PLASMA SOMATOMEDIN

STUDIES ON SOME OF ITS CHARACTERISTICS AND
ON ITS RELATIONSHIP WITH GROWTH HORMONE.

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM,
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INTRODUCTION

Growth is the most characteristic feature of childhood. It also represents the best index of a child's well-being since so many prerequisites must be fullfilled for its harmonious course and such a multitude of pathological factors can interfere with it. It is not surprizing that careful monitoring of growth is common practice for all who are responsible for the health of children. Registering abnormality of a growth pattern is relatively easy. Discovering the cause of the disturbance amongst the vast array of possibilities however, is frequently very difficult.

Amongst the major prerequisites for normal growth we should first mention the intactness of the dividing capacity of all cells and the integrity of specific functions such as e.g. collagen production by the chondrocytes. Equally important is the supply of nutrients: the availability of enough food of good quality, normal absorption, adequate transformation into elementary molecules, and normal transport into the cells.

Disturbances in these functions, which can best be characterized as potentially rate limiting, represent the most frequent causes of stunted growth.

The role of hormones is quite different since they are rate setting, and thus exert the overall control of growth. Their own secretion is regulated in most instances by the central nervous system, which integrates stimuli of a wide variety: 'substrate' signals (such as glucose with respect to GH-secretion), hormone signals (e.g. feedback-regulation), integrated stimuli from within the body (e.g. sleep) and from the outside world ranging from simple visual impressions to complex ones such as emotional deprivation. Certain nuclei of the hypothalamus translate these integrated impulses into the synthesis and secretion of specific releasing hormones which reach the anterior pituitary gland through the portal system and stimulate the secretion of selected pituitary hormones.

All anterior pituitary hormones are involved in the regulation of growth rate. The most important amongst them is growth hormone (GH). It has long been thought that GH exerted a direct action on the growth rate of tissues. The discovery of sulfation factor (SF), later termed somatomedin (SM), and Daughaday's hypothesis on its role as the mediator of GH's growth promoting effect, places GH in line with the other anterior pituitary hormones which all have their major effect through secondary hormones. If this hypothesis proves to be correct, SM would represent the major regulator of growth rate. In this thesis our own work on SM is reported and related to the progress made by others. The investigations of Daughaday and of Salmon leading to the discovery of somatomedin and of its potential importance for human pathology are summarized in chapter one.

The results of our own and collaborative work make up chapter two. Initially we have addressed ourselves to the characteristics of somatomedin in human plasma (section A). Intrigued by the question which organ or organs are producing SM, the early localization of ^{125}I growth hormone in the rat was studied (section B).

Meanwhile, many different in vitro biological effects had been assigned to this substance.

This prompted a group of investigators to propose a change of the operational term 'sulfation factor' into somatomedin (section C).
Both from a physiological and from a practical standpoint, it seemed important to compare the species differences of SM with those of GH. The results of these studies are described in section D.
The imprecision and laboriousness of the SM bioassay is notorious and has limited progress in purification as well as in biological and clinical studies. Attempts to improve the technique resulted in the description of our method in section E.
Using this assay technique and in an attempt to define a model for an appraisal of hormone-target organ interactions in clinical conditions, a pilot study on the dose-response relationship between GH, plasma SM and total urinary OH-proline in short children with varying plasma GH levels was carried out (section F).
An extreme example of such a disturbed interaction is described in section G. It describes a patient similar to those first reported by Laron et al. (1966) in whom a lack of SM-generation is most likely to be the cause of their growth failure. The paper describes his responsiveness to GH-treatment and some characteristics of his GH-molecule.
Chapter three summarizes the present status of our knowledge about somatomedin. It describes the problems of its measurement, what is known about its molecular characteristics, its possible sources, the regulation of its synthesis, its biological actions and possible relationship with other 'growth factors'.

Chapter one

DISCOVERY OF 'SULFATION FACTOR' (SOMATOMEDIN)

In 1957, Salmon and Daughaday reported that serum from growth hormone (GH) treated hypophysectomized rats stimulated *in vitro* chondroitin sulfate synthesis in cartilage, whereas growth hormone itself or serum from untreated hypophysectomized animals or the combination of both lacked this capacity. This growth hormone dependent biological activity in serum was termed sulfation factor. Their findings, which were later confirmed by many investigators (Almqvist, 1960; Kogut et al., 1963 and others), introduced a totally new view on GH-action. Salmon and Daughaday's paper had been preceeded by a series of reports leading to this breakthrough point.

In 1949, Dziewiatkowski et al., demonstrated that ^{35}S-sodiumsulfate, when injected into normal young rats, was highly concentrated in the cartilage. Two years later Dziewiatkowski (1951) demonstrated that it was incorporated into chondroitin sulfate. Around this time, purification of growth hormone had reached a stage where its metabolic effects could be investigated.

In 1921, Evans and Long had proven the hypothesis formulated by Harvey Cushing in 1910, that the pituitary produces a growth stimulating substance. This was accomplished by producing gigantism in normal rats injected with crude extracts from beef pituitary glands. In 1933, Smith arrested growth in young rats by hypophysectomy and restored it by injection of crude pituitary extracts. The development of the tibia-assay as a sensitive method for measuring growth hormone activity stimulated progress in the purification of pituitary extracts. In 1944, Li and Evans isolated highly purified bovine growth hormone. Ellis, Hublé and Simpson, using a similar preparation, demonstrated in 1953 that the *in vivo* incorporation of ^{35}S-sulfate into costal cartilage of young rats was drastically reduced after hypophysectomy and that 25 μg of growth hormone, when administered for three consecutive days, partially restored it. This was subsequently confirmed by Denko and Bergenstal (1955).

Murphy, Daughaday and Hartnett (1956) systematically explored this phenomenon. They found the lowest *in vivo* uptake of ^{35}S-sulfate by rat cartilage eight to ten days after hypophysectomy. They further established that the stimulation of chondroitin sulfate synthesis by growth hormone was dose related: a linear log dose-response curve was obtained with dosages ranging from 10 to 250 μg twice daily.

Meanwhile Layton (1950 a and b) had published data on the sulfate fixation *in vitro* in many tissues of mammals and chicken embryo's, and in 1952 Boström and Månsson described a technique for measuring *in vitro* ^{35}S-sulfate incorporation into calf costal cartilage, using Krebs' solution as incubation medium. It was essentially this technique,

but incubating costal cartilage from hypophysectomized rats, which Salmon and Daughaday used for their first experiments with serum, mentioned above.

They found that *in vitro* incorporation of ^{35}S-sulfate into cartilage from hypophysectomized (hypox-) rats was lower than in control animals. Bovine growth hormone added *in vitro* to hypox rat serum in concentrations up to 0.14 μg/ml had no effect. In the absence of serum, the same preparation exerted only a minimal effect even at a concentration of 50 μg/ml. However, when plasma from normal rats or from hypox rats, treated with growth hormone was used, a dose related response resulted with doubling or even tripling of the ^{35}S-incorporation over the controls. This GH dependent biological activity was called sulfation factor.

With their *in vitro* assay technique Daughaday and his collaborators then started measuring 'sulfation factor' activity in the human (Daughaday et al., 1959; Daughaday and Parker, 1963). They found low values in hypopituitary patients which could be restored by growth hormone treatment. High values were found in acromegalics. This suggested an important role of 'sulfation factor' in the regulation of normal and abnormal growth.

Not only the synthesis of chondroitin sulfate in cartilage but also its collagen synthesis (Daughaday and Mariz, 1962) