) was mixed with 2.5g/100 ml bovine serum albumin (BSA) (fraction V) (Miles, Biochemicals, Cape Town) and 90 mg/100 ml D (+) glucose and the pH adjusted to 7.4 with a few drops of 1M NaOH. Glucose was omitted in those experiments to test the gluconeogenic capacity of the livers.

c) The erythrocyte pellet and KHB-BSA mixture were mixed in a ratio of 1:9 to give a final haematocrit of 8-10%. The addition of another 0.7-1.0 ml 1M $NaHCO_3$ was required to bring the final perfusate pH to 7.38-7.50 after equilibration through the apparatus for 20 minutes at $37^{o}C$.

d) This provided an easily prepared, uniformly reproducible perfusate, the characteristics of which are summarised in Table 8.1.

TABLE 8.1

PERFUSATE COMPOSITION (MEAN OF 10)

		Methodological Appendix
Whole perfusate		
Haemoglobin (g/100 ml)	2.8 ± 0.5	B
Haematocrit (%)	8 ± 1	B
Glucose (mg/100 ml)		
added (standard)	85 ± 6	B
not added (gluconeogenic experiments)	< 2.5	B
Supernatant		
Free haemoglobin (g/100 ml)	< 0.005	B
Albumin (g/100 ml)	2.5 ± 0.1	B
Lactate (mMol/L)	< 1	B

iv) The operative technique of liver perfustion: Anaesthesia was induced with 20% diethylether (Natal Cane Byproducts Ltd., Natal) in 95% O_2:5% CO_2 from an ether dispenser, flow rate 3-5 L/min. Once surgical anaesthesia was achieved, this was maintained using 5-10% ethylether in the same gas mixture and flow rate. The rat was positioned on its back with its head away from the operator and the limbs gently abducted by elastic bands from the feet to pins on the operating board. The abdominal cavity was opened by a midline incision from pubis to xiphoid process and extended laterally on each side just below the costal margin. Care was exercised to avoid excessive haemorrhage from the internal mammary arteries or damage to the liver and diaphragm. The abdominal viscera were displaced to the left of the animal and enclosed in a layer of gauze moistened with "Plasmalyte B" (Na^+ 130, K^+4, Mg^{++} 1.5, Cl^-109 and HCO^-_3 28mM in sterile water). This allowed easy

access to the portal vein, bile duct, inferior vena cava and right kidney. Better display of the portal vein and bile duct was obtained by gentle traction applied by "mosquito" forceps on the pyloroduodenal junction, the duodenal wall at the level of the ampulla of Vater and the descending colon.

The bile duct was opened with fine scissors and cannulated with fine polyethylene tubing OD 0.75mm (Portex Ltd., U.K.) with a sharp point and made rigid by placing in crushed ice and secured with one 4-0 silk suture. The fine strands of connective tissue between the right lobe of the liver and the inferior vena cava were divided and a loose linen ligature (A) placed around the vena cava above the level of the right renal vein with dissecting forceps. A loose 4-0 silk suture (B) was inserted under the portal vein 4mm from the point where the main trunk divides into the branches entering the different lobes of the liver. Another suture (C) was inserted under the portal vein 6mm distal to suture B. Suture C was tied.

The portal vein was opened with fine scissors and a cannula introduced. The cannula was connected to an intravenous giving set and a bottle of "Plasmalyte B" warmed to 36-37°C. Great care was taken to exclude air bubbles from the system. Cannulae were constructed from 8-inch infant feeding tubes (Pharmaseal Inc., U.S.A.) with a tip of polyethylene tubing, Size C PE190 (Clay Adams Inc., N.J., U.S.A.) for rats in the weight range 90-130g. For protein malnourished rats (weights 27-40g) the cannulae had tips of polyethylene tubing, size B, PE60. The portal vein cannula was secured by tying suture B, the cannula tip thus lying well below the point where the main portal trunk divides. The long ends of suture C were also tied around the cannula to ensure firm positioning.

The "Plasmalyte B" was allowed to flow at 2-4 ml/min and the liver checked for non-perfused areas (which remained the normal red-brown colour while perfused segments were yellow-brown). If the cannulation was satisfactory, the diaphragm was opened along the costal margin taking care not to damage the intrathoracic inferior vena cava or liver. The anterior chest wall was widely removed to give adequate exposure of the thoracic viscera. The thymus was excised. A loose linen ligature (D) was passed under the inferior vena cava above the diaphragm. The inflow of "Plasmalyte B" was briefly increased to 4-8 ml/min to distend the vena cava and right heart chambers but distension of the liver itself was avoided. The right atrium was incised and a second cannula introduced into the inferior vena cava and secured in place by tying ligature D. Flow-rate was restored to 2-4 ml/min. The abdominal inferior vena cava was ligated by tying ligature A.

Blood was swabbed from the body cavity with a moist gauze swab and the animal transferred to a glass platform which was then fitted into the top of the perfusate reservoir (later cannulations were performed directly on the platform). The portal venous cannula was disconnected from the intravenous giving set and connected to lung outflow without the introduction of air bubbles. The initial volume of effluent was drained into a measuring cylinder. After this initial "washout" phase the out-flow cannula was inserted into the reservoir. The vertical distance from the liver to the end of the out-flow cannula was kept at 3-4 cm. The bile duct cannula was placed in a tube (volume 1 ml graduated in 10 μl divisions). The liver was covered with a gauze swab moistened with warm "Plasmalyte B". Flow rate was adjusted by altering the lung outflow valve, the lung height or the secondary pump. Care was exercised to avoid liver swelling or transudation from the liver surface.

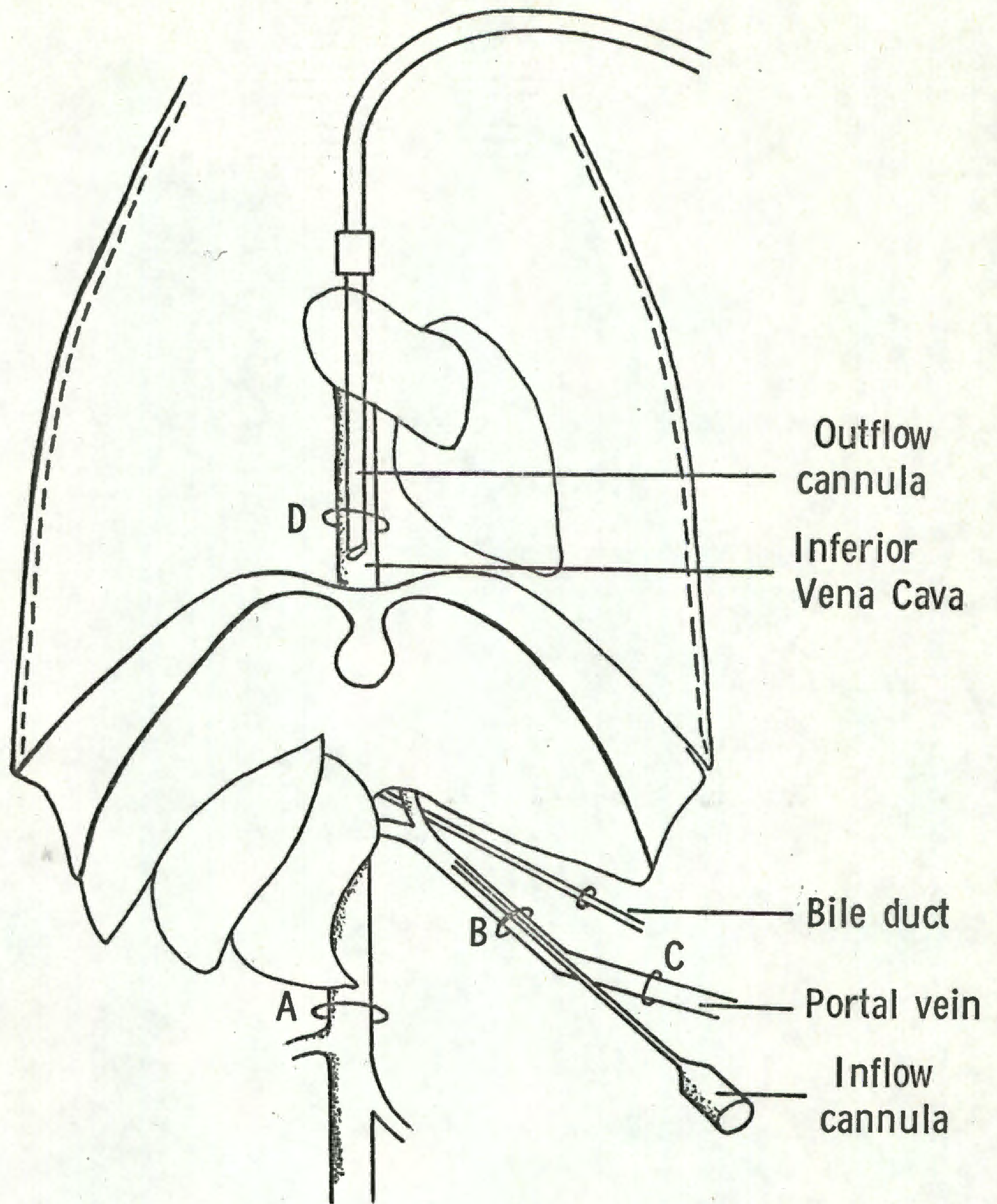


Fig. 8.2 Operative procedure of liver perfusion.

The total procedure took 10-14 minutes, of which the "ischaemic time" was 3.5-4.5 minutes (i.e. the time from portal vein ligature to establishment of perfusate flow).

v) Assessment of liver function and viability: In all perfusions the following were assessed:

a) Macroscopic liver appearance: Only perfusions in which livers were uniformly perfused, showing a normal reddish-brown appearance with no swelling, distension or exudation from the surface were considered satisfactory.

b) Bile volume: Bile was collected during the perfusion (except as discussed under operative technique in the very small protein malnourished rats). Mean bile production from 20% protein fed and normal diet fed rats was 63-66 µl/hr/g wet liver weight. Although bile flow has been frequently cited as a criterion for liver function in perfused livers, it is an insensitive index (Ross, 1972). Mechanical problems such as twisting and curling of the bile duct can give falsely low bile flow rates and are very difficult to correct once perfusion has commenced.

c) Flow rate: Flow rate was measured by timing the initial 100 ml perfusate (in the case of 20% protein fed and normal diet rats) or 30 ml (in the case of 4% protein fed rats) through the liver. Throughout the perfusion the flow rate was monitored by counting the drop rate from the cannula in the inferior vena cava at frequent intervals. (The drop volume of perfusate from such cannulae had been shown to be very uniform). Just before the termination of the experiment another timed collection of 15 ml was made. A constant flow rate was maintained, the adjustments described in Section I.B iii being made if necessary. See Table 8.2.

TABLE 8.2

CHARACTERISTICS OF PERFUSED LIVERS

	4% Protein Diet	20% Protein Diet	Standard Diet
Macroscopic appearance	Satisfactory	Satisfactory	Satisfactory
Histology	Satisfactory	Satisfactory	Satisfactory
Bile flow rate (μ/h/g)	-	66.0 ± 3.3 (20)	63.3 ± 3.4 (29)
Perfusate flow rate (ml/min/g)	1.60 ± 0.06 (30)	2.20 ± 0.07 (30) 1.69 ± 0.08 (9)	2.14 ± 0.07 (30)
O_2 consumption (μmol/min/g)			
without lactate	2.43 ± 0.31 (8)	2.31 ± 0.28 (8)	2.45 ± 0.40 (6)
with lactate	3.74 ± 0.40 (3)	3.83 ± 0.31 (3)	3.84 ± 0.41 (3)
Wet/dry weight ratio			
Unperfused	4.09 ± 0.08 (8)	3.55 ± 0.03 (8)	3.58 ± 0.07 (8)
Perfused	4.15 ± 0.09 (30)	3.66 ± 0.05 (30)	3.68 ± 0.06 (30)
Lactate gluconeogenesis (μmol/min/g)	0.71 ± 0.19 (7)	0.71 ± 0.11 (6)	1.02 ± 0.16 (8)
Glucagon induced glycogenolysis	-	-	min. $6x10^{-11}M$ max. $2.4x10^{-9}M$ ½max. $1.2x10^{-10}M$

Mean ± SEM (number)

d) Oxygen consumption: Specimens of perfusate were drawn into 2 ml syringes via 21 gauge needles inserted into the portal vein and inferior vena cava cannulae and immediately analysed for pO_2 by the method described in Appendix B. Such sampling was conducted at 30 minute intervals over the 2 hour perfusion. With knowledge of flow rate, perfusate haemoglobin content ($\sim$ 2.8 g/100 ml) and the portal/hepatic pO_2 difference, the oxygen consumption could be calculated. (See Table 8.2).

e) Wet/dry liver weight ratio: Livers were excised and weighed immediately after perfusion (a sample then being removed for histology in some cases) and dried to constant weight at 100°C. The wet/dry weight ratios were then calculated, taking the weight of the histological sample into account if necessary. The ratio did not differ significantly from those of unperfused livers (see Table 8.2

f) Histology: A total of 70 livers were examined histologically by means of 7 µ fixed paraffin embedded sections stained with haematoxylin and eosin (see Appendix A for method). The histological appearance of the perfused livers did not differ from that of unperfused livers described in Chapter 4.

g) Glucagon-induced glycogenolysis. Using the identical apparatus, perfusate and techniques but livers from fed normal animals, Sacks, Waligora, Matthews, Pimstone (1977) found a glucagon concentration of 6×10^{-11}M sufficient to produce detectable glycogenolysis. The maximal rate of glycogenolysis was achieved with 2.4×10^{-9}M and the ½ max with 1.2×10^{-10}M. These results represent a glucagon responsiveness close to the physiological range.

h) Maximal rate of lactate gluconeogenesis: While all the indices described above are important measures of liver function in the

perfused liver, all are relatively insensitive. A far more sensitive and satisfactory index of metabolic function in the perfused liver is the examination of a biosynthetic process as recommended by Hems, Ross, Berry and Krebs (1966). Maximal rates of urea, protein and glucose synthesis may all be used (Ross, 1972f).

The choice of maximal rate of gluconeogenesis from lactate. The maximal rate of glucose synthesis from media containing 10mM (a saturating concentration) was chosen because this reaction is more sensitive than urea synthesis in that it requires 30% more ATP, mol per mol, and requires a longer chain of enzyme catalysed reactions (Ross, 1972f). Hepatic protein synthesis (usually albumin) requires the use of labelled amino acids, the radioactivity of which would have contaminated the apparatus, or sensitive assays for other liver proteins (e.g. fibrinogen). The rate of albumin (Kelman, Saunders, Wicht, Frith, Corrigall, Kirsch and Terblanche, 1972) and urea (Schimke, 1964) synthesis by the livers of protein depleted rats, is known to be diminished. This fact in itself need not be an objection to their use, providing the rate of the reaction remains constant over the period of the experiment (Sacks, 1977a).

The determination of lactate gluconeogenesis was performed in experiments separate from those for somatomedin generation. Animals from all three nutritional groups were studied:

1) The following protocol was observed: time 0 min was taken as the moment that perfusate flow was achieved, the circuit was primed with 67 ml of glucose free medium, at 28 min 1.0 ml of 0.7M neutral L(+) Lactate (700μmol) was added to the reservoir. By 30 min the lactate concentration of the medium was ∿ 10mM.

2) Lactate sampling at 0, 30, 60, 90 and 120 minutes.

0.6 ml of perfusate was aspirated from the reservoir and deproteinised by the addition of 0.2 ml of ice cold 12% perchloric acid, centrifuged and the supernatant frozen until analysed for lactate (see Appendix B).

3) Glucose sampling at 0, 10, 20, 30, 45, 60, 75, 90 and 120 minutes. 0.6 ml of perfusate was aspirated from the reservoir for glucose analyses (see Appendix B).

4) Perfusate volume was determined at the end of perfusion by allowing the perfusate to drain from the liver and the circuit pumped dry (1 ml was left behind). Thus the initial volume (ml) = volume drained + volume of samples taken + 1. The volume at each time of sampling could be calculated. Total perfusate glucose and lactate at each time point = perfusate volume x concentration. Total glucose and lactate were plotted against time and rates of glucose formation and lactate removal were obtained from the gradients of the plots and expressed as μmol/min/g wet or dry liver weight.

5) Results: The plots from a representative experiment are shown graphically in Fig. 8.3. Glucose appearing in the medium during the initial 30 minute perfusion represented glucose formed from residual liver glycogen by glycogenolysis (Hems, Ross, Berry and Krebs, 1966). When no lactate was added at 30 minutes there was only a slight increase in glucose due to endogenous gluconeogenic substrate in the liver (the glycogenolytic substrate now having been depleted) (Hems, Ross, Berry and Krebs, 1966). When lactate was added (10mM) there was a linear increase of glucose with a reciprocal linear fall in lactate. A delay of up to 15 minutes after the addition of lactate sometimes occurred as previously described (Hems, Ross, Berry and Krebs, 1966). In the case of

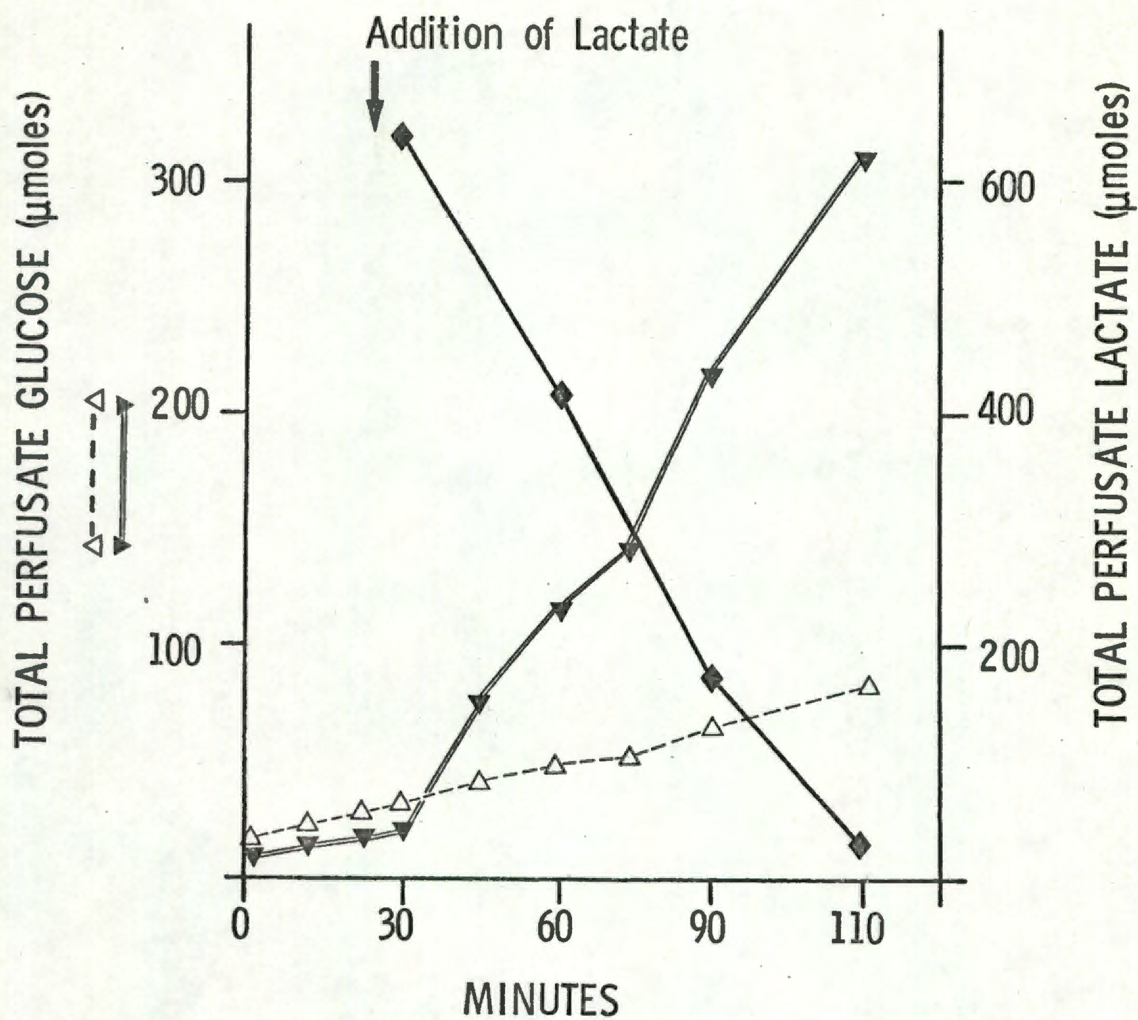


Fig. 8.3 Time course of glucose generation (▼) and lactate clearance (♦) by a perfused normal rat liver. The generation of glucose (Δ) from another liver in the absence of added lactate is also shown.

of larger livers from well-nourished animals, the glucose production diminished between 90 and 120 minutes, at this stage the perfusate lactate had fallen to 4mM (n = 6) and linear glucose output could be restored by the further addition of lactate. In the case of the smaller protein-malnourished livers, glucose output was linear until 120 minutes at which stage the lactate concentration was 9mM (n = 6). These results show that the flattening of glucose output from larger livers was due to substrate depletion and not deteriorating liver function. The maximal rates of gluconeogenesis from lactate are shown in Table 8.2 and Table 8.3. The rates in all 3 nutritional groups were similar, whether expressed in terms of wet or dry liver weight and resemble those of Hems, Ross, Berry and Krebs (1966) (1.0 $\pm$ 0.06 μmol/min/g wet weight), Exton and Park (1967) (1.0 $\pm$ 0.3 μmol/min/g wet weight) and Sacks (1977b) (normal rats 0.85 $\pm$ 0.05 μmol/min/g wet weight, protein-depleted rats 0.75 $\pm$ 0.08 μmol/min/g wet weight).

vi) Summary of tests of hepatic viability (Table 8.2): Table 8.2 summarises the results of the experiments which were used to determine the adequacy of liver function during perfusions of 2 hours. No single test is an adequate test of viability but with satisfactory performance by 8 criteria, especially those of metabolic synthetic function and hormone sensitivity, the livers were considered adequately viable for further experiments on somatomedin generation.

C Discussion

i) The choice of perfusion as a technique to study somatomedin generation: The methods available to study somatomedin gene-

TABLE 8.3

RATES OF GLUCONEOGENESIS WITH AND WITHOUT LACTATE (10 mM)

	4% Protein Diet	20% Protein Diet	Standard Diet
Added Lactate (μmol/min/g wet wt)	0.71 ± 0.19 (7)	0.71 ± 0.11 (6)	1.02 ± 0.16 (8)
No added Lactate (μmol/min/g wet wt)	0.11 ± 0.10 (6)	0.10 ± 0.11 (6)	0.14 ± 0.12 (6)

Mean ± SEM (number in parenthesis)

(No significant difference if results are expressed as μmol/min/g dry weight)

ration by the liver in protein malnutrition include homogenates, subcellular fractions enriched with microsomes, isolated hepatocytes, liver slices (incubated or perifused) or the intact perfused liver. The isolated in situ liver perfusion technique was chosen and the advantages and disadvantages are compared to some of the techniques previously used to study somatomedin generation.

a) When the isolated organ is perfused, the macroscopic and microscopic morphology remain intact, thus the architectural relationships of cells to each other and of intracellular organelles within cells are preserved. Similarly the so-called physiological compartments of the intravascular, interstitial and intracellular spaces are preserved. Cell membranes which are vital permeability barriers maintaining gradients between cells and the interstitial compartment and between organelles within cells are not disrupted as is the case in homogenates and subcellular fractionations of homogenates such as those used by Hall and Uthne (1971).

b) The nutrient and oxygen-carrying medium circulates through the vascular space as it would in life. In the case of liver slices, exchange of nutrients, gases and metabolic substrates is by diffusion across the whole tissue for distances of 0.5 to 0.2 mm.

ii) The choice of the in situ perfusion technique of Hems, Ross, Berry and Krebs (1966): The advantages included:

a) Simple operative technique. The entire procedure could be conducted by a single operator without assistance and the difficult transfer of the isolated liver to an organ-bath is eliminated.

b) Once cannulation and connection to the circuit was completed very little supervision was required and the flow rate remained remarkably constant as the positions of the cannulae were maintained throughout.

c) The liver itself was hardly handled at all, and this atraumatic method has been shown to be important in at least certain effects of insulin on the liver (Haft and Miller, 1958).

d) Should the bile duct not be cannulated there is free flow of bile into the duodenum. In isolated liver perfusions the bile duct must be cannulated to prevent bile flowing into the medium where it may have untoward effects. Alternatively the bile duct may be ligated (Abraham, Dawson, Grasso and Goldberg, 1968) with resultant unphysiological cholestasis.

e) Because of the advantages listed above, Ross (1972b) recommends the in situ technique except where frequent liver tissue samples or liver surface measurements are the primary aim of the perfusion.

iii) How physiological is perfusion via the portal vein alone?: Perfusion via the portal vein alone is not entirely physiological in that in life the portal vein supplies 80% and the hepatic artery ∿ 20% of the liver's blood supply. The portal circulation is low pressure (12-14 cm blood), non-pulsatile and relatively deoxygenated (Ross, 1972c). Of especial interest is the observation that liquid media injected via the portal vein (silicone rubber) did not enter the hepatic arterial system, while injection into the hepatic artery does fill part of the portal venous system and central sinusoids (Hase and Brim, 1966). Most authors agree that perfusion

via the portal vein alone is adequate to provide liver oxygen requirements and that in the rat at least the hepatic arterial supply is not required for normal biochemical function (Ross, 1972d). There are liver perfusion circuits by means of which both circulations may be perfused (Powis, 1970) but these were not considered because their complexity outweighed the theoretical advantages.

iv) The choice of a semisynthetic red cell containing medium: To date somatomedin generation has been studied from livers perfused with synthetic media, usually Waymouth's MB 752/1 with bovine serum albumin (BSA) 0.5-1.0% (See Table 8.5). The reason for the use of a complex tissue culture medium is that it will support the cartilage of the somatomedin bioassay after perfusion (Ross, 1972g). We however chose a semisynthetic medium (KRB, 2.5% BSA), aged human red cells to a final haematocrit of ∿ 8%) for the following reasons:

a) The higher concentration of BSA was much closer to that in vivo and provided a more physiological colloid osmotic pressure for the medium which prevented the liver becoming oedematous (confirmed by wet/dry weight ratios).

b) The aged human red cells greatly increased the oxygen carriage of the medium, ensuring adequate oxygenation of the tissue without the problems of very high flow rates. Aged red cells are unable to glycolyse and do not influence the glucose in the medium in experiments studying gluconeogenesis (Hems, Ross, Berry and Krebs, 1966).

c) The amino acids, other nutrients and antibiotics required to maintain the cartilage fragments in the bioassay were all added to the medium post perfusion after the

centrifugation of the red cells. The addition of these substances preperfusion, as is usually the case, may result in their loss by catabolism during perfusion (especially gluconeogenic amino acids). In addition it was found in preliminary experiments using Waymouth's medium, that bile flow was very poor. Although the mechanism of this phenomenon was never elucidated it was decided to use a semisynthetic medium in the place of Waymouth's medium.

v) Assessment of viability: The perfused livers were judged as being satisfactory by 8 criteria: macroscopic appearance, histology, wet/dry weight ratio, perfusate flow rate, bile flow, oxygen consumption, lactate gluconeogenesis, glucagon induced glycogenolysis, as discussed earlier in this chapter.

In much of the previously published work on the generation of somatomedin by perfused livers there is no reference to the viability of the preparation (see Tables 8.4 and 8.5). Only in the cases of Daughaday, Phillips and Mueller (1976), Phillips, Herington, Karl and Daughaday (1976) and Hill, Francis, Conroy and Ashton (1977), where normal lactate/pyruvate ratios were demonstrated, and Heinrich, Schalch, Miller and Johnson (1977), where the synthesis of 5 plasma proteins was measured, has there been biochemical proof of viability.

In earlier work there is only reference to flow rate (in some cases); these were 3.5-5.0 ml/min (see Table 8.5). For a red cell free medium gassed at atmospheric pressure with 95% O_2:5% CO_2, such flow rates are at best marginally adequate in providing oxygenation. The flow rates described by Daughaday, Phillips and Mueller (1976), Phillips, Herington, Karl and Daughaday (1976) and Hill, Francis, Conroy and Ashton (1977) are three times greater than other workers (15-16 ml/min) and are adequate.

Table 8.4.

AVAILABLE DATA ON SOMATOMEDIN GENERATION BY INCUBATED OR PERIFUSED LIVER (OR KIDNEY)

Authors	Animals	Perfusate	Hormones	Assay	Incubation/ perfusion system	Tests of viability	Volumes	Duration and flow rates
McConaghey (1972) J.Endocrinol. 52: 1	Normal 6 month old male rats. Normal 3 week old rats. Hypox. 3 week old rats.	Waymouth's MB 752/1 0.5% BSA (fraction V).	bGH (NIH-GH-B15) 0.1 µg/ml 1.0 µg/ml 10.0 µg/ml 100.0 µg/ml	Medium frozen in sterile bottles. Assayed for sulphation factor with normal 3 week old rat costal cartilage assay (1 point).	(a) 100-150 mg of pooled 1 mm thick liver slices in flasks in shaking water-bath in room air. (b) As above except slices repetitively frozen and thawed before incubation. (c) As above except slices homogenized before incubation.	-	4 ml changed 2-hourly.	Medium changed 2-hourly for up to 12 hours
Hintz Clemmons & van Wyk (1972) Pediat.Res. 6: 353/93	Rats	TC 199	hGH 100 µg/ml	Assayed for sulphation factor with hypox. rat costal cartilage assay. Non-parallel dose response with rat serum standard Sephadex G75 chromatography: major peak 6000-12000 mw, small peak <2000 mw	700 mg liver slices 0.5 - 1.0 mm thick superfused in 1.5 ml chamber	-	-	1 ml/min
McConaghey & Dehnel (1972) J.Endocrinol. 52: 587	Normal male Sprague-Dawley rats. Hypox. male Sprague-Dawley rats.	Waymouth's MB 752/1 0.5% BSA penicillin 100 u/ml streptomycin 100 µg/ml	bGH (NIH-GH-B15) 10 µg/ml	Sulphation factor assayed with normal rat costal cartilage assay (1 point)	Kidney slices incubated in air in shaking water bath for 2 hours.	-	-	2 hours

Table 8.5. AVAILABLE DATA ON SOMATOMEDIN GENERATION BY THE PERFUSED LIVER (OR KIDNEY)

Authors	Animals	Perfusate	Hormones	Assay	Perfusion system	Tests of viability	Volumes	Duration and flow rates
McConaghey & Sledge (1970) Nature 225:1249	Normal adult rats.	Waymouth's MB 752/1 0.5% BSA penicillin 100 u/ml. streptomycin 100 μg/ml.	bGH 10 μg/ml	Centrifugation, millipore filtration and frozen. Assayed for sulphation factor by hypox. rat costal cartilage assay (1 point) and autoradiography of in vitro ^{35}S uptake by cartilage.	In situ (F.Chavey-Peon unpublished technique), a modification of that of Mortimore (1963) liver perfusion.	-	-	3.5 -4.5 ml/min.
McConaghey (1972) J.Endocrinol. 52: 1	Male Sprague-Dawley rats	Waymouth's MB 752/1 0.5% BSA (fraction V) penicillin 100 u/ml streptomycin 100 μg/ml	bGH (NIH-GH-B15) 10 μg/ml	Centrifugation, millipore filtration and frozen. Assayed for sulphation factor by hypox. rat costal cartilage assay (1 point).	In situ (F.Chavey-Peon) liver perfusion.	-	100 ml washout with hormone-free medium. 100 ml bGH containing medium re-circulated thrice. 100 ml wash-out with hormone-free medium.	3.5 - 4.5 ml/min.
McConaghey & Dehnel (1972) J.Endocrinol. 52: 587	Normal Sprague-Dawley rats. Hypox. Sprague-Dawley rats.	Waymouth's MB 752/1 0.5% B.S.A. penicillin 100 u/ml. streptomycin 100 μg/ml.	bGH (NIH-GH-B15) 10 μg/ml	Centrifugation, millipore filtration and frozen. Assayed for sulphation factor by normal rat costal cartilage assay. (1 point).	In situ kidney perfusion. (Dehnel unpublished technique).	-	100 ml washout with hormone-free medium. 100 ml bGH containing medium re-circulated thrice.	-
Williams & Hughes (1974) J.Endocrinol. 63: 585	Rats	Waymouth's MB 752/1	hGH 10 μg/ml	Assayed for sulphation and thymidine factor by rat costal cartilage assay (4 point). Dose response parallel to rat serum standard.	In situ liver perfusion (technique of McConaghey, 1972).	-	100 ml washout with hormone-free medium. 100 ml bGH containing medium re-circulated thrice.	1 hour. 5 ml/min.
Dehnel, McConaghey & Francis (1974) J.Endocrinol. 62: 355	Adult male Sprague-Dawley rats.	Waymouth's MB 752/1 0.5% BSA (fraction V) penicillin 100 u/ml streptomycin 100 μg/ml	bGH 10 μg/ml	Assayed for sulphation factor by normal rat costal cartilage assay. (1 point). Ultrafiltration with Amicon XM50, UM20E, UM10, UM2.	In situ liver perfusion.	-	100 ml washout with hormone-free medium. 100 ml bGH containing medium re-circulated thrice.	-
Ash & Francis (1975) J.Endocrinol. 62: 56: 71	Adult male Sprague-Dawley rats (Wt.250 g).	Waymouth's MB 752/1 0.5% B.S.A. (fraction V) penicillin 100 u/ml. streptomycin 100 μg/ml	bGH (NIH-GH-B15) 10 μg/ml	Centrifugation, millipore filtration (0.45 μM). Assayed for sulphation factor and thymidine factor by isolated rabbit chondrocytes from articular cartilage and epiphyseal growth plate cartilage.	In situ liver perfusion.	-	100 ml washout with hormone-free medium. 100 ml bGH containing medium re-circulated thrice.	3.5 - 4.5 ml/min.

Table 8.5 (continued) AVAILABLE DATA ON SOMATOMEDIN GENERATION BY THE PERFUSED LIVER (OR KIDNEY)

Author	Animal	Perfusate	Hormones	Assay	Perfusion system	Tests of viability	Volumes	Duration and flow rates
Francis & Hill (1975) Nature 255: 167	Normal male Sprague-Dawley rats (Wt.220-260 g).	Waymouth's MB 752/1 0.5% B.S.A. (fraction V)	bGH (NIH-GH-B17) 0.1, 1.0, 10.0 µg/ml. Ovine prolactin (Sigma or NIH-S10) 5.0, 50, 500 ng/ml.	Centrifugation, millipore filtration and frozen. Assayed for sulphation factor by normal rat costal cartilage assay (1 point).	In situ liver perfusion	-	100 ml washout with hormone-free medium. 100 ml hormone containing medium re-circulated thrice.	-
Phillips, Herington, Karl & Daughaday (1976) Endocrinol. 98: 606.	Normal male Sprague-Dawley rats (Wt.85-150g). Hypox. male rats (Wt.85-110g)	Waymouth's MB 752/1 1% B.S.A. (fraction V)	bGH (NIH-GH-B17) in vitro 25 µg/ml. In vivo to hypox. rats 500 µg 53, 29 and 6 hours ip before perfusion.	Millipore filtration and frozen. Assayed for sulphation factor by hypox. rat costal cartilage. (Supplement of penicillin, streptomycin, amphotericin and polymyxin added before assay).	Ex situ liver perfusion	Lactate/Pyruvate ratio 13.5	40 ml changed every 30 min for 3 hours.	3 hours 15-16 ml/min.
Daughaday, Phillips & Mueller (1976) Endocrinol. 98: 1214	Hypox.male Sprague-Dawley rats (Wt.85-110g)	Waymouth's MB 752/1 1% B.S.A. (fraction V)	bGH (NIH-GH-B17) 0.25 µg/ml 25.0 µg/ml Insulin (Lilly) 100 µU/ml 1000 µU/ml	Assayed for sulphation factor by hypox. rat costal cartilage (Supplement of penicillin streptomycin, amphotericin and polymyxin added before assay).	Ex situ liver perfusion	Lactate/Pyruvate ratio	40 ml changed every 30 min for 3 hours.	3 hours 15-16 ml/min.
Wondergem, Gaspard, Hamamdzic & Klitgaard (1977) Fed.Proc. 36: 497	Normal rats Hypox. rats. Hypox. rats treated with bGH.	-	bGH 10 µg/ml bGH 25 µg/ml	Assayed for sulphation factor by chick cartilage Sephadex G25 chromotography of acid-ethanol extracts showed identity with rat serum sulphation factor.	In situ liver perfusion	-	-	-
Schalch, Heinrich, Johnson, Koch & Miller (1977) Clin.Res. 25: 399A	Normal rats. Hypox. rats Hypox. rats pretreated with GH, T_3, cortisol and insulin.	-	GH T_3 Cortisol Insulin	Assayed for NSILA by competitive protein binding assay.	Perfused liver	-	-	12 hours
Heinrich, Schalch, Miller & Johnson (1977) Acta Endocrin. Suppl. 212:100	Normal rats. Hypox. rats. Hypox. rats pretreated with GH, T_3, cortisol and insulin	-	GH T_3 Cortisol Insulin	Assayed for NSILA by competitive protein binding assay.	Perfused liver	Synthesis of 5 different plasma protein	-	12 hours

Table 8.5 (continued)

AVAILABLE DATA ON SOMATOMEDIN GENERATION BY THE PERFUSED LIVER (OR KIDNEY)

Author	Animal	Perfusates	Hormones	Assay	Perfusion system	Tests of viability	Volumes	Duration and flow rates
Hill, Francis, Conroy & Ashton (1977) Calcif.Tiss. Res. 22 : 378	Male Sprague-Dawley rats (Wt.180-200g) Normal. Sham-operated. Adrenalectomised with or without cortisol 0.2 to 20 μg.	Waymouth MB 752/1 0.5% B.S.A. (fraction V)	Ovine prolactin 50 ng/ml Cortisol 5 μg/ml	Assayed for sulphation factor by normal rat costal cartilage assay.	In situ perfusion (Based on that of Mortimore (1963)	Lactate/Pyruvate ratio <13	150 ml wash-out with hormone-free medium 100 ml hormone containing medium re-circulated thrice.	<30 min 15 ml/min
Hill, Francis, & Milner (1977) J.Endocrinol. 75: 137-143	Male Long-Evans and Sprague-Dawley rats (Wt.180-250 g). Normal and Hypox. In vivo to (150-300g) Hypox. or sham operated. 50 μg/ injection x 5 at 12 hourly intervals.	Waymouth's MB 752/1 0.5% B.S.A. (fraction V) penicillin 100 u/ml. streptomycin 100 μg/ml. HEPES 4.76 mg/ml.	Rat prolactin (B-1) 50ng/ml	Assayed for sulphation and thymidine factors by normal rat costal cartilage assay.	In situ liver perfusion (Based on that of Mortimore 1963)	Lactate/Pyruvate ratio 10-13	150 ml wash-out with hormone-free medium 100 ml hormone containing medium re-circulated thrice.	15 ml/min

SECTION II

A Somatomedin generation by the isolated in situ perfused liver

Somatomedin activity generation by the isolated perfused livers of 4% protein fed, 20% protein fed and standard diet fed rats were studied under five hormonal conditions. (The rats fed standard rat cubes were a pilot study conducted before animals fed special diets were studied):

1. No added hormones
2. Human or bovine growth hormone 10 μg/ml
3. Porcine insulin 1000 μU/ml
4. Porcine insulin 1000 μU/ml + bovine growth hormone 10 μg/ml.

B Methodology

i) The perfusate was prepared and the liver prepared for perfusion as already described.

ii) In the case of 20% protein fed and standard diet fed rats the liver was subjected to an initial "once through washout" of 100 ml of hormone-free perfusate which required about 10 minutes and which was collected in a measuring cylinder rather than being returned to the reservoir. With 4% protein fed rats a similar procedure was carried out with 30 ml of hormone-free perfusate which also required about 10 minutes and was equivalent on a liver weight basis.

iii) At the end of the "washout" phase the outflow cannula was inserted into the reservoir and the perfusion circuit thus converted from a straight through to a recycling system.

iv) In those perfusions in which hormonal additions were made, these were to the reservoir and the remaining 35 ml of perfusate. In the case of growth hormone a single addition was made directly

after the "washout" phase; with insulin the first addition was directly after the "washout" phase and smaller additional amounts of insulin were added at 30 minute intervals to compensate for the rapid clearance of insulin. The amounts of insulin added to compensate for liver clearance were derived from the work of Sacks (1977c).

v) A sample of uncirculated medium from the same batch with the same hormonal supplement was reserved and at the end of the perfusion period (2 hours) a sample of circulated medium was collected for radioimmunoassay of the hormone infused, to confirm the levels, and for bioassay of somatomedin. Samples were prepared as follows: red cells were removed by centrifugation (2000g for 10 minutes) and the supernatant decanted, rapidly frozen and stored at -20°C until assay.

vi) Preparation of samples for assay of somatomedin activity. Samples were thawed and filtered into sterile tubes through (EG 0.2 μm) millipore filters. Circulated and uncirculated media from the same batch were always dealt with in the same assay. The sterile perfusate was then pipetted into the incubation rack at a concentration of 80%, the volume being made up by a supplement of amino acids and antibiotics which gave amino acid and antibiotic concentrations identical to those of the bioassay medium described in Chapter 3 and Appendix B. The amino acids and antibiotics were themselves dissolved in the KHB-BSA medium of the perfusate.

Some samples were subjected to assay (after supplementation as above) at dilutions in assay buffer of 80, 40, 20 and 10% against a pooled rat serum standard of arbitrary (normal) potency 1 U/ml.

All perfusates were assayed in triplicate by measuring the $^{35}SO_4$ uptake into porcine costal cartilage discs as described in Chapter 3. In the case of the "single point" assay the amount of

$^{35}SO_4$ incorporated with uncirculated medium from a given perfusion was taken as a nominal 100% and the $^{35}SO_4$ incorporation occurring with the circulated medium expressed in terms of this.

C Results

The $^{35}SO_4$ incorporation into porcine costal cartilage discs stimulated by circulated perfusate (relative to uncirculated perfusate) from the livers of protein malnourished and control animals is shown in Table 8.6. In dilution samples were parallel to standard.

TABLE 8.6

STIMULATION OF $^{35}SO_4$ INCORPORATION INTO PORCINE COSTAL CARTILAGE DISCS BY PERFUSATES AT AN 80% CONCENTRATION, WITH AMINO ACID AND ANTIBIOTIC SUPPLEMENT

Hormones	4% Protein Diet	20% Protein Diet	Standard Diet
Nil	108.5± 4.9(11)*	124.1± 5.6(12)	148.6±12.1(6)
GH (10μg/ml)	158.8±11.2 (9)§o	243.3± 9.7(12)†	214.8±11.8(14)†
Insulin (1000 μU/ml)	147.3±11.0(10)§o	211.5±10.4(10)†ø	223.0±11.9 (6)‡
GH + insulin	159.9±10.7 (8)§o	251.2±11.9 (8)†	-

1. as bGH and hGH were not significantly different from each other they are presented combined
2. unperfused medium = 100
3. Mean ± SEM (number)
4. Student's unpaired t test

* $p < 0.05$ 4% Protein diet vs 20% Protein diet or Standard diet

§ $p < 0.005$ 4% Protein diet vs 20% Protein diet or Standard diet

† $p < 0.001$ hormonal addition vs hormone free (20%) or Standard diet

‡ $p < 0.002$ hormonal addition vs hormone free Standard diet

o $p < 0.005$ hormonal addition vs hormone free (4%)

ø $p < 0.05$ Insulin vs GH or GH + insulin (20%)

D Discussion

The generation of somatomedin activity by the isolated perfused liver of protein malnourished and control rats.

i) Hormonal stimuli previously shown to stimulate somatomedin generation. Human and bovine growth hormone at 10 μU/ml are concentrations previously shown to stimulate somatomedin generation in perfused liver systems (McConaghey and Sledge, 1970; Daughaday, Phillips and Mueller, 1976). The range of growth hormone concentrations used has been 0.1 to 100 μg/ml (McConaghey, 1972) with the majority of workers using 10 or 25 μg/ml (see Tables 8.4 and 8.5). The lowest level of growth hormone producing detectable somatomedin activity generation is 1 μg/ml (McConaghey, 1972; Francis and Hill, 1975). The somatomedin activity generation dose response relationship with growth hormone remains incompletely explored but at present pharmacological or maximum physiological doses of growth hormone are required to produce detectable somatomedin activity. (In the rat, growth hormone peaks as high as 1 μg/ml can occur (J. Martin, personal communication, 1977). To date, only insulin at the pharmacological dose of 1000 μU/ml, has produced somatomedin activity generation by the perfused liver (Daughaday, Phillips and Mueller, 1976).

The subject of interactions between growth hormone and insulin has only been partly explored (Daughaday, Phillips and Mueller, 1976) but a synergistic action is suggested by the fact that doses of growth hormone (0.25 μg/ml) and insulin (100 μU/ml) neither of which produced significant somatomedin activity generation alone together resulted in significant generation of somatomedin activity. At the high insulin concentration (1000 μU/ml) the suboptimal growth hormone concentration (0.25 μg/ml) had no additive effect probably because the response to insulin was already maximal. To

study the interaction between the somatomedin activity generation dose response relationships to insulin and growth hormone is an immensely complex and laborious task and has not as yet been attempted.

Ovine prolactin at concentrations as low as 50 ng/ml has been shown to result in significant generation of somatomedin activity by the isolated perfused liver (Francis and Hill, 1975). This phenomenon also has a circadian variability in that more somatomedin is generated at 1100 hours than at 1500 hours (Francis and Hill, 1975) and may be related to the circadian variation in glucocorticoids (Hill, Francis, Conroy and Ashton, 1977) although the relevance of ovine prolactin (structurally similar to growth hormone) and the use of cortisone in the rat, whose physiological glucocorticoid is corticosterone, may be questioned.

ii) Hormones used to study somatomedin generation by protein-malnourished livers. The hormones used were selected to seek an answer for the finding of low serum somatomedin activity with normal growth hormone and low insulin in experimental protein malnutrition. Would the malnourished liver respond in vitro to pharmacological levels of growth hormone (when the intact animal failed to do so) and what role did the hypoinsulinaemia play?

The method of hormone addition. Only a single dose of growth hormone was added to the perfusate to give an initial concentration of 10 μg/ml; this is in keeping with the work of others (see Tables 8.4 and 8.5) and the relatively slow clearance of growth hormone by perfused livers in this system (Sacks, 1977, personal communication). The addition of supplementary insulin at 30 minute intervals using the insulin clearance by normal and malnourished livers studied in an identical system (Sacks, 1977c) compensates for the relatively rapid clearance of insulin. This corres-

ponds in some ways to the intermittent changes of insulin containing medium by Daughaday, Phillips and Mueller (1976). Haemolysis is a major cause of insulin degradation in perfusion systems using red cell-containing media, in all experiments great care was taken to prevent haemolysis and that which occurred was within the range shown not to cause excessive insulin degradation (Sacks, 1977c). The high albumin content of the perfusate prevented excessive adsorbance of hormone to glass.

iii) The "washout" phase of perfusion. This served to remove residual blood and thus any already extracellular somatomedin activity trapped in the liver and is a widely practised technique (Table 8.4). The volume used in the 20% protein fed and standard diet fed rats was comparable with those used by other investigators for animals of approximately similar weight. The lesser volume used for the 4% protein fed rats provided a similar volume washout on a gm liver weight basis and required a similar time (∿ 10 minutes).

iv) The period of perfusion, volumes of perfusate and expression of results. In work published to date two approaches have been used; following a washout phase a small volume (40 ml) was recirculated and changed every 30 minutes for three hours which allows for the study of the time-course of somatomedin activity generated (Phillips, Herington, Karl and Daughaday, 1976; Daughaday, Phillips and Mueller, 1976) or larger volumes (100 ml) were recirculated three times through the liver, multiple recirculation being required before a significant generation of somatomedin activity could be demonstrated (see Table 8.5).

The approach in studying the malnourished rats and their controls was somewhat different, a smaller volume of perfusate (35 ml)

was recirculated through the liver for 2 hours. The multiple recirculation of a small volume was to maximise changes in somatomedin activity generated. The time period of 2 hours was one in which variability of the liver preparation was proven.

A comparison between the somatomedin activity generated in such a system by livers with such different weights and flow rates is difficult. The following factors may influence apparent somatomedin activity generation:

a) Perfusate volume (constant in this system)

b) Duration of perfusion (constant in this system)

c) Washout phase (adequate and equal on a liver weight basis in this system)

d) Perfusate flow rate (approximately equal in the different livers on a liver weight basis in this system)

e) Mass of liver tissue (a major difference which was partly compensated for in some of the factors above).

Thus values were expressed per liver wet weight. Corrections for flow rate, while somewhat altering values, did not cause major overall changes. Previous workers have not taken liver or animal weight, variations in flow rate into account, even where animals of different weights (e.g. hypophysectomised and control rats) were used (Phillips, Herington, Karl and Daughaday, 1976). Only in the case of Schalch, Heinrich, Johnson, Koch and Miller (1977) was body surface area taken into account in a study of NSILA generation.

v) The preparation of samples for assay. Centrifugation of red cells, storage at -20°C and sterilisation by millipore filtration are standard techniques in studying somatomedin activity generation in incubation, perifusion and perfusion systems. Micro-

biological contamination of the sample may result in degradation of hormones in it or may interfere with the cartilage fragment incubation which is vital to the somatomedin bioassay. Antibiotics were added to the medium post perfusion for the same reason. Most workers add antibiotics to the medium pre-perfusion (except Daughaday, Phillips and Mueller, 1976) and this may result in alteration in antibiotic concentration and possibly adversely affect the quality of the liver preparation. It was impossible to sterilise the perfusion apparatus but it was at all times kept meticulously clean, and with millipore filtration and antibiotic addition, frequent microbiological surveillance showed contamination by microorganisms not to be a problem.

The addition of the amino acids to the perfusate post perfusion provided known and adequate concentrations, which may have been altered by processes such as gluconeogenesis, if such amino acids had been added before perfusion. Waymouth's medium 752/1 also does not contain optimal concentrations of serine and glutamine for somatomedin bioassay and the release of these amino acids may contribute to the low molecule weight (∿ Daltons) somatomedin activity described in liver perfusates by Dehnel, McConaghey and Francis, (1974).

vi) The assay of somatomedin activity in perfusates.

a) Because of the large numbers of perfusate samples generated by the perfusion programme, all samples were initially assayed for somatomedin activity by a simplified assay at only one concentration (80%) in triplicate. The incorporation of $^{35}SO_4$ occurring with uncirculated perfusate was given a nominal value of 100 and circulated perfusate of the same batch compared with this in the same assay.

Results were expressed as:

CPM $^{35}SO_4$ incorporated/mg cartilage/g wet liver weight = 100 for un-
circulated perfusate

or

CPM $^{35}SO_4$ incorporated/mg cartilage/g wet liver weight/flow rate
(ml/min) = 100 for uncirculated perfusate

A similar approach has been used by the majority of investigators to date (Tables 8.4 and 8.5) and is probably a satisfactory method of estimating somatomedin activity of perfusates.

Assay in serial dilutions of perfusates against a standard of pooled rat serum was carried out on a limited number of perfusates selected at random and in all cases parallelism of perfusate to serum standard was found in keeping with the findings of Wondergem, Gaspard, Hamamdzic and Klitgaard (1977) and Williams and Hughes (1974).

b) The majority of workers have used $^{35}SO_4$ incorporation into rat costal cartilage as a bioassay, using either hypophysectomised rats or immature intact rats (see Tables 8.4 and 8.5). Only Wondergem, Gaspard, Hamamdzic and Klitgaard have used the chick cartilage sulphation assay and Ash and Francis (1975) a rabbit chondrocyte assay examining $^{35}SO_4$ and tritiated thymidine incorporation. The porcine costal cartilage assay has not been used by others to date. It has an additional advantage in that the SO_4 content of the medium, which may change during perfusion (Phillips, Herington, Karl and Daughaday, 1976) does not influence the assay if the $^{35}SO_4$ stage of the assay is performed in the "Community Bath". NSILA has been measured using a competitive binding protein assay (Heinrich, Schalch, Miller and Johnson, 1977).

vi) Somatomedin activity generation by protein malnourished and control animals:

a) The livers of 20% protein fed and standard diet fed rats perfused with a hormone free medium showed a significant generation of somatomedin activity over two hours. In time-course experiments, Phillips, Herington, Karl and Daughaday (1976) have shown the major fraction of somatomedin generated in a hormone-free perfusion to occur early (up to 90 min). Schalch, Heinrich, Johnson, Koch and Miller (1977) reported similar results for the generation of NSILA. This probably represents a carryover of the growth hormone and other hormonal factors present in vivo in that they showed reduced somatomedin generation from the livers of hypophysectomised rats which could be restored to normal by pretreatment in vivo with growth hormone. Thus the somatomedin activity generated by hormone-free perfusions may be taken as an indication of the in vivo stimulatory milieu for somatomedin generation and the liver's capacity to respond to this. In contrast to the significant generation of somatomedin activity by control livers in a hormone-free perfusion, that generated by the livers of protein malnourished animals was less and only just detectable. This observation may indicate that the low serum somatomedin activity observed in protein malnourished rats is due to reduced hepatic generation. Earlier workers were unable to show somatomedin activity generation from hormone-free perfusions (e.g. McConaghey and Sledge, 1970) but the explanation for this offered by Phillips, Herington, Karl and Daughaday (1976), namely that due to low flow rate the preparation was sub-optimally oxygenated, seems valid.

b) Human and bovine growth hormones, both known to be bioactive in the rat, caused significant and similar stimulation of

somatomedin generation in 20% and standard diet fed control animals, and while a response occurred in the 4% protein fed rat livers perfused with these hormones, it was significantly smaller than that of the control animals. This observation is consistent with the failure of protein malnourished rats to grow or their serum somatomedin to increase after high dose exogenous growth hormone administration. It represents an end-organ resistance at liver level to an action of growth hormone in protein malnutrition. The only similar resistance of the perfused liver to a stimulus for somatomedin generation is that produced by cortisol on prolactin-induced somatomedin generation (Hill, Francis, Conroy and Ashton, 1977).

c) The perfusion of well nourished control livers with insulin (1000 μU/ml) resulted in a generation of somatomedin activity significantly less than that achieved with growth hormone but not out of keeping with the findings of Phillips, Herington, Karl and Daughaday (1976). As with growth hormone, the response of the protein malnourished livers to insulin was less than that of well nourished controls but unlike the controls the responses to growth hormone and insulin were of equal magnitude - this may represent a relatively greater sensitivity to insulin of the malnourished livers (possibly a result of the in vivo hypoinsulinaemia in these animals).

d) The combination of growth hormone (10 μg/ml) and insulin (1000 μU/ml) was equal to growth hormone alone in stimulating somatomedin activity generation from well nourished and protein malnourished livers. The failure to obtain an additive effect was probably due to the high concentrations of hormones used, each already giving maximal stimulation and was in keeping with the findings of Daughaday, Phillips and Mueller (1976).

e) The mechanism for the reduced somatomedin generation by protein malnourished livers may be:

Specific, as a homeostatic response to protein malnutrition by which the rapid direct metabolic effects of growth hormone may be dissociated from the effects of somatomedins which growth hormone usually induces. Somatic growth may be seen as non-adaptive in a time of restricted substrates as occurs in protein energy malnutrition. Insulin, and possibly other hormones (e.g. glucocorticoids) may be amongst the factors operative.

Due to a generalised depletion of amino acid substrates for hormone synthesis. If this was the case then it was not due to differences in the circulating amino acids because all livers were perfused with an essentially amino acid free perfusate. However a depletion of the intracellular utilisable amino acid pool is probably present, but as the requirement for hormonal synthesis is such a minute fraction of hepatic protein synthesis, simple substrate deficiency seems a superficial explanation. In human protein energy malnutrition elevated growth hormone levels (and the low somatomedins associated with them) are inversely correlated to certain amino acids and infusions of these amino acids do not lower the growth hormones (Pimstone, Becker and Hansen, 1973).

Non-specific as a response to the deranged liver function which is a consequence of protein malnutrition. The livers were macroscopically fatty, had increased extractable lipid and the characteristic abnormal histology.

f) The term somatomedin activity generation has been used because the presence of somatomedin activity in the perfusates from stimulated or unstimulated livers may be due to release from preexistent stores or de novo synthesis. To date no work with inhibi-

tors of protein synthesis has been performed to elucidate this. Some workers (Williams and Hughes, 1974) have extrapolated somatomedin activity generation by perfused livers to synthetic rates and compared these to that obtained from the $T_{\frac{1}{2}}$ of somatomedin in the rat. This requires considerable assumptions and as the $T_{\frac{1}{2}}$ of somatomedin in the malnourished rat may differ from normal, no such extrapolations were made.

g) The nature of somatomedin activity in liver perfusates. The somatomedin activity of liver perfusates shares many characteristics with serum somatomedin (see Table 8.5).

It stimulates $^{35}SO_4$ incorporation into hypophysectomised and normal rat cartilage, chick cartilage and as demonstrated here, into porcine cartilage. Thymidine uptake into rat cartilage has also been demonstrated.

In those cases where multiple point bioassay has been conducted dose response curves of liver perfusate and rat serum are parallel (Wondergem, Gaspard, Hamamdzic and Klitgaard, 1977; Williams and Hughes, 1974). The non-parallelism discussed by Hintz, Clemmons and Van Wyk (1972) refers to fractions from chromatography only.

Liver perfusate somatomedin activity competes for binding on placental membranes similarly to serum somatomedin (Daughaday, Phillips and Mueller, 1976) and perfusate NSILA binds to specific NSILA binding protein (Heinrich, Schalch, Miller and Johnson, 1977).

Preliminary characterisation by Sephadex chromatography. (Hintz, Clemmons and Van Wyk, 1972; Wondergem, Gaspard, Hamamdzic and Klitgaard, 1977) and by Amicon molecular filters (Dehnel, McConaghey and Francis, 1974) shows a pattern not dissimilar from that found in serum.

E Conclusions

With a well validated in situ isolated perfused liver system significant stimulation of somatomedin activity generation was achieved with growth hormone, insulin and the two hormones in combination in normal rats.

In protein malnourished rats the somatomedin generation was reduced in hormone-free and growth hormone and insulin stimulated perfusions. These observations suggest that the reduced serum somatomedin activity of protein malnourished rats may be due to decreased hepatic generation. The mechanism for reduced somatomedin generation may thus in part be due to the hypoinsulinaemia of protein malnutrition.

CHAPTER NINE

SUMMARY OF CONCLUSIONS

"Ignorabimus, dubitemus" (Where we do not know, let us be cautious) *Emil DuBois-Reymond*

A satisfactory assay for the measurement of somatomedin activity in rat serum was established and its specificity, sensitivity and reproducibility documented.

A model of protein-energy malnutrition in the rat was chosen which has in common with the human syndrome, growth failure, hypoproteinaemia, hypoalbuminaemia and fatty infiltration of the liver. This model was found to have narrow, histologically inactive epiphyseal growth cartilage, the matrix of which did not differ histochemically from wellnourished controls, and reduced $^{35}SO_4$ incorporation into cartilage (as shown by autoradiography of sections of long bones and by liquid scintillation counting of dissolved costal cartilage). Exogenously administered growth hormone (known to be biologically active in the rat) failed to cause weight gain, increase in length or increase in tibial epiphyseal width.

Serum somatomedin activity was reduced in the protein malnourished rats, no obvious inhibitor could be demonstrated either by non-parallelism of dose-response compared to wellnourished standard or by mixing experiments. Low serum somatomedin activity was present in the face of normal growth hormone levels. Exogenously administered growth hormone failed to stimulate serum somatomedin levels in the protein malnourished animals. Insulin levels were low and this may be in part responsible for low somatomedin activity.

The cartilage of protein malnourished animals was found in vivo to incorporate $^{35}SO_4$ similarly to that of age-matched controls both basally and in response to normal rat serum while malnourished rat

serum hardly stimulated either type of cartilage, suggesting that cartilagenous end-organ resistance to somatomedin was not a major factor in the reduced growth rate of protein-energy malnutrition.

A method of isolated in situ liver perfusion was validated for protein malnourished and control animals in which the somatomedin activity generated by the livers of protein malnourished animals was less than controls either in the absence of hormonal stimuli, with growth hormone, with insulin and a combination of insulin and growth hormone. A slightly greater relative sensitivity of the malnourished animal's livers to insulin may reflect the importance of insulin as a factor in the maintenance of normal somatomedin activity. Reduced liver generation of somatomedin activity is postulated as being a possible cause for the reduced somatomedin activity of serum in protein-energy malnutrition, this may be either a non-specific reflection of deranged liver function or may represent an adaptive homeostatic mechanism whereby the direct actions of growth hormone might be permitted at a time that stimulation of growth is disadvantageous.

APPENDICES

"If you have built a perfect demonstration do not remove all traces of the scaffolding by which you have raised it"

Clark Maxwell.

INDEX

		Page
APPENDIX A : HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES		
A.	Routine staining with haemalum and eosin	181
B.	Metachromatic staining with toluidine blue	181
C.	Sweet's resorcin-crystal violet and picro-fuchsin stain	182
D.	Alcian blue (pH 1.0) for sulphate groups of mucopolysaccharides	183
E.	Alcian blue (pH 2.5) for acid groups (COOH and OSO_3H) of mucopolysaccharides	184
F.	Alcian blue : critical electrolyte concentration technique	184
G.	Digestion with testicular hyaluronidase	185
H	Oil Red O stain for fat	186
APPENDIX B : CHEMICAL AND BIOCHEMICAL METHODS		
A.	Measurement of serum and perfusate albumin	187
B.	Measurement of total serum protein	187
C.	Measurement of perfusate glucose	188
D.	Determination of extractable liver lipid	190
E.	Measurement of haemoglobin and haematocrit in perfusates	190
F.	Measurement of perfusate lactate	191
G.	Determination of serum sulphate	193
H.	Measurement of perfusate pO_2	196
I.	Programming of Packard Tricarb 2650 for the counting of $^{35}SO_4$	196
J.	Preparation of incubation medium	197
K.	Radioimmunoassays of rat growth hormone and insulin	198

Page

APPENDIX C : STATISTICAL METHODS

A. Parametric tests 207

B. Bioassay statistics 208

C. The bioassay computer programme 211

APPENDIX A

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

(A) Routine staining with haemalum and eosin (Culling, 1974).

1. Mayer's haemalum was prepared after the method of Culling (1974).
2. Dewaxed sections were brought to water through graded alcohols.
3. Stained in Mayer's haemalum for 15 minutes.
4. Washed in tap water until blue.
5. Differentiated in 1% HCl in ethanol (about 10 secs).
6. Again washed in tap water until blue.
7. Counterstained with 1% aqueous eosin for 3 minutes.
8. Washed in tap water for 3 minutes.
9. Dehydrated in graded alcohols, cleared in xylol and mounted in Depex (Gurr, England).

(B) Metachromatic staining with toluidine blue (Culling, 1974).

Reagents A: Toluidine blue (BDH, Poole, England) 0.25g in 100 ml Michaelis' Veronal acetate - hydrochloric acid buffer pH 4.5.

B: Veronal acetate solution

Sodium acetate 1.943g

Sodium barbiturate 2.943g

Distilled water to 100 ml

C: For pH 4.5 Michaelis' Veronal acetate - hydrochloric acid buffer

5 ml veronal acetate solution

11 ml 0.1N hydrochloric acid

9 ml distilled water

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Stained in solution A for 10 seconds.
3. Rinsed in distilled water.
4. Blotted dry with fluffless blotting paper, then air dried.
5. Dehydrated in graded alcohols, cleared in xylol and mounted in Depex (Gurr, England).

Results:

Metachromatic substances red, pink or purple. Nuclei blue.

(C) Sweet's resorcin-crystal violet and picro-fuchsin stain (Sweet, 1968).

Reagents A: Harris' haematoxylin (Gurr, England) prepared according to the method of Culling (1974).

B: Crystal violet (BDH, 3043960, Poole, England) 1g, resorcin (Hopkins and Williams Ltd., England), distilled water 60 ml were all boiled for 3 min with constant stirring after which 30 ml of a 30% w/v solution of $FeCl_3$ was added and boiling continued for another 3 min. The solution was filtered and the filtrate reserved, the precipitate was washed with 50 ml distilled water and 100 ml of absolute alcohol was added to the second filtrate. Both filtrates were combined and boiled for 5 min, filtered again and the precipitate discarded, finally 2 ml of concentrated (32% w/w) HCl was added to the filtrate which was cooled before use.

C: Van Gieson's picro-fuchsin. 100 ml saturated aqueous picric acid was mixed with 10 ml 1% acid fuschin (Gurr, England).

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Stained in solution A for 5 min.
3. Washed in tap water until blue.
4. Stained in solution B for 1 hour.
5. Briefly washed in tap water (about 30 secs).
6. Stained in solution C for 2 min.
7. Graded alcohols served to differentiate and dehydrate the section.
8. Cleared in xylol and mounted in Depex (Gurr, England).

Results:

Epiphyseal cartilage stained blue and articular cartilage pink. The deepest layer of the articular cartilage may contain scanty pericellular blue staining material.

(D) Alcian blue (pH 1.0) for sulphate groups of mucopolysaccharides (Culling, 1974).

Reagents A: Alcian blue 8GX (Gurr, England) 1% w/v in 0.1N HCl.

B: 0.1N HCl.

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Stained in solution A for 30 min.
3. Rinsed in 0.1N HCl for 10 secs.
4. Blotted dry, dehydrated in graded alcohols, cleared

in xylol and mounted in Depex (Gurr, England).

Results:

Sulphated mucopolysaccharides stained blue.

(E) Alcian blue (pH 2.5) for acid groups (COOH and OSO_3H) of mucopolysaccharides (Culling, 1974).

Reagents A: Alcian blue 8GX (Gurr, England) 1% w/v in 3% v/v acetic acid (pH 2.5).

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Stained in solution A for 30 min.
3. Rinsed in distilled water for 30 secs.
4. Dehydrated in graded alcohols, cleared in xylol and mounted in Depex (Gurr, England).

Results:

Acid mucopolysaccharides stained blue.

(F) Alcian blue: critical electrolyte concentration technique (Quintarelli, Scott, Dellovo, 1964; Culling, 1974).

Reagents A: Alcian blue 8GX 0.1% w/v in 0.05M sodium acetate buffer (pH 5.7).

B: $MgCl_2$ was added to aliquots of solution A to give final molar concentrations of 0.1, 0.7 and 1.0M.

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Serial sections were each stained with one of the four solutions described above for 30 min.
3. Rinsed in tap water for 30 secs.

4. Dehydrated in graded alcohols, cleared in xylol and mounted in Depex (Gurr, England).

Results:

Sulphated mucopolysaccharides stained blue at all concentrations of $MgCl_2$ while carboxylated mucopolysaccharides did not at $MgCl_2$ concentrations of 0.7 and 1.0M.

(G) Digestion with testicular hyaluronidase (Culling, 1974)

Reagents A: Ovine testicular hyaluronidase EC 3.2.1.35 (Boehringer, Mannheim 15005). Solution: 50 mg/100 ml 0.1M sodium acetate buffer pH 6.0.

B: Sodium acetate buffer, 0.1M pH 6.0.

C: Alcian blue 8GX 1.0% w/v in 0.1N HCl.

Method:

1. Dewaxed sections were brought to water through graded alcohols.
2. Sections were covered with solution A, prewarmed to 37°C, and incubated for 6 hours at 37°C.
3. Control sections were similarly incubated in solution B.
4. Slides were rinsed in solution B and then tap water (1 min each).
5. Stained with solution C as described above.
6. Dehydrated in graded alcohols, cleared in xylol and mounted in Depex (Gurr, England).

Results:

Loss of blue staining (relative to buffer control) indicated removal of hyaluronic acid and/or chondroitin sulphates A and/or B.

(H) Oil Red O stain for fat (Lillie and Ashburn, 1943)

Reagents A: Saturated solution of Oil red O (Gurr, England) in iso-propanol.

B: Solution A was diluted 6:4 with distilled water, allowed to stand for 10 mins and filtered. The filtrate must be used within 1-2 hours.

C: 60% Alcohol.

D: Mayer's haemalum (Gurr, England).

Method:

1. Tissue was fixed for 24 hours in phosphate buffered formalin.
2. Frozen sections, 10 µm thick, were cut on an Oxford freezing microtome and fixed to gelatinised slides.
3. Sections were stained in solution B for 10 mins.
4. Differentiated in solution C for 15 sec.
5. Counterstained in solution D for 3 min.
6. Blued in tap water.
7. Mounted in glycerine jelly.

Results:

Fat droplets were stained orange-red and nuclei blue.

APPENDIX B

CHEMICAL AND BIOCHEMICAL METHODS

(A) Measurement of serum and perfusate albumin (Doumas, Watson and Biggs, 1971).

Principle: The dye bromocresol green binds specifically to albumin without reacting with globulins.

Reagents A: A commercial preparation, Albustrate (Warner-Lambert Co., U.S.A.) of bromocresol green, surface autoreagent, buffer and preservative.

B: Commercial serum standards of known albumin concentration were used (Calibrate, Warner-Lambert Co., U.S.A.).

Method: All assays were carried out in duplicate as follows:

1. 0.5 ml Albustrate reagent.
2. 2.0 ml Distilled water.
3. 5 ml Sample or Standard.
4. Absorbance was read after 30 min at 630 nm by means of a Zeiss PMZD spectrophotometer.

Results:

Absorbance was linear over the working range (1.5-4.6 g/100 ml).

Reproducibility:

Interassay coefficient of variation was 1.8% (n = 8).

(B) Measurement of total serum proteins (Lowry, Rosebrough, Farr and Randall, 1951).

Principle: The Folin-Ciocalteu reagent was used which reacts with tyrosine and tryptophan residues in proteins.

Reagents A: 2% w/v Na_2CO_3 in 0.1N NaOH.

B: 0.5% w/v $CuSO_4 \cdot 5H_2O$ in 1% w/v potassium sodium tartrate.

C: Before use 50 ml of solution A was mixed with 1 ml of reagent B.

D: Folin-Ciocalteu reagent (Merck, Darmstadt) diluted 1:1 with distilled water.

E: 0.9% w/v NaCl in distilled water.

F: Standard was as for albumin (Calibrate, Warner-Lambert Co., U.S.A.).

Method: All assays were carried out in duplicate as follows:

1. 5 µl Standard or Sample.
2. 1 ml 0.9% NaCl.
3. .5 ml of Lowry reagent C.
4. After 10 min 0.5 ml of the diluted Folin-Ciocalteu reagent (solution D) was added and thoroughly mixed.
5. After standing at room temperature for 30 min absorbance was read at 500 nm by means of a Zeiss PMZD spectrophotometer.

Results:

Absorbance was linear over the working range (2.46-7.4 g/100ml)

Reproducibility:

Interassay coefficient of variation was 2.1% (n = 8).

(C) Measurement of perfusate glucose.

Principle: The sample was deproteinised with perchloric acid and the glucose content of the supernatant determined by the glucose oxidase/peroxidase/O-dianisidine reagent of Lavine, Chick, Like and Makdisi (1971).

Reagents A: 2% Perchloric acid (Merck, Darmstadt).

B: 1% w/v O-dianisidine dihydrochloride (Sigma, U.S.A.).

C: i) 0.2M Potassium phosphate buffer (34.836g $K_2U_2PO_4$ g/L, KH_2PO_4 27.200 g/L adjusted to pH 7.0).

ii) Horseradish peroxidase EC 1.11.1.7 (Sigma, U.S.A.) 10 mg in 500 ml phosphate buffer.

iii) Glucose oxidase, EC 1.1.3.4 activity 15,000 μ/g (Sigma, U.S.A.) 133.3 mg in 500 ml phosphate buffer.

D: Glucose Standard: 20 mg D (+) glucose (BDH, Poole, England) was made up with double distilled water to a volume of 10 ml (= 200 mg/100 ml^{-1}). With double distilled water secondary standards were made up (200, 150, 100, 50, 25 mg 100 ml^{-1}).

Method:

1. 60 μl of sample or standard was added to 460 μl cold 2% $HClO_4$ and the protein-free supernatant separated by centrifugation at 1000g for 10 min.
2. 100 μl supernatant was added to 1 ml reagent.
3. Incubated for 30 min at 30°C.
4. Absorbance was read at 436 nm against a reagent block by means of a Zeiss PMZN spectrophotometer.

Results:

Absorbance was linear over the range 0-200 mg 100 ml^{-1}.

Reproducibility:

Interassay coefficient of variation was 1% (n = 8).

(D) Determination of extractable liver lipid (Hazlewood and Nichols (1969).

Principle: Lipids were extracted from liver by petroleum ether.

Method:

1. Three samples of liver (300-500 mg) were taken from each liver and immediately weighed in glass-stoppered centrifuge tubes and again after 8 days drying (open) at 110°C.
2. The dry tissue was then extracted with 10 ml of petroleum ether (BP 40°-60°C BDH Chemicals Ltd., Cape Town) for 24 hours, while being shaken mechanically (60 cycles/min, 5 cm oscillation).
3. This extraction procedure was repeated twice after which the tissue was freed of residual petroleum ether by drying at 37°C for 24 hours and again weighed.
4. The lipid content was expressed as:-

$$\frac{\text{(Dry weight) - (Extracted weight)}}{\text{Dry Weight}} \times 100$$

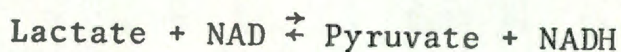
(E) Measurement of haemoglobin and haematocrit in perfusates.

a) Perfusate total haemoglobin content was measured by the cyanmethaemoglobin method, and the "free" haemoglobin in the perfusate supernatant by the benzidene method (both as described by Dacie and Lewis, 1963). These analyses were performed by the Cape Provincial Blood Transfusion Laboratory, Cape Town.

b) Perfusate haematocrit was measured in heparinised capillary tubes after centrifugation at 12,000 rpm for 3 min in a high speed microhaematocrit centrifuge (Hawksley, England).

(F) Measurement of perfusate lactate.

Principle: Perfusate lactate concentrations were determined enzymatically using lactate dehydrogenase by the method of Hohorst (1964) in the enzyme catalyses the reaction:



$$\text{Lactate} + \text{NAD} \rightleftarrows \text{Pyruvate} + \text{NADH}$$

Under the chosen conditions of incubation the equilibrium is directed towards pyruvate and NADH. The NADH is thus formed in direct proportion to the lactate concentration.

Reagents A: Glycine buffers: This was prepared from two stock solutions (i) and (ii) in a ratio of approximately 1:1 to give a pH of 9.4-9.5, just before assay.

Solution (i) 2N NaOH

Solution (ii) 20.8g Hydrazine Sulphate, 30.0g Glycine, 0.8g EDTA in 200ml double distilled water

B: Lactate standard solution, 1mM (Boehringer, Mannheim).

C: NAD (Miles Laboratories, U.K.) 1% w/v in double distilled water.

D: Lactate dehydrogenase EC 1.1.1.27 (32,000 U/10 ml) Miles, Laboratories, U.K.). The required concentration of 36,000 U/ml was obtained by adding 1.125 ml of the stock enzyme solution to 10 ml double distilled water.

Method:

1. Perfusate was deproteinised by the addition of perchloric

acid (2% w/v) and centrifugation of the precipitated proteins.

2. The resulting supernatant was neutralised by adding a known volume of 0.1N KOH containing a trace of BDH universal indicator.

3. Assays were performed at 20-22°C in clean 3.0 ml cuvettes and the absorbance read at 340 nm with air as reference by means of a Unicam SP 1700 spectrophotometer.

4. The experiment was set up as follows:

Reagents	Sample (ml)	Blank (ml)
Buffer	1.50	1.50
NAD	0.20	0.20
Distilled H_2O	1.10 or 1.20	1.30
Sample or Standard	0.05 or 0.20	-
LDH	0.10	0.10
Total	∿ 3.00	∿ 3.00

5. The reaction was started by the addition of 0.01 ml LDH solution.

6. The absorbance was read every 10 min until stable. The optical density change (ΔOD) at 340 nm of the blanks was subtracted from those of samples or standards. Where high lactate concentrations were anticipated (> 2nM) 0.05 ml of sample was used, whereas for those with low concentrations 0.20 ml was used.

Calculations: The cuvette having a volume of 3 ml and a 1 cm light path, the expected ΔOD produced by 0.05, 0.10 and 0.20 ml of 1 mM lactate are directly proportional to each other.

Thus 0.05 ml 1mM Lactate produces ΔOD 0.1305 absorbance units

0.10 ml 1mM Lactate produces ΔOD 0.207 absorbance units

0.20 ml 1mM Lactate produces ΔOD 0.414 absorbance units

then with 0.05 ml Sample

$$\text{Lactate (mM)} = \frac{\Delta\text{OD Sample} - \Delta\text{OD Blank}}{0.1035}$$

with 0.10 ml Sample

$$\text{Lactate (mM)} = \frac{\Delta\text{OD Sample} - \Delta\text{OD Blank}}{0.207}$$

and with 0.20 ml Sample

$$\text{Lactate (mM)} = \frac{\Delta\text{OD Sample} - \Delta\text{OD Blank}}{0.414}$$

The concentration of lactate in each sample before neutralisation was then calculated

$$\%\ \text{Recovery} = \frac{\Delta\text{OD 0.05 ml Standard} - \Delta\text{OD Blank}}{0.1035} \times 100$$

on this basis; mean ± SEM recovery was 95 ± 2% (n = 10)

(G) Determination of Serum Sulphate.

Principle: Serum sulphate was determined by a modification (Henry, 1964) of the method of Kleeman, Taborsky and Epstein (1956).

Serum was deproteinized with uranyl acetate, which simultaneously removed inorganic phosphate. The sulphate in the filtrate is precipitated as benzidene sulphate and the benzidene quantitated by photometric measurement after reaction with β naphthoquinone-4-sulphonate.

Reagents A: 1% Benzidene (BDH, Poole, England) w/v ethanol was prepared freshly on the day of assay from twice recrystallised benzidene.

B: 0.4% w/v aqueous uranyl acetate (Merck, Darmstadt).

C: 1% w/v Sodium borate in 0.1N NaOH (must be stored in polyethylene bottle).

D: 2:1 v/v ethanol, diethylether wash reagent.

E: Sodium β naphthoquinone-4-sulphonate, 15 mg in 10 ml double distilled water was prepared on the day of assay.

F: Stock sulphate standard 1.090g K_2SO_4/L double distilled water.

G: Working sulphate standard 1:10 dilution of stock standard. Contains 20 μg S/ml.

H: Powdered glass. Pyrex glass was ground to a fine powder with a mortar and pestle, washed twice with hot, dilute HNO_3 and repeatedly washed with distilled water and then dried.

Method:

1. 0.5 ml serum was added to 1.5 ml of reagent B, mixed and then precipitated by centrifugation (2000g for 5 min).
2. In 8 ml glass stoppered centrifuge tubes the following were made up:

	Blank	Standard	Unknown
Water	0.25 ml	-	-
Standard (5 μg S)	-	0.25 ml	-
Sample (Supernatant)	-	-	1.00 ml
Uranyl acetate solution	0.75 ml	0.75 ml	-
Glacial acetic acid	0.25 ml	0.25 ml	0.25 ml
Benzidene solution	2.25 ml	2.25 ml	2.25 ml
Powdered glass (a pinch)	∿ 5 mg	∿ 5 mg	∿ 5 mg

3. Tubes were stoppered and stood upright at $4^{o}C$ for 18 hours.
4. After centrifugation (2000g/5 min) the supernatant was aspirated and the tubes drained by inversion on absorbent paper for 3 min.
5. The inside of the tube was rinsed with 1 ml of absolute alcohol without disturbing the precipitate, centrifuged and aspirated as before.
6. 8 ml of alcohol-ether wash was added, the tube stoppered and vigorously shaken, centrifuged and aspirated.
7. 0.5 ml of alcohol was added without disturbing the precipitate, centrifuged and aspirated.
8. 0.25 Sodium borate solution was added and the tubes heated to $60^{o}C$ in a waterbath for 10 min.
9. After cooling, 0.25 ml double distilled water and 0.25 ml β naphthaquinone-4-sulphonate solution were added, mixed and incubated for exactly 5 min then 0.5 ml acetone was added, mixed and the tubes centrifuged as before.
10. The supernatant was transferred to clean tubes and the absorbance read by means of a Zeiss PMZN spectrophotometer at 485 nM.

Calculation:

$$\text{mg Sulphate/100} = \frac{\text{Absorbance unknown - absorbance blank}}{\text{Absorbance of standard - absorbance of blank}} \times 6$$

Absorbance was linear over the range 1-7 mg/100 ml.

Reproducibility:

Interassay coefficient of variation was 6.9% (n = 5).

Comment:

In view of the known carcinogenicity of benzidene and the radioactivity or uranylacetate, the greatest care was exercised with this assay, gloves and apron being worn at all times and in addition a mask was worn during the weighing of benzidene. All glassware was used only for this assay and was separately and specially cleaned.

(H) Measurement of Perfusate pO_2.

1. Perfusate pO_2 was measured by means of a Radiometer (Copenhagen) pO_2 electrode Type E 5046, pH meter Type PHM27 and gas monitor PHA 927. The instrument was calibrated against Radiometer S4150 pO_2 zero solution and distilled water equilibrated with room air. The scale 0-1200 mmHg was used for high pO_2 perfusates leaving the oxygenator and the scale 0-120 mmHg for low pO_2 perfusates leaving the liver.
2. All samples were drawn into plastic syringes and analysed within 5 min.

(I) Programming of Packard Tricarb 2650 for the counting of $^{35}SO_4$

1 cycle/tray

1st vial is a background

Lower limit reject (Red, green) 0, 0

Lower limit discriminator	(Red, green)	50, 50
Upper limit discriminator	(Red, green)	1000, 1000
% Gain	(Red, green)	4.5, 4.5
Count mode	ES	
Calibration	No	
Curve 1D	CPM	
Count/Sample	1	
Divide constant	1.00	
Reset time (Red, green)	5 min, 5 min	
Preset counts (Red, green)	8×10^6, 8×10^6	

(J) Preparation of incubation medium (Daughaday and Reeder, 1966) 400 ml required (preincubation 75 ml, dilution of samples 150 ml, isotope incubation 175 ml).

Method:

1. Glucose 800 mg.
2. Phosphate buffer (5 x final concentration) 80.0 ml

   15775g Na_2HPO_4 dissolved in 850 ml double distilled water

   1N HCl to pH 7.4

   Double distilled water to 1000 ml

   Frozen in aliquots of 81 ml
3. Salt solution (20 x final concentration) 20.0 ml

   133.5g NaCl

   6.8g KCl

   2.8g $MgSO_4$

   Double distilled water to 1000 ml

   Frozen in aliquots of 21 ml
4. Amino acid solution (10 x final concentration) 40.0 ml

   L glutamine 833.5 mg   L Valine 293.0 mg

L Lysine	292.5 mg	L Arginine	173.5 mg
L Leucine	170.0 mg	Glycine	157.5 mg
L Threonine	142.5 mg	L Histidine	124.0 mg
L Cystine	120.0 mg	L Tyrosine	108.5 mg
L Tryptophan	102.0 mg	L Isoleucine	92.0 mg
L Phenylalanine	44.5 mg	L Methionine	82.5 mg

Cystine and methionine were dissolved in 300 ml of double distilled water with heating, the phenylalanine and tyrosine were added while the solution was hot and after cooling the remaining amino acids were added and the volume made up to 1000 ml with double distilled water and the resultant solution frozen in aliquots of 41 ml.

5. Buffered Na penicillin 40,000u (Novo Industries (Pharmaceuticals) (Pty) Ltd., South Africa.
6. Streptomycin SO_4 40 mg (Glaxo Allenburys (S.A.) (Pty) Ltd., South Africa.
7. Kanamycin 40 mg (Novo Industries (Pharmaceuticals) (Pty) Ltd., South Africa.
8. Double distilled water to 400 ml.
9. The resulting solution was sterilized by filtration through a 0.2μ Millipore filter (EGWPO2500).

Dextrose and salts AR grade BDH, Poole, England.
Amino acids, Grade I, Sigma Biochemicals, U.S.A.

(K) Radioimmunoassays of rat growth hormone and insulin.

(a) General principle of radioimmunoassay (Yalow and Berson, 1964).

Such assays depend on an antibody directed against and specifically binding to the hormone to be measured. A fixed amount of radioactively labelled hormone competes with unlabelled hormone

(either Standard or unknown) for binding sites on a limited amount of antibody.

The amount of labelled hormone bound to antibody diminishes with increasing amounts of unlabelled hormone. Separation of the labelled hormone bound to antibody from that unbound ("free") is possible by a variety of processes, in these instances a double antibody precipitated the first along with the hormone bound to it.

The concentration of hormone in the unknown is determined by comparing the displacement of labelled hormone produced by the unknown with that produced by a series of known concentrations of pure hormone standard in a dose-response curve.

(b) Radioimmunoassay of rat insulin.

The method of Weinkove, Weinkove and Pimstone (1974) was used. Bound labelled insulin was separated from free labelled hormone by the double antibody technique of Morgan and Lazarow, 1963.

Reagents A: Assay buffer. 0.05M Veronal buffer pH 8.6 containing 0.25% w/v crystalline bovine serum albumin (BDH, Poole, England) was used to dilute reagents, standards and if necessary, serum samples. In the case of precipitating second antibody and guinea pig carrier plasma the buffer also contained 0.01M EDTA.

B: Standards. Crystalline rat insulin biopotency 2114/mg, separated from proinsulin and related substances (b-component) by Sephadex chromatography by the manufacturers, Novo Research Institute, Denmark (lot number R171).

C: ^{125}I labelled insulin. Porcine monocomponent insulin (Novo, lot number S823058) was labelled by the method of Hunter and Greenwood (1962) to a

specific activity of 250-300 μCi/μg.

D: Guinea pig antiporcine insulin antibody (Burroughs Wellcome Ltd., U.K., Lot number K2357).

E: Rabbit anti-guinea pig antiserum. Made by Pentex U.K., was used as second precipitating antibody.

F: Carrier plasma was obtained from healthy 3 month old guinea pigs.

Method:

The assay was carried out at 4°C in 400 μl Beckman microfuge tubes. The optimal concentrations of antibodies and carrier plasma and the time required to reach steady-state binding at 4°C were all determined before using the assay for determining insulin content of unknown sera.

After addition of each reagent, tubes were closed and centrifuged in a Beckman microfuge in order to mix them.

1. Preincubation of standards and unknowns with insulin antibody. 5 μl of serum sample or standards (40, 20, 10, 5, 2.5, 1.25, 0.625 ng/ml) were pipetted into the tubes followed by 50 μl of guinea pig anti-insulin antibody (K2357) diluted 1/100,000. Incubation was conducted at 4°C for 24 hours.
2. Addition of ^{125}I-insulin. 20 pg ^{125}I-insulin in 50 μl buffer ($\pm$ 8000 cpm) was added, mixed and incubated at 4°C for 24 hours.
3. Separation of bound from free ^{125}I-insulin. 50 μl of rabbit anti-guinea pig precipitation serum diluted 1/8 and 50 μl of guinea pig plasma, diluted 1/5000 were added to the tubes, mixed and incubated at 4°C for 48 hours.
4. Centrifugation in the Beckman microfuge for 5 min

(17,5000g) produced a firmly adherent precipitate. The supernatant was "flicked" out of the tube and drainage completed by being left inverted for 1 hour at $4^{o}C$.

5. The tip containing the precipitate was cut off with shears and placed in a counting vial and counted for a minimum of 10,000 counts or 20 minutes in a Packard autogamma scintillation spectrometer (efficiency 56%).

Calculation:

1. Total counts added, 6 tubes containing labelled hormone alone were counted. The mean being taken as the total counts for the purpose of calculation.
2. "Non-specific" binding or blank. This reflects the label adsorbed onto the tube and trapped in the precipitate in the absence of the specific binding of the anti-insulin antiserum and was estimated from tubes containing all the reagents detailed above except that buffer replaced antibody in Step 1 of the method. The blank counts were 1-2% of the total counts added.
3. Bound counts (B) = counts in sample or standard-counts in blank.
4. Free counts (F) = Total counts - B.
5. B/F ratio was plotted semilogarithmically as a function of the concentration of insulin standards to generate a standard curve (see Fig. B.1). Serum insulin concentrations were determined from this curve.

Sensitivity: This was 0.5 ng/ml.

Reproducibility:

Intra-assay coefficient of variation = 8% in the midrange (n = 6)
Interassay coefficient of variation, low concentration 24% (n=13)
Midrange 16% (n = 12) and high concentration 13% (n = 8).

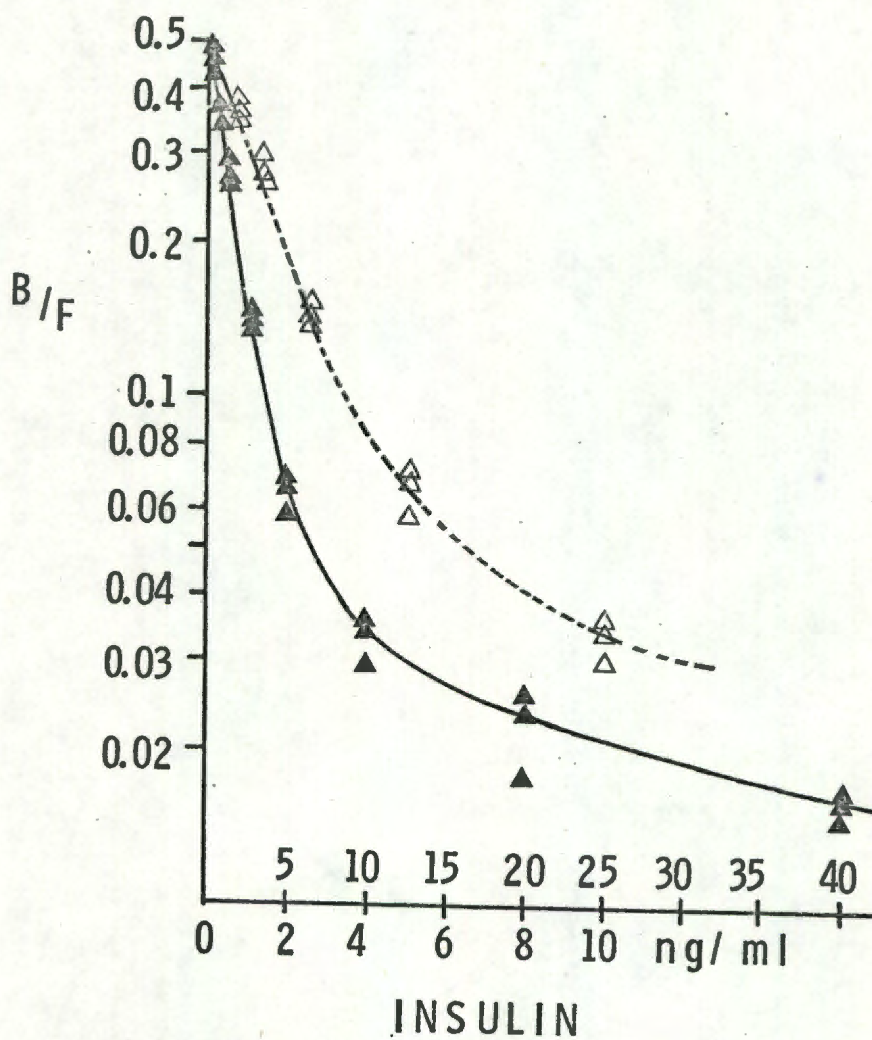


Fig. B.1 A representative insulin immunoassay standard curve.

(c) Radioimmunoassay of rat growth hormone.

This assay made use of reagents kindly donated by the National Institute for Arthritis, Metabolism and Digestive Diseases of the National Institutes of Health (U.S.A.) Rat Pituitary Hormone Distribution Program.

Reagents A: Assay buffer 0.025M EDTA phosphosaline buffer containing 1% w/v crystalline bovine serum albumin (BDH, Poole, England) and 0.1% w/v sodium azide pH 7.6.

B: Standard: Rat growth hormone reference preparation for radioimmunoassay (NIAMDD-Rat GH-RP-1). Biological potency = 0.6 International units (Bovine GH) per mg.

C: ^{125}I labelled rat growth hormone. Purified rat rat growth hormone (NIAMDD-rat GH-1-3). The lyophilised powder was dissolved in 0.01M $NaHCO_3$ and 25 µl aliquots (concentration 1 mg/ml) stored at -20°C until iodination.

D: Monkey antiserum to rat growth hormone NIAMDD-anti-Rat GH-Serum-3). The lyophilized antiserum was diluted 1/1000 in the assay buffer and frozen in 100 µl aliquots.

E: Rabbit anti-monkey gamma globulin serum. A serum with high affinity was prepared in our laboratory and was used as a second precipitating antibody.

F: Carrier plasma was obtained from healthy Rhesus monkeys.

Method:

1. Radioiodination: ^{125}I-NaI (The Radiochemical Centre,

Amersham, U.K. Specific activity mCi/mg).

0.5M NaH_2PO_4 buffer pH 7.6.

Chloramine-T, a solution of 35 mg in 10 ml 0.05M NaH_2PO_4 buffer was prepared immediately before use.

Sephadex G-50, equilibrated with 0.05 barbital buffer, pH 8.6 was packed into a 0.9 x 20 cm column, coated with a few drops of 5% bovine serum albumin and washed with barbital buffer before use.

2. Labelling procedure: In a reaction tube 100 µl 0.5M phosphate buffer, 2 mCi ^{125}I, 20 µl of NIAMDD-Rat GH-1-3 (1 mg/ml) and 10-20 µl chloramine-T solution were added in this sequence. 45 seconds after the addition of the chloramine-T, the reaction mixture was placed on the Sephadex column and eluted with 0.05M barbital buffer pH 8.6. Fractions of 0.5 ml were collected into bovine serum albumin coated plastic tubes.

3. Results: Two distinct peaks were obtained, the first radioactive peak contained the labelled hormone and was diluted in 1% BSA-phosphaline buffer pH 7.6, aliquoted and stored at -20°C until use. The second peak contained the free iodine and was discarded. On the day of assay a portion of the labelled hormone was repurified on a Sephadex G-100 column 1.5 x 50 cm equilibrated in 0.05M barbital buffer pH 8.6 1×10^6 cpm was applied to the column which was then eluted with barbital buffer and 2 ml fractions collected. Three radioactive peaks were obtained, the first being aggregated poorly immunoreactive material, the second being the labelled hormone used in the assay, and the third small molecules. The first and third peaks were discarded.

4. Radioimmunoassay procedure:

i) 300 μl of buffer, 100 μl of standards in duplicate (at 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0 ng/100 μl) or 0.05 and 0.025 ml of unknown serum sample (the 0.025 ml sample being made up to 0.05 ml with buffer) were added to 10 x 75 mm disposable test tubes in that sequence.

ii) 100 μl of the repurified labelled rat growth hormone was added such that each tube received 5000 to 8000 cpm.

iii) 100 μl of monkey anti-rat growth hormone serum diluted 1:20,000 was added to all but 3 non-specific binding tubes which received 100 μl of assay buffer in its place.

iv) The mixture was incubated for 72 hours at 4°C.

v) 25 μl Sodium heparin (1000 USP units/ml), 100 μl carrier plasma diluted 1:1600 and 100 μl of rabbit anti-monkey precipitating serum were added in that sequence and the incubation continued for 24 hours at 4°C.

vi) The precipitate was separated by centrifugation for 30 min at 1000g at 4°C and the supernatant discarded, the radioactivity in the precipitate was counted in a Packard automatic gamma scintillation spectrometer (efficiency 56%) for a minimum of 10,000 counts or 20 minutes.

Calculations: These were as for the rat insulin radioimmunoassay.

Sensitivity: This was 2-5 ng.

Reproducibility:

(Midrange) Intra-assay coefficient variation 5% (n = 7)

Interassay coefficient variation 13% (n = 8).

APPENDIX C

STATISTICAL METHODS

"A judicious man looks at statistics not to get knowledge but to save himself from having ignorance foisted on him" *Thomas Carlyle.*

All statistical analysis of data was performed using the appropriate programme for a Hewlett-Packard computer, model 9830A.

(A) Parametric tests:

a) The mean, $\bar{x} = \frac{1}{n} \Sigma x$

where n = number of observations

b) Standard Deviation, S.D. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n-1}}$

where $n < 30$

c) Standard error of the mean, SEM $= \frac{S.D.}{\sqrt{n}}$

d) Coefficient of Variation, $= \frac{S.D.}{\bar{x}} \times 100$

e) Least Square Regression Line

a line $y = a + b\,x$ was fitted to pairs of data (x_1y_1, x_2y_2, x_ny_n)

$$\text{when } a = \frac{(\Sigma y)(\Sigma x^2) - (\Sigma x)(\Sigma xy)}{n\Sigma x^2 - (\Sigma x)^2}$$

$$b = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

f) Correlation Coefficient

$$r = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{n\Sigma x^2 - (\Sigma x)^2 \quad n\Sigma y^2 - (\Sigma y)^2}}$$

g) "Student's" t test:

To compare the means $\bar{x}$ and $\bar{x}$ of 2 small samples n and n from normal populations (with unknown variances which are assumed to be equal)

$$t = \frac{\bar{x} - \bar{x}}{S\sqrt{\frac{1}{n} + \frac{1}{n}}}$$

where S is an estimate of the standard deviation based on both samples together

$$S^2 = \frac{1}{n+n-2} \quad \Sigma x^2 - \frac{(\Sigma x)^2}{n} + \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

Degrees of freedom = n+n - 2

h) Levels of significance

Values of p were determined from the appropriate tables for t tests and correlation coefficients.

Two-tailed tests were used in the determination of p, where p exceeded 0.05 ($p > 0.05$) the differences between sample means were regarded as non-significant.

(B) Bioassay Statistics:

a) Data from the somatomedin bioassay was subjected to analysis by means of a computer programme designed by Phillips and Herington (1975) and a Hewlett-Packard 9810 (model 10) desk top computer with the following options: 001 (111 data-storage registers), 003 (2036 programme steps), 004 (printer), read only memory (ROM) plug-in instruction blocks 11214A (statistics), 11261A (plotter printer alpha), 11262A (peripheral control cassette memory), 9862A (calculator plotter), 2748B (punch tape reader) and 9865A (cassette memory).

The programme was entered on magnetic cards. Data could be

entered manually or by means of paper tape and the dose responses automatically plotted. The programme accepted dose-response lines made up of up to 30 points at up to 5 concentrations (the number of points for each concentration must be constant).

The automatic plotting of dose-response lines allowed easy examination and rapid selection of the appropriate portions for analysis, which could then proceed without re-entry of the raw data.

Data was processed with the response represented in the form:

$$z = K_1 K_2 \frac{(x - K_3)}{y}$$

where K_1 = specific activity

K_2 = the dilution factor

K_3 = the background CPM

x = the uncorrected response CPM

y = the cartilage weight

Error was minimised by specific instructions prior to the entry of each datum or option. Assay number, date, estimated maximal and minimal responses, background CPM and standard CPM, total $\mu g SO_4$/tube (a constant), dilution factor (to convert specific activity to the chosen units), options for variable or constant cartilage weight and log-log or semi-log transformation, maximum t value for fiducial limits calculations (for minimum n and chosen probability), the number of points per group (a constant) and the potency of the standard.

Data for each sample were then entered in sequence. The line number and total number of points entered manually, followed by the uncorrected CPM entered in sequence, either manually or read from paper tape. Concentrations for each sample were entered manually in sequence. The full dose response lines were then plotted and the means and standard errors of the means printed out. If parallel

line analysis was required, the standard line was entered first.

After inspection of the whole dose reponse lines, concentrations were chosen for a trial pair of standard and test lines, those portions appearing linear and parallel being selected. If a test line was obviously unsatisfactory, analysis was omitted entirely and a new test sample requested. If a trial comparison was unsatisfactory, analysis with different concentrations was performed until the best possible comparison was obtained, after which the next test line was analysed as before.

The schematic flow chart of the programme is represented in Fig. C.1.

b) Equations

i) General form of data $Z = K_1 K_2 \frac{(x-K_3)}{y}$

where K_1 = specific activity

K_2 = dilution factor

K_3 = background CPM

x = uncorrected response CPM

y = cartilage weight

ii) Specifically for the somatomedin bioassay

Incorporated SO_4 (as μg/100 mg cartilage) =

$$\frac{CPM_i - BKgd}{Wt_i} \times \frac{\text{total } \mu g\ SO_4}{CPM_{SA} - BKgd} \times \text{conversion factor}$$

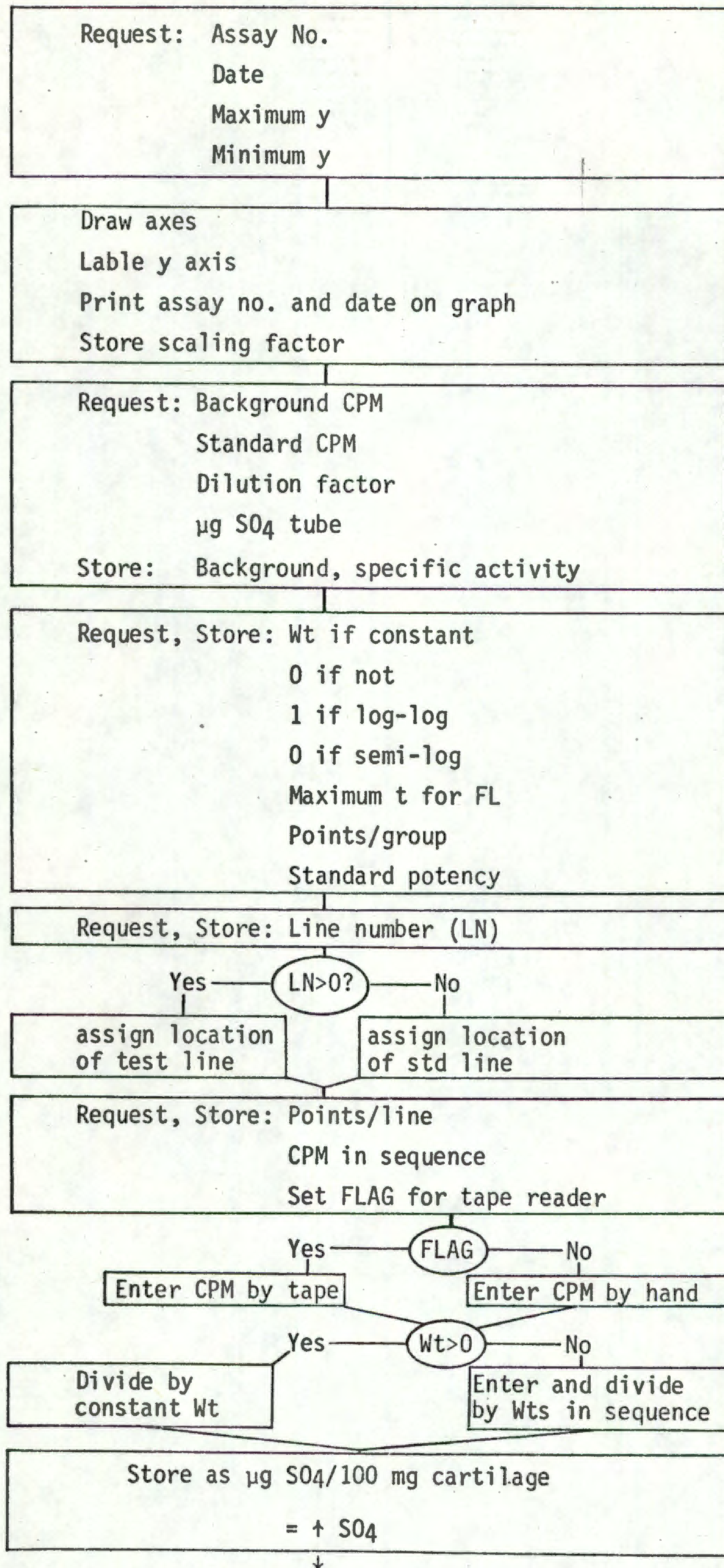
where total μg SO_4 =

(tube volume x $\frac{\text{Sample concentration(\%)}}{100}$ x Sample SO_4 conc. (μg/ml)

(tube volume x $\frac{\text{100-Sample concentration(\%)}}{100}$ x Buffer SO_4 conc.)

Wt_i may be constant or vary for each CPM_i

Fig. C1 Program Flow Chart (Phillips & Herington, 1975)



Recall analysis code

yes (log-log) — >0? — no (semi-log)

Take log of each ↑ SO_4

yes — LN>0? — No

assign location of test concentration

assign location of std concentration

Request, store as log: concentrations in sequence

Print group means and SEMs
Plot dose response line, Print LN on graph

(A) Yes (Std) — LN=0

Request new line

No

Code location of Std ↑ SO_4 values
Request concentration numbers for Std line
Enter 0 for test line, when done, set FLAG

(B)

(C) FLAG — Yes — (D) Line analysis

No

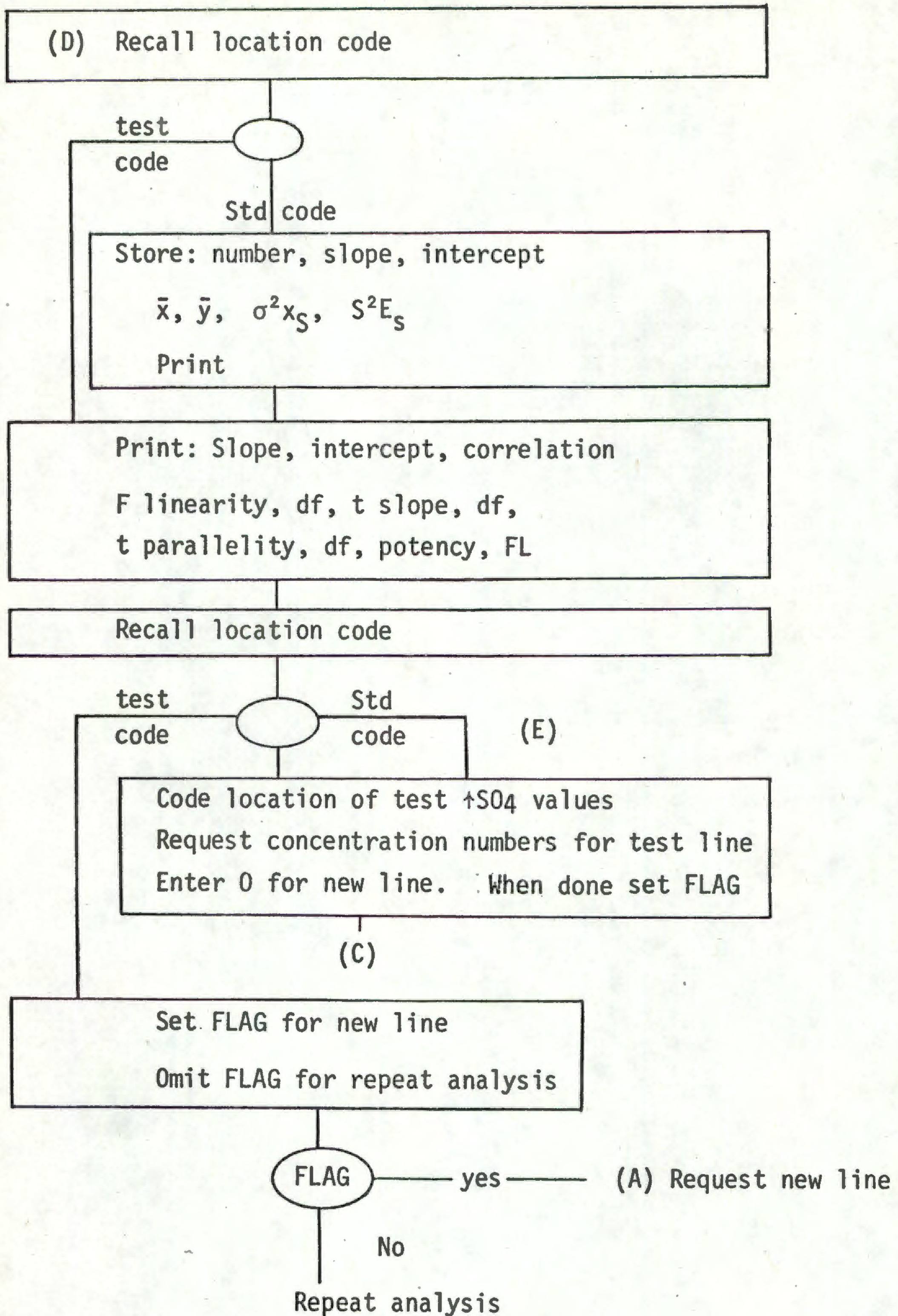
=0? — Yes — Recall location code

Std code — (E) Use previous Std line
Request test concentration numbers

No

Recall concentration corresponding to number
Form regression sums

Test code — (A) Request new line



(A) (B) (C) (D) (E) denote important locations in the program

$BKgd$, CPM_{SA}, conversion factor are all constant
Total μgSO_4 depends on relative volumes and SO_4 concentrations of sample and buffer (or buffer only if the $^{35}SO_4$ incubation follows the serum incubation as in the method of Van den Brande and Du Caju (1974).

iii) Dose response lines

Each dose reponse line was designated a line number to distinguish standard (=0) and unknown test sample line (≠0).

Each line would consist of several concentrations, each with a series of associated data points. The number of data points was constant.

The standard had a constant potency. The potency of the unknown test sample was expressed in terms of the standard ("potency ratio").

The appropriate portions of standard and unknown test sample dose response lines were selected for derivation of potency ratios.

All concentrations were subjected to logarithmic transformation (semi-log): $y = b \log x + a$. Responses (data points) could be transformed logarithmically if this provided a more linear dose response line (log-log): $\log y = b \log x + a$.

iv) Least-squares analysis

1. Intercept = $a = \bar{y}_i - b\bar{x}_i$

2. Slope = $b = \dfrac{\{\Sigma x_i y_i - (\Sigma x_i)(\Sigma y_i/n\}}{\{\Sigma x_i^2 - (\Sigma x_i)^2/n\}}$

3. Within each line, $\sigma x^2 = \dfrac{\{\Sigma x_i^2 - (\Sigma x_i)^2/n\}}{n-1}$

4. Similarly, $\sigma y^2 = \dfrac{\{\Sigma y_i^2 - (\Sigma y_i)^2/n\}}{n-1}$

5. Residual mean square for each line = S_E^2

$$= \frac{\{\sigma y^2 - b^2 \sigma x^2\}(n-1)}{(n-2)}$$

6. Significance of b for any line (t-test)

$$t = \frac{b}{\{S_E/(\sigma x \sqrt{n-1})\}}$$

with (n-2) degrees of freedom (d_f)

7. F-test for linearity of any line = F

$$F = \frac{S_2^2}{S_3^2}$$ with d_f S_2^2 : K-2; S_3^2 : n-K

where n = total number of points and K = number of groups.

$$S_3^2 = \frac{\{\Sigma y_i^2 - \Sigma(T_k^2/n_j)\}}{n - K}$$

$T_k^2 = (\Sigma y_i)^2$ for each group of size n_j

$$S_2^2 = \frac{\{(n-1)\sigma y^2 - (n-K)S_3^2 - (n-1)\sigma x^2\}}{K - 2}$$

8. Correlation coefficient for any line = r

$$r = b \frac{\sigma y}{\sigma x}$$

9. Index of precision = ℓ

$$\ell = \frac{S_E}{b}$$ (for standard line only)

v) Comparison of Standard and test lines

1. Pooled slope $b_{ST} = \frac{(b_S + b_T)}{2}$

where b_S = slope of standard line

b_T = slope of test line

2. Potency ratio

$$m = \bar{x}_S - \bar{x}_T - \frac{(\bar{y}_S - \bar{y}_T)}{b_{ST}}$$

3. Pooled $S_E^2 = S_p^2$

$$Sp^2 = \frac{\{(n_S-2)S^2{}_{ES} + (n_T-2)S^2{}_{ET}\}}{\{n_S + n_T - 4\}}$$

4. Pooled variance of x = $S^2{}_{xp}$

$$S^2{}_{xp} = (n_S-1)\sigma x^2{}_S + (n_T-1)\sigma x^2{}_T + n_S\bar{x}_S{}^2 + n_T\bar{x}^2{}_T - \frac{\{n_S\bar{x}_S + n_T\bar{x}_T\}^2}{\{n_S + n_T\}}$$

5. Finney's $g = \frac{\{t^2 . S^2{}_p\}}{\{b_{ST}{}^2 . S^2{}_{xp}\}}$

where t is chosen for appropriate limits for (n_S+n_T-2) degrees of freedom and preselected degree of confidence usually ~ 2

6. Parallelity t

$$t = \frac{|b_S - b_T|}{Sp\sqrt{\{1/(n_S-1)\sigma x_S{}^2\} + \{1/(n_T-1)\sigma x_T{}^2\}}}$$

degrees of freedom = $n_S + n_T - 4$

7. Test potency = Standard potency·antilog M.

8. Fiducial limits =

$$\text{Standard potency}\cdot\text{antilog } \bar{x}_S - \bar{x}_T + \frac{(m-\bar{x}_S+\bar{x}_T)}{(1-g)} \pm \frac{(t \cdot S_p)}{b_{ST}(1-g)} \cdot \left\{(1-g)\left(\frac{1}{n_S} + \frac{1}{n_T}\right) + (m-\bar{x}_S+\bar{x}_T)^2\right\}^{\frac{1}{2}}$$

(C) The Bioassay Computer Programme

1) Plotter options

Steps 0000-0331

1. Set up plotter first, with lower left and upper right chosen.
2. Card was entered.
3. The programme requested assay number, date, expected minimum and maximum value.
4. Axes were then drawn and labelled, ordinates divided into 20 parts and abscissa incremented.
5. The computer automatically started for entry of assay programme.

ii) Assay programme

Steps 0000-1974

1. Data was requested and analysed as described above.

iii) Plotter option listing (magnetic card 1).

iv) Assay programme listing (magnetic card 2).

v) Important steps in somatomedin assay programme

1 Set decimal

2 Request background

30 Store background

34	Request standard CPM (CPM_{SA})
63	Request total SO_4
84	Request dilution factor
105	Store specific activity
109	Request weight of constant
146	Store weight or 0
150	Request 1 if Log-Log
184	Store 1 or 0
188	Request maximum t for fiducial limits
209	Store t
213	Request points per group
230	Store
234	Request potency for standard line
251	Store
255	Request line number
273	Store
277	Determine location of CPM for standard or test S/R (a)
292	Request number of points
310	Store
319	Request CPM, FLAG for tape reader
356	Correct CPM for background
361	Convert CPM to μg SO_4
373	Store as μg
384	Reset location
392	Test for constant weight
403	If not, request individual weights
425	÷ μg SO_4 by weight, store
444	÷ μg SO_4 by weight, store
486	Test for Log-Log
492	If Log-Log, take log of each μg/100mg S/R (xto)

497	Request concentrations
514	Determine location
562	Store concentration
573	Redetermine location of μg/100mg
626	Form sums for groups
641	Print mean
650	Scale mean for plot
661	Increment x coordinate
675	Test for first group
696	Pen down to mean if not first
709	Pen up to mean if first
731	Standard error
749	Determine location for line number plot
773	Pen up to location
784	Print line number
791	Test: Standard or test line
804	Request concentration numbers for standard
868	Determine location of μg/100mg
885	Calculate sum for linearity
913	Test if line number = 0 (Standard)
939	Test for FLAG
946	Where to store concentration number
985	Form regression sums
1010	Test: if Standard or test line
1024	Store for Standard: n, a_1, a_0, $\bar{x}_S$, $\bar{y}_s$, $\sigma^2 x_S$
1055	Calculate $(n-2)SE^2$ $S/R(^1/x)$
1059	Calculate ℓ
1093	Slope, intercept, correlation
1134	Calculate $(n-2)S^2{}_E$
1138	Store

1142	Calculate F for linearity and degrees of freedom
1217	Calculate t for slope
1281	Calculate $S^2{}_p$
1318	Calculate S^2x_p
1409	Store
1413	Calculate t parallelity
1504	Calculate mean slope
1513	Store
1517	Calculate $M-\bar{x}_S + \bar{x}_t$
1528	Store
1532	Calculate M (potency ratio) and somatomedin
1559	SM value
1566	Calculate (1-g)
1591	Store
1593	Calculate 2nd term for fiducial limits
1641	Store
1645	Calculate 1st term for fiducial limits
1644	Fiducial limits
1682	Upper limit calculation
1703	Lower limit calculation
1724	Test: Standard or test line
1748	Request concentration numbers for test line
1821	Choose new line or repeat analysis
1880	Set location for test values
1887	Tape reader instruction (here = 2nd of 3)
1928	Take log of each µg/100mg
1952	Calculate $(n-2)S^2{}_E$

REFERENCES

Abraham, R., Dawson, W., Grasso, P. and Goldberg, L. (1968)
Lysosomal changes associated with hypoxia in the isolated perfused rat liver.
Experimental and Molecular Pathology 8, 370-387.

Adams, P. and Berridge, F.R. (1969)
Effects of kwashiorkor on cortical and trabecular bone.
Archives of Diseases in Childhood 44, 705-709.

Adamson, L.F. and Anast, C.S. (1966)
Amino acid, potassium and sulfate transport and incorporation by embryonic chick cartilage: the mechanism of the stimulatory effects of serum.
Biochimica et Biophysica Acta 121, 10-20.

Adibi, S.A. and Drash, A.L. (1970)
Hormone and aminoacid levels in altered nutritional states.
Journal of Laboratory and Clinical Medicine 76, 722-732.

Alford, F.P., Bellair, J.T., Burger, H.G. and Lovett, N. (1972)
A simplified assay for somatomedin.
Journal of Endocrinology 54, 365-366.

Almqvist, S. (1961)
Studies on sulfation factor (SF) activity of human serum.
Acta Endocrinologica 36, 31-50.

Almqvist, S. and Falkheden, T. (1961)
Studies on sulfation factor (SF) activity of human serum. Rate of decrease of serum SF after hypophysectomy.
Acta Endocrinologica 37, 315-320.

Almqvist, S., Ikkos, D. and Luft, R. (1961)
Studies on sulfation factor (SF) activity of human serum. Serum SF in hypopituitarism and acromegaly.
Acta Endocrinologica 36, 577-595.

Almqvist, S. and Rune, I. (1961)
Studies on sulfation factor (SF) activity of human serum. The variation of serum SF with age.
Acta Endocrinologica 36, 566-576.

Angeletti, P.U., Salvi, M.L., Capani, F. and Frati, L. (1965)
Granulocytosis-inducing factor from the mouse submaxillary gland.
Biochimica et Biophysica Acta 111, 344-346.

Ash, P. and Francis, M.J.O. (1975)
Response of isolated rabbit articular and epiphyseal chondrocytes to rat liver somatomedin.
Journal of Endocrinology 66, 71-78.

Ashton, I.K., Matheson, J.A., Vesey, J. and Francis, M.J.O. (1978)
Somatomedin in human foetal and adolescent growth.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure (Genoa, Italy) March 1-3, 1978, p. 48.

Ashworth, A., Bell, R., James, W.P.T. and Waterlow, J.C. (1968)
Calorie requirements of children recovering from protein-calorie malnutrition.
Lancet ii, 600-603.

Bala, R.M., Hankins, C. and Smith, G.R. (1975)
A somatomedin assay using normal rabbit cartilage in clinical studies.
Canadian Journal of Physiology and Pharmacology 53, 403-409.

Beas, F., Contreras, I., Maccioni, A. and Arenas, S. (1971)
Growth hormone in infant malnutrition: the arginine test in marasmus and kwashiorkor.
British Journal of Nutrition 26, 169-175.

Beaton, G.R. (1976)
Studies on serum somatomedin activity and cartilage responsiveness in the regulation of growth.
Ph.D. Thesis, University of the Witwatersrand, Johannesburg.

Beaton, G.R., Sagel, J. and Distiller, L.A. (1975)
Somatomedin activity in cerebrospinal fluid.
Journal of Clinical Endocrinology and Metabolism 40, 736-737.

Becker, D.J., Pimstone, B.L. and Hansen, J.D.L. (1975)
The relationship between insulin secretion, glucose tolerance, growth hormone and serum proteins in protein-calorie malnutrition.
Pediatric Research 9, 35-39.

Becker, D.J., Pimstone, B.L., Hansen, J.D.L. and Hendricks, S. (1971)
Insulin secretion in protein-calorie malnutrition. I. Quantitative abnormalities and response to treatment.
Diabetes 20, 542-551.

Becker, D.J., Pimstone, B.L., Hansen, J.D.L. and Hendricks, S. (1971)
Serum albumin and growth hormone relationships in kwashiorkor and the nephrotic syndrome.
Journal of Laboratory and Clinical Medicine 78, 865-871.

Becker, D.J., Pimstone, B.L., Kronheim, S. and Weinkove, E. (1975)
The effect of alanine infusions on growth hormone, insulin and glucose in protein-calorie malnutrition.
Metabolism 24, 953-958.

Becks, H., Simpson, M.E., Evans, H.M., Ray, R.D., Li, C.H. and Asling, C.W. (1946)
Response to pituitary growth hormone and thyroxin of the tibias of hypophysectomised rats after long postoperative intervals.
Anatomical Record 94, 631-648.

Berridge, F.R. and Prior, K.M. (1954)
The skeletal development of the children at the beginning and end of the period of experimental feeding.
Special Report Series of the Medical Research Council (London) No. 287, 119.

Binoux, M., Schimpff, R.M. and Donnadieu, M. (1977)
Serum somatomedin activity depressed after glucagon administration in man.
Journal of Clinical Endocrinology and Metabolism 44, 1006-1009.

Birge, C.A., Peake, G.T., Mariz, I.K. and Daughaday, W.H. (1967)
Radioimmunoassayable growth hormone in the rat pituitary gland: effects of age, sex and hormonal state.
Endocrinology 81: 195-204.

Blizzard, R.M. and Alberts, M. (1956)
Hypopituitarism, hypoadrenalism, and hypogonadism in the newborn infant.
Journal of Pediatrics 48, 782-792.

Boström, H. (1952)
On the metabolism of the sulfate group of chondroitin sulphuric acid.
Journal of Biological Chemistry 196, 477-481.

Boström, H., Roden, L. and Westermark, A. (1955)
Glutamine as an accelerator of chondroitin sulphate synthesis.
Nature 176, 601-602.

Chan, J.S.D., Robertson, H.A. and Friesen, H.G. (1976)
The purification and characterization of ovine placental lactogen.
Endocrinology 98, 65-76.

Chesley, L.C. (1962)
Growth hormone activity in human pregnancy. I. Serum sulfation factor.
American Journal of Obstetrics and Gynecology 84, 1075-1080.

Chochinov, R.H. and Daughaday, W.H. (1976)
Current concepts of somatomedin and other biologically related growth factors.
Diabetes 25, 994-1007.

Chochinov, R.H. and Daughaday, W.H. (1976)
Unpublished observation quoted in Current concepts of somatomedin and other biologically related growth factors.
Diabetes 25, 994-1007.

Chochinov, R.H., Mariz, I.K. and Daughaday, W.H. (1977)
Isolation of a somatomedin from plasma of rats bearing growth hormone secreting tumours.
Endocrinology 100, 549-556.

Chochinov, R.H., Mariz, I.K., Hajek, A.S. and Daughaday, W.H. (1977)
Characterization of a protein in mid-term human amniotic fluid which reacts in the somatomedin-C radioreceptor assay.
Journal of Clinical Endocrinology and Metabolism 44, 902-908.

Cohen, K.L. and Nissley, S.P. (1975)
Comparison of somatomedin activity in rat serum and lymph.
Endocrinology 97, 654-658.

Cohen, K.L., Short, P.A. and Nissley, S.P. (1975)
Growth hormone-dependent serum stimulation of DNA synthesis in chick embryo fibroblasts in culture.
Endocrinology 96, 193-198.

Cohen, S. (1959)
Purification and metabolic effects of a nerve growth-promoting protein from snake venom.
Journal of Biological Chemistry 234, 1129-1137.

Cohen S. (1962)
Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal.
Journal of Biological Chemistry 237, 1555-1562.

Cohen, S., Carpenter, G. and Lembach, K.J. (1975)
Interaction of epidermal growth factor (EGF) with cultured fibroblasts.
Advances in Metabolic Disorders 8, 265-284.

Costin, G., Kogut, M.D., Phillips, L.S. and Daughaday, W.H. (1976)
Craniopharyngioma: the role of insulin in promoting postoperative growth.
Journal of Clinical Endocrinology and Metabolism 42, 370-379.

Culling, C.F.A. (1974)
In: Handbook of Histopathological and Histochemical Techniques.
3rd Edition, pp. 259-314. Butterworth & Co., London.

Dacie, J.V. and Lewis, S.M. (1963)
In: Practical Haematology. 5th Edition, pp. 36 and 42.
J. & H. Churchill, London.

Daniels, J.S., Mariz, I.K. and Daughaday, W.H. (1977)
Immunologic characterization of a rat somatomedin.
Endocrine Society 59th Annual Meeting, Chicago, June 8-10, 1977 (Abstract 163).

Daughaday, W.H. (1969)
Growth hormone assay in acromegaly, gigantism, dwarfism and hypopituitarism.
Postgraduate Medicine 46: 84-91.

Daughaday, W.H. (1971)
Regulation of skeletal growth by sulfation factor.
Advances in Internal Medicine 17, 237-263.

Daughaday, W.H. (1975)
Somatomedin levels in human beings.
Advances in Metabolic Disorders 8, 159-170.

Daughaday, W.H. and Garland, J.T. (1972)
The sulfation factor hypothesis: recent observations.
Excerpta Medica ICS 244 (Growth and Growth Hormone) 168.

Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D., van den Brande, J.L. and van Wyk, J.J. (1972)
Somatomedin: proposed designation for sulphation factor.
Nature 235, 107.

Daughaday, W.H. and Kipnis, D.M. (1966)
The growth-promoting and anti-insulin actions of somatotropin.
Recent Progress in Hormone Research 22, 49-99.

Daughaday, W.H., Laron, Z., Pertzelan, A. and Heins, J.N. (1969)
Defective sulfation factor generation: a possible etiological link in dwarfism.
Transactions of the Association of American Physicians 82, 129-140.

Daughaday, W.H. and Mariz, I.K. (1962)
Conversion of proline-U-C^{14} to labeled hydroxyproline by rat cartilage in vitro: effects of hypophysectomy, growth hormone, and cortisol.
Journal of Laboratory and Clinical Medicine 59, 741-752.

Daughaday, W.H., Mariz, I.K., Daniels, J.S., Jacobs, J.W. and Rubin, J.S. (1978)
Studies on rat somatomedin.
International Symposium on Somatomedins and Growth, Santa Margherita Ligure (Genoa, Italy) March 1-3, 1978, p. 11.

Daughaday, W.H., Phillips, L.S. and Herington, A.C. (1975)
Measurement of somatomedin by cartilage in vitro.
Methods in Enzymology 37, 93-109.

Daughaday, W.H., Phillips, L.S. and Mueller, M.C. (1976)
The effects of insulin and growth hormone on the release of somatomedin by the isolated rat liver.
Endocrinology 98, 1214-1219.

Daughaday, W.H. and Reeder, C. (1966)
Synchronous activation of DNA synthesis in hypophysectomised rat cartilage by growth hormone.
Journal of Laboratory and Clinical Medicine 68, 357-368.

Daughaday, W.H., Salmon, W.D. and Alexander, F. (1959)
Sulfation factor activity of sera from patients with pituitary disorders.
Journal of Clinical Endocrinology and Metabolism 19, 743-758.

Davies, D.V. and Coupland, R.E. (1967)
In: Grey's Anatomy, 34th Edition, pp. 32-47, Longmans, London.

Dehnel, J.M. and Francis, M.J.O. (1972)
Somatomedin (sulphation factor)-like activity of homocystine.
Clinical Science 43, 903-906.

Dehnel, J.M., McConaghey, P.D. and Francis, M.J.O. (1974)
Fractionation of liver somatomedin activity by ultrafiltration.
Journal of Endocrinology 62, 355-361.

Deo, M.G., Sood, S.K. and Ramalingaswami, V. (1965)
Experimental protein deficiency. Pathological features in the Rhesus monkey.
Archives of Pathology 80, 14-23.

D'Ercole, A.J., Underwood, L.E. and van Wyk, J.J. (1977)
Serum somatomedin-C in hypopituitarism and in other disorders of growth.
Journal of Pediatrics 90, 375-381.

Dickerson, J.W.T. and John, P.M.V. (1969)
The effect of protein-calorie malnutrition on the composition of the human femur.
British Journal of Nutrition 23, 917-924.

Doumas, B.T., Watson, W.A. and Biggs, H.G. (1971)
Albumin standards and the measurement of serum albumin with bromcresol green.
Clinica Chimica Acta 31, 87-96.

Draznin, B., Schalch, D., Heinrich, U.E. and Schlueter, R.J. (1978)
Physicochemical and biological characteristics of insulin-like Growth Factor Carrier Protein.
International Symposium on Somatomedins and Growth, Santa Margherita Ligure, (Genoa, Italy) March 1-3, 1978, p.29.

Du Caju, M.V.L. and van den Brande, J.L. (1976)
Personal communication, quoted in: Beaton, G.R. Ph.D. Thesis, University of the Witwatersrand.

Dziewiatkowski, D.D. (1951)
Radioautographic visualisation of sulfur-35 disposition in the articular cartilage and bone of suckling rats following injection of labeled sodium sulfate.
Journal of Experimental Medicine 93, 451-458.

Dziewiatkowski, D.D. (1954)
Effect of age on some aspects of sulfate metabolism in the rat.
Journal of Experimental Medicine 99, 283-298.

Dziewiatkowski, D.D. (1964)
Effect of hormones on the turnover of polysaccharides in connective tissues. In: Connective Tissue. Intercellular macromolecules (Proceedings of a Symposium sponsored by the New York Heart Association), 1st Edition, pp. 215-238.
Little Brown and Co. Boston.

Dziewiatkowski, D.D., Di Ferrante, N., Bronner, F. and Okinaka, G. (1957)
Turnover of S^{35}-sulfate in epiphyses and diaphyses of suckling rats. Nature of the S^{35}-labelled compounds.
Journal of Experimental Medicine 106, 509-524.

Edozien, J.C. (1968)
Experimental kwashiorkor and marasmus.
Nature 220, 917-919.

Elders, M.J., Garland, J.T., Daughaday, W.H., Fisher, D.A., Whitney, J.E. and Hughes, E.R. (1973)
Laron's dwarfism: studies on the nature of the defect.
Journal of Pediatrics 83, 253-263.

Elders, M.J., Wingfield, B.S., McNatt, M.L., Clarke, J.S. and Hughes, E.R. (1975)
Glucocorticoid therapy in children.
American Journal of Diseases of Childhood 129, 1393-1396.

Enberg, G., Fryklund, L. and Hall, K. (1978)
Purification of somatomedin. A binding protein.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa-Italy), March 1-3, 1978, p. 36.

Engfeldt, B. and Hjertquist, S-O. (1967)
The effect of various fixatives on the preservation of acid glycosaminoglycans in tissues.
Acta Pathologica et Microbiologica Scandinavica 71, 219-232.

Enwonwu, C.O. and Sreebny, L.M. (1970)
Experimental protein-calorie malnutrition in rats: biochemical and ultrastructural studies.
Experimental and Molecular Pathology 12, 332-353.

Eshet, R., Laron, Z., Brown, M. and Arnon, R. (1973)
Immunoreactive properties of the plasma hGH from patients with the syndrome of familial dwarfism and high plasma IR-hGH.
Journal of Clinical Endocrinology and Metabolism 37, 819-821.

Evans, H.M., Simpson, M.E., Marx, W. and Kibrick, E. (1943)
Bioassay of the pituitary growth hormone. Width of the proximal epiphysial cartilage of the tibia in hypophysectomized rats.
Endocrinology 32, 13-16.

Eversmann, T., Glöckner, B., Gottsmann, M., Weidl, G. and v. Werder, K. (1977)
Somatomedin-B in acromegaly, liver cirrhosis and renal failure.
Acta Endocrinologica Supplement 221, 186 (Abstract 320).

Exton, J.H. and Park, C.R. (1967)
Control of gluconeogenesis in liver. I. General features of gluconeogenesis in the perfused livers of rats.
Journal of Biological Chemistry 242, 2622-2636.

Finkelstein, J.W., Kream, J., Ludan, A. and Hellman, L. (1972)
Sulfation factor (somatomedin): An explanation for continued growth in the absence of immunoassayable growth hormone in patients with hypothalamic tumors.
Journal of Clinical Endocrinology and Metabolism 35, 13-17.

Finney, D.J. (1964)
Statistical method in biological assay. 2nd Edition.
Charles Griffin and Co., London.

Follis, R.H. (1967)
A kwashiorkor-like syndrome observed in monkeys fed maize.
Proceedings of the Society for Experimental Biology and Medicine 96, 523-528.

Francis, M.J.O. and Hill, D.J. (1975)
Prolactin-stimulated production of somatomedin by rat liver.
Nature 255, 167-168.

Franklin, R.C., Pepperel, R.J., Burger, H.G. and Cameron, D.P. (1978)
Non-suppressible insulin-like activity - soluble (NSILA-S): Studies of normal human pregnancy.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, p. 46.

Freud, J., Levie, L.H. and Kroon, D.B. (1939)
Observations on growth (chondrotrophic) hormone and localization of its point of attack.
Journal of Endocrinology 1, 56-64.

Friedman, R.C. and Reichlin, S. (1965)
Growth hormone content of the pituitary gland of starved rats.
Endocrinology 76, 787-788.

Froesch, E.R., Bürgi, H., Müller, W.A., Humbel, R.E., Jakob, A. and Labhart, A. (1967)
Nonsuppressible insulinlike activity of human serum: purification, physicochemical and biological properties and its relation to total serum ILA.
Recent Progress in Hormone Research 23, 565-605.

Froesch, E.R., Schlumpf, U., Heimann, R., Eigenmann, E. and Zapf, J. (1975)
NSILA-S, physiologic and pharmacologic significance as an insulin-like hormone and as a growth-promoting hormone.
Advances in Metabolic Disorders 8, 237-245.

Froesch, E.R., Schlumpf, U., Heimann, R., Zapf, J., Humbel, R.E. and Ritschard, W.J. (1975)
Purification procedures for NSILA-S.
Advances in Metabolic Disorders 8, 203-210.

Froesch, E.R., Zapf, J., Meuli, C., Mäder, M., Waldvogel, M., Kaufmann, U. and Morell, B. (1975)
Biological properties of NSILA-S.
Advances in Metabolic Disorders 8, 211-235.

Fryklund, L., Skottner, A. and Forsman, A. (1978)
Somatomedin A and B: Chemistry and biology.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure (Genoa, Italy), March 1-3, 1978, p. 8.

Fryklund, L., Uthne, K. and Sievertsson, H. (1974)
Identification of two somatomedin A active polypeptides and in vivo effects of a somatomedin A concentrate.
Biochemical and Biophysical Research Communications 61, 957-962.

Fryklund, L., Uthne, K., Sievertsson, H. and Westermark, B. (1974)
Isolation and characterization of polypeptides from human plasma enhancing the growth of human normal cells in culture.
Biochemical and Biophysical Research Communications 61, 950-956.

Fujisawa, Y. (1964)
Studies on sulfation factor in serum.
Folia Endocrinologica Japonica (Kyoto) 40, 203-208.

Furlanetto, R.W., Underwood, L.E., van Wyk, J.J. and D'Ercole, A.J. (1977)
Estimation of somatomedin-C levels in normals and patients with pituitary disease by radioimmunoassay.
Journal of Clinical Investigation 60, 648-657.

Garland, J.T. and Daughaday, W.H. (1972)
Feedback inhibition of pituitary growth hormone in rats infected with spirometra mansonoides.
Proceedings of the Society for Experimental Biology and Medicine 139, 497-499.

Garland, J.T., Jennings, J., Levitsky, L.L. and Buchanan, F. (1976)
Stimulation of DNA synthesis in isolated chondrocytes by somatomedin. II. Validation of the assay for clinical use and comparison with the stimulation of protein synthesis.
Journal of Clinical Endocrinology and Metabolism 43, 847-851.

Garland, J.T., Lottes, M.E., Kozak, S. and Daughaday, W.H. (1972)
Stimulation of DNA synthesis in isolated chondrocytes by sulfation factor.
Endocrinology 90, 1086-1090.

Garland, J.T., Ruegamer, W.R. and Daughaday, W.H. (1971)
Induction of sulfation factor activity by infection of hypophysectomized rats with spirometra mansonoides.
Endocrinology 88, 924-927.

Giordano, G., Foppiani, E., Minuto, F. and Perroni, D. (1976)
Growth hormone and somatomedin behaviour in the newborn.
Acta Endocrinologica 81, 449-454.

Giordano, G., Foppiani, E., Minuto, F., Perroni, D., DiCicco, M. (1975)
Somatomedin (Sm) behaviour in patients with chronic liver diseases.
Acta Endocrinologica Supplement 199, p. 262.

Gluckman, P.D. and Brinsmead, M.W. (1976)
Somatomedin in cord blood: Relationship to gestational age and birth size.
Journal of Clinical Endocrinology and Metabolism 43, 1378-1381.

Gordon, A.S. (1971)
The current status of erythropoietin.
British Journal of Haematology 21, 611-616.

Gospodarowicz, D., Rudland, P., Lindstrom, J. and Benirschke, K. (1975)
Fibroblast growth factor: Its localization, purification, mode of action, and physiological significance.
Advances in Metabolic Disorders 8, 301-335.

Graham, G.G., Cordano, A., Blizzard, R.M. and Cheek, D.B. (1969)
Infantile malnutrition: Changes in body composition during rehabilitation.
Pediatric Research 3, 579-589.

Grant, D.B., Hambley, J., Becker, D. and Pimstone, B.L. (1973)
Reduced sulphation factor in undernourished children.
Archives of Diseases in Childhood 48, 596-600.

Greenspan, F.S., Li, C.H., Simpson, M.E. and Evans, H.M. (1949)
Bioassay of hypophyseal growth hormone: the tibia test.
Endocrinology 45, 455-463.

Gregory, H. (1975)
Isolation and structure of urogastrone and its relationship to epidermal growth factor.
Nature 257, 325-327.

Hadden, D.R. (1967)
Glucose, free fatty acid and insulin interrelations in kwashiorkor and marasmus.
Lancet ii, 589-593.

Hadden, D.R. and Rutishauser, I.H.E. (1967)
Effect of human growth hormone in kwashiorkor and marasmus.
Archives of Disease in Childhood 42, 29-33.

Haft, D.E. and Miller, L.L. (1958)
Alloxan diabetes and demonstrated direct action of insulin on metabolism of isolated perfused rat liver.
American Journal of Physiology 192, 33-42.

Hall, K. (1970)
Quantative determination of the sulphation factor activity in human serum.
Acta Endocrinologica 63, 338-350.

Hall, K. (1971)
Effect of intravenous administration of human growth hormone on sulphation factor activity in serum of hypopituitary subjects.
Acta Endocrinologica 66, 491-497.

Hall, K. (1972)
Human somatomedin: Determination, occurrence, biological activity and purification.
Acta Endocrinologica Suppl. 163.

Hall, K. and Bozovic, M. (1969)
Stimulation of ^{35}S incorporation into embryonic chick cartilage by extract from rat muscle.
Hormone and Metabolic Research 1, 235-240.

Hall, K., Brant, J., Enberg, G. and Fryklund, L. (1978)
Somatomedin A by radioimmunoassay and radioreceptor assay and its relation to carrier protein.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, p. 24.

Hall, K. and Filipsson, R. (1975)
Correlation between somatomedin A in serum and body height development in healthy children and children with certain growth disturbances.
Acta Endocrinologica 78, 239-250.

Hall, K. and Olin, P. (1972)
Sulphation factor activity and growth rate during long-term treatment of patients with pituitary dwarfism with human growth hormone.
Acta Endocrinologica 69, 417-433.

Hall, K., Takano, K., Fryklund, L. and Sievertsson, H. (1975)
Somatomedins.
Advances in Metabolic Disorders 8, 19-46.

Hall, K. and Uthne, K. (1971)
Some biological properties of purified sulfation factor (SF) from human plasma.
Acta Medica Scandinavica 190, 137-143.

Hambley, J., Howell, A. and Grant, D.B. (1974)
Absence of sulphation factor (somatomedin) activity in preparations of colony stimulating factor and nerve growth factor.
Experientia 30, 1225-1226.

Harper, A.E. (1959)
Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance.
Journal of Nutrition 68, 405-418.

Hase, T. and Brim, J. (1966)
Observation on the microcirculatory architecture of the rat liver.
Anatomical Record 156, 157-174.

Hazlewood, C.F. and Nichols, B.L. (1969)
Changes in muscle sodium, potassium, chloride, water and voltage during maturation in the rat: An experimental and theoretical study.
Johns Hopkins Medical Journal 125, 119-133.

Heard, C.R.C. and Turner, M.R. (1967)
Glucose tolerance and related factors in dogs fed diets of sub-optimal protein value.
Diabetes 16, 96-107.

Heinrich, U.E., Draznin, B., Johnson, C.J. and Schalch, D.S. (1978)
NSILA (Non Suppressible insulin-like activity) and fetal growth.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, p. 47.

Heinrich, U.E., Schalch, D.S., Miller, L.L. and Johnson, C.G. (1977)
Hormonal regulation of NSILA release from the isolated perfused rat liver.
Acta Endocrinologica Supplement 221, 100 (Abstract 150).

Heins, J.N., Garland, J.T. and Daughaday, W.H. (1970)
Incorporation of ^{35}S-sulfate into rat cartilage explants in vitro: effects of aging on responsiveness to stimulation by sulfation factor.
Endocrinology 87, 688-692.

Hems, R., Ross, B.D., Berry, M.N. and Krebs, H.A. (1966)
Gluconeogenesis in the perfused rat liver.
Biochemical Journal 101, 284-292.

Henry, R.J. (1964)
In: Clinical Chemistry, Principles and Techniques. 1st Edition, pp. 417-421, Hoeber Medical Division, Harper and Row, New York.

Herbai, G. (1970)
Incorporation and disappearance of sulfate in different regions of mouse and rat costal cartilage in vivo and in vitro.
Acta Physiologica Scandinavica 79, 541-551.

Herington, A.C., Adamson, L.F. and Bornstein, J. (1972)
Differentiation on the basis of glucose requirements between the effects of somatomedin on protein synthesis and sulfate incorporation in embryonic chick cartilage.
Biochimica et Biophysica Acta 286, 164-174.

Higginson, J. (1954)
Studies on human bone from South African Bantu subjects. II. Histological changes in the ribs of South African Bantu infants.
Metabolism 3, 392-399.

Hill, D.J. (1977)
Somatomedin production by rat prolactin depends on pituitary integrity.
Acta Endocrinologica Supplement 221, 99 (Abstract 148).

Hill, D.J., Francis, M.J.O., Conroy, P.D. and Ashton, K. (1977)
The control of somatomedin release from the perfused rat liver.
Proceedings of the XIIth European Symposium on Calcified Tissues.
Calcified Tissue Research (Supplement to Vol.22), 378-380.

Hintz, R.L., Clemmons, D.R., Underwood, L.E. and van Wyk, J.J. (1972)
Competitive binding of somatomedin to the insulin receptors of adipocytes, chondrocytes, and liver membranes.
Proceedings of the National Academy of Sciences, USA 69, 2351-2353.

Hintz, R.L., Clemmons, D.R. and van Wyk, J.J. (1972)
Growth hormone-induced somatomedin-like activity from liver.
Pediatric Research 6, 353/93.

Hintz, R.L., Seeds, J.M. and Johnsonbaugh, R.E. (1974)
Somatomedin and growth hormone in the newborn.
Pediatric Research 8, 369/95.

Hintz, R.L., Suskind, R., Amatayakul, K. and Olsen, R. (1976)
Somatomedin in children with protein calorie malnutrition.
Pediatric Research 10, 339 (Abstract 229).

Hintz, R.L., Suskind, R., Amatayakul, K., Thanangkul, O. and Olson, R. (1978)
Plasma somatomedin and growth hormone values in children with protein-calorie malnutrition.
Journal of Pediatrics 92, 153-156.

Hoffenberg, R., Howell, A., Epstein, S., Pimstone, B.L., Fryklund, L., Hall, K., Schwalbe, S. and Rudd, B.T. (1977)
Increasing growth with raised circulating somatomedin but normal immunoassayable growth hormone.
Clinical Endocrinology 6, 443-448.

Hogue-Angeletti, R.A., Bradshaw, R.A. and Frazier, W.A. (1975)
Nerve growth factor: Structure and mechanism of action.
Advances in Metabolic Disorders 8, 285-299.

Hohorst, H.J. (1963)
L (+) lactate. Determination with lactic dehydrogenase and DPN.
In: Methods of Enzymatic Analysis. H.U. Bergmeyer, Editor.
pp. 266-270. Academic Press, New York.

Humbel, R.E. and Rinderknecht, E. (1977)
Insulin-like growth factor - isolation and aminoacid sequence.
Acta Endocrinologica Supplement 212, 20 (Abstract S23).

Hunter, W.M. and Greenwood, F.C. (1962)
Preparation of iodine-131 labeled human growth hormone of high specific activity.
Nature 194, 495-496.

Hurley, T.W., D'Ercole, A.J., Handwerger, S., Underwood, L.E., Furlanetto, R.W. and Fellows, R.E. (1977)
Ovine placental lactogen induces somatomedin: A possible role in fetal growth.
Endocrinology 101, 1635-1638.

Ingalls, T.H. and Hayes, D.R. (1941)
Epiphyseal growth: The effect of removal of the adrenal and pituitary glands on the epiphyses of growing rats.
Endocrinology 29, 720-724.

Jacobs, L.S., Sneid, D.S., Garland, J.T., Laron, Z. and Daughaday, W.H. (1976)
Receptor-active growth hormone in Laron dwarfism.
Journal of Clinical Endocrinology and Metabolism 42, 403-406.

Jakob, A., Hauri, Ch. and Froesch, E.R. (1968)
Nonsuppressible insulin-like activity in human serum. III. Differentiation of two distinct molecules with nonsuppressible ILA.
Journal of Clinical Investigation 47, 2678-2688.

James, R., Niall, H., Kwok, S. and Bryant-Greenwood, G. (1977)
Primary structure of porcine relaxin: Homology with insulin and related growth factors.
Nature 267, 544-546.

Jha, G.J., Deo, M.G. and Ramalingaswami, V. (1968)
Bone growth in protein deficiency. A study in Rhesus monkeys.
American Journal of Pathology 53, 1111-1121.

Kalk, W.J. and Pimstone, B.L. (1974)
Calcium-binding protein and vitamin D metabolism in experimental protein malnutrition.
British Journal of Nutrition 32, 569-578.

Kelman, L., Saunders, S.J., Wicht, S., Frith, L., Corrigall, A., Kirsch, R.E. and Terblanche, J. (1972)
The effect of amino acids on albumin synthesis by the isolated perfused rat liver.
Biochemical Journal 129, 805-809.

Kenny, F.M., Guyda, H.J., Wright, J.C. and Friesen, H.G. (1973)
Prolactin and somatomedin in hypopituitary patients with "catch-up" growth following operations for craniopharyngioma.
Journal of Clinical Endocrinology and Metabolism 36, 378-380.

Kenny, F.M., Iturzaeta, N.F., Mintz, D., Drash, A., Garces, L.Y., Susen, A. and Askari, H.A. (1968)
Iatrogenic hypopituitarism in craniopharyngioma: Unexplained catch-up growth in three children.
Journal of Pediatrics 72, 766-775.

Keret, R., Schimpff, R.M. and Girard, F. (1976)
The inhibitory effect of hydrocortisone on the chicken embryo cartilage somatomedin assay.
Hormone Research 7, 254-259.

Kirsch, R.E., Brock, J.F. and Saunders, S.J. (1968)
Experimental protein-calorie malnutrition.
American Journal of Clinical Nutrition 21, 820-826.

Kirsch, R.E., Saunders, S.J. and Brock, J.F. (1968)
Animal models and human protein-calorie malnutrition.
American Journal of Clinical Nutrition 21, 1225-1228.

Kleeman, C.R., Taborsky, E. and Epstein, F.H. (1956)
Improved method for determination of inorganic sulfate in biological fluids.
Proceedings of the Society for Experimental Biology and Medicine 91, 480-483.

Kogut, M.D., Kaplan, S.A. and Shimizu, C.S.N. (1963)
Growth retardation: Use of sulfation factor as a bioassay for growth hormone.
Pediatrics 31, 538-551.

Koumans, J. and Daughaday, W.H. (1963)
Amino acid requirement for activity of partially purified sulfation factor.
Transactions of the Association of American Physicians 76, 152-162.

Laron, Z., Pertzelan, A., Karp, M., Kowadlo-Silbergeld, A. and Daughaday, W.H. (1971)
Administration of growth hormone to patients with familial dwarfism with high plasma immunoreactive growth hormone: measurement of sulfation factor, metabolic and linear growth responses.
Journal of Clinical Endocrinology and Metabolism 33, 332-342.

Laron, Z., Pertzelan, A. and Mannheimer, S. (1966)
Genetic pituitary dwarfism with high serum concentration of growth hormone. A new inborn error of metabolism?
Israel Journal of Medical Sciences 2, 152-155.

Lavine, R.L., Chick, W.L., Like, A.A. and Makdisi, T.W. (1971)
Glucose tolerance and insulin secretion in neonatal and adult mice.
Diabetes 20, 134-139.

Lecornu, M. (1973)
Le facteur serique de sulfatation (somatomedine) dans les retards de croissance, le gigantisme cerebral et l'acromegalie.
Archives Francaises de Pédiatrie 30, 595-608.

le Roith, D. and Pimstone, B.L. (1973)
Bone metabolism and composition in the protein-deprived rat.
Clinical Science 44, 305-319.

Levi-Montalcini, R. and Angeletti, P.U. (1968)
Nerve growth factor.
Physiological Reviews 48, 534-569.

Levine, L.S., Wiedemann, E., Saenger, P., Schwartz, E. and New, M.I. (1976)
Low somatomedin activity (SM) and lack of response to hGH in glycogen storage disease.
Paediatric Research 10, 340 (Abstract 238).

Liberti, J.P. (1975)
Purification of bovine somatomedin.
Biochemical and Biophysical Research Communications 67, 1226-1233.

Lillie, R.D. and Ashburn, L.L. (1943)
Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degenerations not shown by Herxheimer technic.
Archives of Pathology 36, 432-435.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951)
Protein measurement with the Folin phenol reagent.
Journal of Biological Chemistry 193, 265-275.

Lunn, P.G., Whitehead, R.G., Hay, R.W. and Barker, B.A. (1973)
Progressive changes in serum cortisol, insulin and growth hormone concentrations and their relationship to the disordered amino acid pattern during the development of kwashiorkor.
British Journal of Nutrition 29, 399-422.

MacGillivray, M.H., Hastings, C. and Brown, J.A. (1975)
Growth hormone dependent effects of human serum on the in vitro growth characteristics of human skin fibroblasts.
Journal of Clinical Endocrinology and Metabolism 40, 62-69.

Magyesi, K., Kahn, C.R., Roth, J., Neville, D.M., Nissley, S.P., Humbel, R.E. and Froesch, E.R. (1975)
The NSILA-S receptor in liver plasma membranes. Characterization and comparison with the insulin receptor.
Journal of Biological Chemistry 250, 8990-8996.

Martin, J.B., Durand, D., Gurd, W., Faille, G., Audet, J. and Brazeau, P. (1978)
Neuropharmacological regulation of episodic growth hormone and prolactin secretion in the rat.
Endocrinology 102, 106-113.

Martin, J.M. and Jambrosic, J. (1977)
Growth hormone secretion under various nutritional conditions.
Acta Endocrinologica Supplement 212, 97 (Abstract 143).

McCance, R.A. and Widdowson, E.M. (1966)
Protein deficiencies and calorie deficiencies.
Lancet ii, 158-159.

McConaghey, P. (1972)
The production of "sulphation factor" by rat liver.
Journal of Endocrinology 52, 1-9.

McConaghey, P. and Dehnel, J. (1972)
Preliminary studies of "sulphation factor" production by rat kidney.
Journal of Endocrinology 52, 587-588.

McConaghey, P. and Sledge, C.B. (1970)
Production of "sulphation factor" by the perfused liver.
Nature 225, 1249-1250.

Meuli, C. and Froesch, E.R. (1977)
Insulin and nonsuppressible insulin-like activity (NSILA-S) stimulate the same glucose transport system via two separate receptors in rat heart.
Biochemical and Biophysical Research Communications 75, 689-695.

Milner, R.D.G. (1971a)
Metabolic and hormonal responses to glucose and glucagon in patients with infantile malnutrition.
Pediatric Research 5, 33-39.

Milner, R.D.G. (1971b)
Metabolic and hormonal responses to oral amino acids in infantile malnutrition.
Archives of Disease in Childhood 46, 301-305.

Mönckeberg, F., Donoso, G., Oxman, S., Pak, N. and Meneghello, J. (1963)
Human growth hormone in infant malnutrition.
Pediatrics 31, 58-64.

Morgan, C.R. and Lazarow, A. (1963)
Immunoassay of insulin: Two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats.
Diabetes 12, 115-126.

Moses, A.C., Nissley, S.P., Rechler, M.M., Short, P.A. and Podskalny, J.M. (1978)
The purification and characterization of multiplication stimulating activity (MSA) from media conditioned by a rat liver cell line.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, pp. 16-17.

Mosier, H.D., Dearden, L.C., Jansons, R.A. and Hill, R.R. (1977)
Growth hormone, somatomedin and cartilage sulfation in failure of catch-up growth after propylthiouracil-induced hypothyroidism in the rat.
Endocrinology 100, 1644-1651.

Mosier, H.D., Jansons, R.A., Hill, R.R. and Dearden, L.C. (1976)
Cartilage sulfation and serum somatomedin in rats during and after cortisone-induced growth arrest.
Endocrinology 99, 580-589.

Mulinos, M.G. and Pomerantz, L. (1940)
Pseudohypophysectomy: a condition resembling hypophysectomy produced by malnutrition.
Journal of Nutrition 19, 493-504.

New, M.I., Schwartz, E., Parks, G.A., Landey, S. and Wiedemann, E. (1972)
Pseudohypopituitary dwarfism with normal plasma growth hormone and low serum sulfation factor.
Journal of Pediatrics 80, 620-626.

Padwaldesai, S.R., Ninjoor, V., Ramakrishnan, T.V., Sawant, P.L., Kumta, U.S. and Sreenivasan, A. (1969)
Protein deficiency in spaced-fed rats.
British Journal of Nutrition 23, 745-753.

Peschle, C., Rappaport, I.A., Sasso, G.F., Gordon, A.S. and Condorelli, M. (1972)
Mechanism of growth hormone (GH) action on erythropoiesis.
Endocrinology 91, 511-517.

Phillips, L.S. and Herington, A.C. (1975)
A general bioassay program for desktop computer: Application to somatomedin bioassay.
Computers and Biomedical Research 8, 362-369.

Phillips, L.S., Herington, A.C. and Daughaday, W.H. (1974)
Somatomedin stimulation of sulfate incorporation in porcine costal cartilage discs.
Endocrinology 94, 856-863.

Phillips, L.S., Herington, A.C., Karl, I.E. and Daughaday, W.H. (1976)
Comparison of somatomedin activity in perfusates of normal and hypophysectomized rat livers with and without added growth hormone.
Endocrinology 98, 606-614.

Phillips, L.S. and Orawski, A.T. (1976)
Dietary control of somatomedin.
Clinical Research 24, 530A.

Phillips, L.S. and Young, H.S. (1976a)
Nutrition and somatomedin. I. Effect of fasting and refeeding on serum somatomedin activity and cartilage growth activity in rats.
Endocrinology 99, 304-314.

Phillips, L.S. and Young, H.S. (1976b)
Nutrition and somatomedin. II. Serum somatomedin activity and cartilage growth activity in streptozotocin-diabetic rats.
Diabetes 25, 516-527.

Pierson, R.W. and Temin, H.M. (1972)
The partial purification from calf serum of a fraction with multiplication-stimulating activity for chicken fibroblasts in cell culture and with non-suppressible insulin-like activity.
Journal of Cellular Physiology 79, 319-330.

Pimstone, B. (1976)
Endocrine function in protein-calorie malnutrition.
Clinical Endocrinology 5, 79-95.

Pimstone, B., Barbezat, G., Hansen, J.D.L. and Murray, P. (1967)
Growth hormone and protein-calorie malnutrition. Impaired suppression during induced hyperglycaemia.
Lancet ii, 1333-1334.

Pimstone, B.L., Barbezat, G., Hansen, J.D.L. and Murray, P. (1968)
Studies on growth hormone secretion in protein-calorie malnutrition.
American Journal of Clinical Nutrition 21, 482-487.

Pimstone, B.L., Becker, D.J. and Hansen, J.D.L. (1972)
Human growth hormone in protein calorie malnutrition.
In: Growth and Growth Hormone. Proceedings of the Second International Symposium on Growth Hormone, Milan, May 5-7, 1971, pp.389-401, Pecile, A. and Müller, E.E. Editors, Exerpta Medica, Amsterdam.

Pimstone, B.L., Becker, D.J. and Hansen, J.D.L. (1973)
Human growth hormone and sulphation factor in protein-calorie malnutrition. In: Endocrine Aspects of Malnutrition, pp. 73-90. L.I. Gardner and P. Amacher, Editors. Kroc Foundation Symposia, Number 1, Santa Ynez, California.

Pimstone, B.L., Becker, D. and Kronheim, S. (1975)
Disappearance of plasma growth hormone in acromegaly and protein-calorie malnutrition after somatostatin.
Journal of Clinical Endocrinology and Metabolism 40, 168-171.

Pimstone, B.L., Wittmann, W., Hansen, J.D.L. and Murray, P. (1966)
Growth hormone and kwashiorkor. Role of protein in growth-hormone homeostasis.
Lancet ii, 779-780.

Platt, B.S., Heard, C.R.C. and Stewart, R.J.C. (1964)
Experimental protein-calorie deficiency. In: Mammalian Protein Metabolism, Vol. 2, pp. 456-466. Munro, H.N. and Allison, J.B. Editors. Academic Press, New York and London.

Platt, B.S. and Stewart, R.J.C. (1962)
Transverse trabeculae and osteoporosis in bones in experimental protein-calorie deficiency.
British Journal of Nutrition 16, 483-495.

Poffenbarger, P.L. (1975)
The purification and partial characterization of an insulin-like protein from human serum.
Journal of Clinical Investigation 56, 1455-1463.

Poffenbarger, P.L. (1978)
Chemistry and physiology of a human serum nonsuppressible insulin-like protein (NSILP).
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa-Italy), March 1-3, 1978, p.19.

Powell, G.F., Brasel, J.A. and Blizzard, R.M. (1967)
Emotional deprivation and growth retardation stimulating idiopathic hypopituitarism.
New England Journal of Medicine 276, 1271-1278.

Powis, G. (1970)
Perfusion of rat's liver with blood; transmitter overflow and gluconeogenesis.
Proceedings of the Royal Society B174, 503-515.

Quintarelli, G., Scott, J.E. and Dellovo, M.C. (1964)
The chemical and histochemical properties of Alcian blue. II. Dye binding of tissue polyanions.
Histochemie 4, 86-98.

Ramalingaswami, V. and Deo, M.G. (1968)
Experimental protein-calorie malnutrition in the Rhesus monkey.
In: Calorie Deficiencies and Protein Deficiencies.
Proceedings of a Colloquium, Cambridge, April, 1967, pp. 265-275.
McCance, R.A. and Widdowson, E.M. Editors, Churchill, London.

Rappaport, R. and Prevot, C. (1977)
Normal plasma somatomedin activity in hydrocortisone-treated congenital adrenal hyperplasia.
Journal of Pediatrics 90, 963-964.

Ray, R.D., Evans, H.M. and Becks, H. (1941)
Effect of the pituitary growth hormone on the epiphyseal disk of the tibia of the rat.
American Journal of Pathology 17, 509-528.

Reber, K. and Liske, R. (1976)
Radioimmunoassay for non-suppressible insulin-like activity.
Hormone Research 7, 201-213.

Rechler, M.M., Podskalny, J.M. and Nissley, S.P. (1976)
Interaction of multiplication-stimulating activity with chick embryo fibroblasts demonstrates a growth receptor.
Nature 259, 134-136.

Reeves, R.D., Dickinson, L., Lee, J., Kilgore, B., Branham, B. and Elders, M.J. (1977)
Effects of dietary composition on somatomedin activity in growing rats.
Federation Proceedings 36, (Abstract 4540).

Reeves, R.D., Lee, J.A., Dickinson, L., Kilgore, B., Branham, B. and Elders, M.J. (1977)
Influence of dietary protein and fat on somatomedin activity in growing rats.
Clinical Research 25, 68A.

Reid, J.D. (1960)
Congenital absence of the pituitary gland.
Journal of Pediatrics 56, 658-664.

Rinderknecht, E. and Humbel, R.E. (1976)
Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell growth promoting activities: Evidence for structural homology with insulin B chain.
Proceedings of the National Academy of Sciences USA 73, 4379-4381.

Ross, B.D. (1972a)
In: Perfusion Techniques in Biochemistry. First Edition, p. 190, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972b)
In: Perfusion Techniques in Biochemistry. First Edition, p. 197, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972c)
In: Perfusion Techniques in Biochemistry. First Edition, p. 139, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972d)
In: Perfusion Techniques in Biochemistry. First Edition, pp. 139 and 145, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972e)
In: Perfusion Techniques in Biochemistry. First Edition, p. 199, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972f)
In: Perfusion Techniques in Biochemistry. First Edition, pp. 192-193, Clarendon Press, Oxford, U.K.

Ross, B.D. (1972g)
In: Perfusion Techniques in Biochemistry. First Edition, p. 27, Clarendon Press, Oxford, U.K.

Rothenberg, L.H., Hintz, R. and VanCamp, M. (1977)
Assessment of physical maturation and somatomedin levels during puberty.
American Journal of Orthodontics 71, 666-677.

Russell, J.A. (1957)
Effects of growth hormone on protein and carbohydrate metabolism.
American Journal of Clinical Nutrition 5, 404-416.

Sacks, H. (1977)
In: Insulin Metabolism by Livers from Normal, Protein-depleted and Starved Rats.
Ph.D. Thesis, University of Cape Town, 1977.

Sacks, H. (1977a)
In: Insulin Metabolism by Livers from Normal, Protein-depleted and Starved Rats.
Ph.D. Thesis, University of Cape Town, p.88.

Sacks, H. (1977b)
In: Insulin Metabolism by Livers from Normal, Protein-depleted and Starved Rats.
Ph.D. Thesis, University of Cape Town, p. 92.

Sacks, H. (1977c)
In: Insulin Metabolism by Livers from Normal, Protein-depleted and Starved Rats.
Ph.D. Thesis, University of Cape Town, pp. 94-111.

Sacks, H., Pimstone, B., Waligora, K., Peires, L., Weinkove, E. and Saunders, S. (1977)
Differences between insulin degradation by soluble fractions and insulin clearance by perfused livers of normal, protein-depleted and starved rats.
Diabetes 26, 956-966.

Sacks, H., Waligora, K., Matthews, J. and Pimstone, B. (1977)
Inhibition by somatostatin of glucagon-induced release from the isolated perfused rat liver.
Endocrinology 101, 1751-1759.

Saenger, P., Levine, L.S., Wiedemann, E., Schwartz, E. and New, M.I. (1976)
Somatomedin in cerebral gigantism.
Journal of Pediatrics 88, 155-156.

Saenger, P., Schwartz, E., Wiedemann, E., Levine, L.S., Tsai, M. and New, M.I. (1976)
The interaction of growth hormone, somatomedin and oestrogen in patients with Turner's syndrome.
Acta Endocrinologica 81, 9-18.

Saenger, P., Wiedemann, E., Schwartz, E., Korth-Schutz, S., Lewy, J.E., Riggio, R.R., Rubin, A.L., Stenzel, K.H. and New, M.I. (1974)
Somatomedin and growth after renal transplantation.
Pediatric Research 8, 163-169.

Salmon, W.D. (1975)
Interaction of somatomedin and a peptide inhibitor in serum of hypophysectomised and starved, pituitary-intact rats.
Advances in Metabolic Disorders 8, 183-199.

Salmon, W.D. and Daughaday, W.H. (1956)
Sulfation factor, a serum component mediating the action of growth hormone in stimulating incorporation of sulfate into cartilage.
Journal of Clinical Investigation 35, 733.

Salmon, W.D. and Daughaday, W.H. (1957)
A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro.
Journal of Laboratory and Clinical Medicine 49, 825-836.

Salmon, W.D. and Daughaday, W.D. (1958)
The importance of amino acids as dialyzable components of rat serum which promote sulfate uptake by cartilage from hypophysectomised rats in vitro.
Journal of Laboratory and Clinical Medicine 57, 167-173.

Salmon, W.D. and DuVall, M.R. (1970a)
A serum fraction with "sulfation factor activity" stimulates in vitro incorporation of leucine and sulfate into protein-polysaccharide complexes, uridine into RNA and thymidine into DNA of costal cartilage from hypophysectomized rats.
Endocrinology 86, 721-727.

Salmon, W.D. and DuVall, M.R. (1970b)
In vitro stimulation of leucine incorporation into muscle and cartilage protein by a serum fraction with sulfation factor activity: differentiation of effects from those of growth hormone and insulin.
Endocrinology 87, 1168-1180.

Salmon, W.D. and Hosse, B.R. (1971)
Stimulation of HeLa cell growth by a serum fraction with sulfation factor activity.
Proceedings of the Society for Experimental Biology and Medicine 136, 805-808.

Salter, J. and Best, C.H. (1953)
Insulin as a growth hormone.
British Medical Journal ii, 353-356.

Samaan, N., Stillman, D. and Fraser, R. (1962)
Abnormalities of serum insulin-like activity in liver disease.
Lancet ii, 1287-1290.

Schalch, D.S., Heinrich, U.E., Johnson, C.J., Koch, J.G. and Miller, L.L. (1977)
Hepatic synthesis of somatomedin NSILA is under hormonal control.
Clinical Research 25, 399A.

Schimke, R.T. (1964)
The importance of both synthesis and degradation in the control of arginase levels in rat liver.
Journal of Biological Chemistry 239, 3808-3817.

Schimpff, R.M., Donnadieu, M., Glasinovic, J.C., Warnet, J.M. and Girard, F. (1976)
The liver as a source of somatomedin: an in vivo study in the dog.
Acta Endocrinologica 83, 365-372.

Schimpff, R.M., Donnadieu, M., Gourmelen, M. and Girard, F. (1974)
The effects of hGH treatment on somatomedin levels in the serum (as determined by ^{35}S uptake into cartilage).
Hormone and Metabolic Research 6, 494-498.

Schnieden, H., Hendrickse, R.G. and Haigh, C.P. (1958)
Studies in water metabolism in clinical and experimental malnutrition.
Transactions of the Royal Society of Tropical Medicine and Hygiene 52, 169-175.

Schwabe, C. and McDonald, J.K. (1977)
Relaxin: A disulfide homolog of insulin.
Science 197, 914-915.

Shapiro, B. and Pimstone, B.L. (1977)
A phylogenetic study of sulphation factor activity in 26 species.
Journal of Endocrinology 74, 129-135.

Shapiro, B. and Pimstone, B.L. (1978)
Sulphation factor (somatomedin activity) in experimental protein malnutrition in the rat.
Journal of Endocrinology 77, 233-240.

Sidransky, H. and Verney, E. (1970)
Skeletal muscle protein metabolism changes in rats force-fed a diet inducing an experimental kwashiorkor-like model.
American Journal of Clinical Nutrition 23, 1154-1159.

Sievertsson, H., Fryklund, L., Uthne, K., Hall, K. and Westermark, B. (1975)
Isolation and chemistry of human somatomedins A and B.
Advances in Metabolic Disorders 8, 47-60.

Silberberg, M. and Silberberg, R. (1940)
Changes in cartilage and bone of immature female guinea pigs due to undernourishment. With consideration of the processes of repair following a period of refeeding.
Archives of Pathology 30, 675-688.

Simmons, D.J. (1964)
Periodicity of S^{35} uptake in rat femurs.
Experientia 20, 137-138.

Simmons, D.J. (1968)
Daily Rhythm of S^{35} incorporation into epiphyseal cartilage in mice.
Experientia 24, 363-364.

Smith, G.L. and Temin, H.M. (1974)
Purified multiplication-stimulating activity from rat liver cell conditioned medium: Comparison of biological activities with calf serum, insulin, and somatomedin.
Journal of Cellular Physiology 84, 181-192.

Sogani, R.K., Matsushita, S., Mueller, J.F. and Raben, M.S. (1972)
Stimulation of ornithine decarboxylase activity in rat tissues by growth hormone and by serum growth factor from rats infested with spargana of Spirometra mansonoides.
Biochimica et Biophysica Acta 279, 377-386.

Srebnik, H.H. and Nelson, M.M. (1962)
Anterior pituitary function in male rats deprived of dietary protein.
Endocrinology 70, 723-730.

Stead, R.H. and Brock, J.F. (1972)
Experimental protein-calorie malnutrition: rapid induction of protein depletion signs in early-weaned rats.
Journal of Nutrition 102, 1357-1366.

Steelman, S.L., Morgan, E.R., Cuccaro, A.J. and Glitzer, M.S. (1970)
Growth hormone-like activity in hypophysectomized rats implanted with Spirometra mansonoides spargana.
Proceedings of the Society for Experimental Biology and Medicine 133, 269-273.

Stuart, M.C. and Lazarus, L. (1975)
Somatomedins.
Medical Journal of Australia 1, 816-820.

Stuart, M.C., Lazarus, L., Moore, S.S. and Smythe, G.A. (1976)
Somatomedin production in the neonatal rat.
Hormone and Metabolic Research 8, 442-445.

Svan, H., Hall, K., Ritzén, M., Takano, K. and Skottner, A. (1977)
Somatomedin A and B in serum from neonates, their mothers and cord blood.
Acta Endocrinologica 85, 636-643.

Sweet, M.B.E. (1968)
Differentiation of articular and epiphysial cartilage, based on resorcin-crystal violet and picro-fuchsin staining of chondroitin sulphate and keratosulphate.
Stain Technology 43, 79-81.

Takano, K., Hall, K., Fryklund, L. and Sievertsson, M. (1976)
Binding of somatomedins and insulin to plasma membranes prepared from rat and monkey tissue.
Hormone and Metabolic Research 8, 16-24.

Takano, K., Hizuka, N. and Shizume, K. (1977)
The effects of GH and fasting on the serum levels of somatomedin in rats and men determined by radioreceptor assay.
Acta Endocrinologica Supplement 212, 189 (Abstract 326).

Tato, L., DuCaju, M.V.L., Prêvot, C. and Rappaport, R. (1975)
Early variations of plasma somatomedin activity in the newborn.
Journal of Clinical Endocrinology and Metabolism 40, 534-536.

Tell, G.P.E., Cuatrecasas, P. van Wyk, J.J. and Hintz, R.L. (1973)
Somatomedin: Inhibition of adenylate cyclase activity in subcellular membranes of various tissues.
Science 180, 312-315.

Trowell, H.C., Davies, J.N.P. and Dean, R.F.A. (1954)
In: Kwashiorkor. 1st Edition, pp. 69-73, Edward Arnold, London.

Tsushima, T., Friesen, H.G. Chang, T.W. and Raben, M.S. (1974)
Identification of sparganum growth factor by a radioreceptor assay for growth hormone.
Biochemical and Biophysical Research Communications 59, 1062-1068.

Underwood, L.E., Hintz, R.L., Voina, S.J. and van Wyk, J.J. (1972)
Human somatomedin, the growth hormone-dependent sulfation factor, is anti-lipolytic.
Journal of Clinical Endocrinology and Metabolism 35, 194-198.

Urdanivia, E., Mataverde, A. and Cohen, M.P. (1975)
Growth hormone secretion and sulfation factor activity in pseudohypoparathyroidism.
Journal of Laboratory and Clinical Medicine 86, 772-776.

Uthne, K. (1973)
Human somatomedins - Purification and some studies on their biological actions.
Acta Endocrinologica Supplement 175, 1-35.

Uthne, K. (1975)
Preliminary studies of somatomedin in vitro and in vivo in rats.
Advances in Metabolic Disorders 8, 115-126.

Uthne, K. and Uthne, T. (1972)
Influence of liver resection and regeneration on somatomedin (sulphation factor) activity in sera from normal and hypophysectomized rats.
Acta Endocrinologica 71, 255-264.

Van Buul, S., Dumoleijin, L., Korteland, A.M., Van de Klundert, P. and van den Brande, J.L. (1978)
The Snell-dwarfmouse: Interrelationship of growth in length and weight, serum somatomedin activity and sulfate incorporation in costal cartilage during growth hormone and thyroxin treatment.
International Symposium on Somatomedins and Growth, Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, p. 55.

Van den Brande, J.L, and DuCaju, M.V.L. (1973)
Plasma somatomedin activity in children with growth disturbances.
In: Advances in Human Growth Hormone Research, Raiti, S. Editor, pp. 98-115. DHEW Publication No. (NIH) 74-612.

Van den Brande, J.L. and DuCaju, M.V.L. (1974)
An improved technique for measuring somatomedin activity in vitro.
Acta Endocrinologica 75, 233-242.

Van den Brande, J.L., Kootte, F., Tielenburg, R., van der Wilk, M. and Huyser, T. (1974)
Studies on plasma somatomedin activity in different animal species.
Acta Endocrinologica 75, 243-248.

Van den Brande, J.L., van Buul, S., Heinrich, U., van Roon, F., Zurcher, T. and van Steirtegem, A.C. (1975)
Further observations on plasma somatomedin activity in children.
Advances in Metabolic Disorders 8, 171-181.

Van den Brande, J.L., van Wyk, J.J., Weaver, R.P. and Mayberry, H.E. (1971)
Partial characterization of sulphation and thymidine factors in acromegalic plasma.
Acta Endocrinologica 66, 65-81.

van Wyk, J.J., Hall, K., van den Brande, J.L. and Weaver, R.P. (1971)
Further purification and characterisation of sulfation factor and thymidine factor from acromegalic plasma.
Journal of Clinical Endocrinology 32, 389-403.

van Wyk, J.J., Hall, K. and Weaver, R.P. (1969)
Partial purification of sulphation factor and thymidine factor from plasma.
Biochimica et Biophysica Acta 192, 560-562.

van Wyk, J.J., Underwood, L.E., Baseman, J.B., Hintz, R.L., Clemmons, D.R. and Marshall, R.N. (1975)
Explorations of the insulinlike and growth-promoting properties of somatomedin by membrane receptor assays.
Advances in Metabolic Disorders 8, 127-150.

van Wyk, J.J., Underwood, L.E., Hintz, R.L., Clemmons, D.R., Voina, S.J. and Weaver, R.P. (1974)
The somatomedins: A family of insulinlike hormones under growth hormone control.
Recent Progress in Hormone Research 30, 259-318.

Wasteson, Å., Uthne, K. and Westermark, B. (1973)
A novel assay for the biosynthesis of sulphated polysaccharide and its application to studies on the effects of somatomedin on cultured cells.
Biochemical Journal 136, 1069-1074.

Wasteson, A., Westermark, B. and Uthne, K. (1975)
Somatomedin A and B: Demonstration of two different somatomedin-like components in human plasma.
Advances in Metabolic Disorders 8, 101-113.

Weinkove, C. (1974)
Glucose tolerance and insulin release in malnourished rats.
Ph.D. Thesis, University of Cape Town.

Weinkove, C., Weinkove, E.A. and Pimstone, B.L. (1974)
Micro assays for glucose and insulin.
South African Medical Journal 48, 365-368.

Weinkove, C., Weinkove, E.A. and Pimstone, B.L. (1976)
Glucose tolerance and insulin release in malnourished rats.
Clinical Science and Molecular Medicine 50, 153-163.

White, A. and Goldstein, A.L. (1975)
The endocrine role of the thymus and its hormone, thymosin, in the regulation of the growth and maturation of host immunological competence.
Advances in Metabolic Disorders 8, 359-374.

Widdowson, E.M. and McCance, R.A. (1957)
Effect of a low-protein diet on the chemical composition of the bodies and tissues of young rats.
British Journal of Nutrition 11, 198-206.

Wiedemann, E. and Schwartz, E. (1972)
Suppression of growth-dependent human serum sulfation factor by estrogen.
Journal of Clinical Endocrinology and Metabolism 34, 51-58.

Williams, J.P.G. and Hughes, S. (1974)
Somatomedin activity from rat livers perfused with human growth hormone.
Journal of Endocrinology 63, 585-586.

Wondergem, R., Gaspard, T., Hamamdzic, M. and Klitgaard, H.M. (1977)
Chick cartilage somatomedin activity released from the perfused rat liver.
Federation Proceedings 36 (Abstract 497).

Wu, A., Grant, D.B., Hambley, J. and Levi, A.J. (1974)
Reduced serum somatomedin activity in patients with chronic liver disease.
Clinical Science and Molecular Medicine 47, 359-366.

Yalow, R.S. and Berson, S.A. (1964)
Immunoassay of plasma insulin. In: Methods of Biochemical Analysis. Vol. 12, pp. 69-96, D. Glick, Editor, Interscience Publishers, New York.

Yalow, R.S., Hall, K., and Luft, R. (1975a)
Immunoreactive somatomedin B in urine.
Journal of Clinical Endocrinology and Metabolism 41, 638-639.

Yalow, R.S., Hall, K. and Luft, R. (1975b)
Radioimmunoassay of somatomedin B. Application to clinical and physiologic studies.
Journal of Clinical Investigation 55, 127-137.

Yde, H. (1968)
A simplified technique for the determination of growth hormone dependent sulfation factor, using intact animals.
Acta Endocrinologica 57, 557-564.

Yde, H. (1969)
A comparative study of the sulfation factor and immunodetectable growth hormone in serum.
Acta Endocrinologica 62, 49-55.

18 DEC 1978

Zapf, J. and Froesch, E.R. (1978)
Binding protein (S) for NSILA in serum, protein binding assay and serum NSILA levels in health and disease.
International Symposium on Somatomedins and Growth. Santa Margherita Ligure, (Genoa, Italy), March 1-3, 1978, pp. 26-27.

Zapf, J., Kaufmann, U. and Froesch, E.R. (1977)
Growth hormone dependence of nonsuppressible insulin-like activity (NSILA) and of the NSILA-carrier protein in the rat.
Acta Endocrinologica Supplement 212, 99 (Abstract 147).

Zapf, J., Mäder, M., Waldvogel, M., Schalch, D.S. and Froesch, E.R. (1975)
Specific binding of nonsuppressible insulinlike activity to chicken embryo fibroblasts and to a solubilized fibroblast receptor.
Archives of Biochemistry and Biophysics 168, 630-637.

Zapf, J., Schönle, E. and Froesch, E.R. (1977)
Biological effects and receptor binding of two species of insulin-like growth factor (IGF) I and II.
Acta Endocrinologica Supplement 212, 21, (Abstract S24).

Zapf, J., Waldvogel, M. and Froesch, E.R. (1975)
Binding of nonsuppressible insulin-like activity to human serum. Evidence for a carrier protein.
Archives of Biochemistry and Biophysics 168, 638-645.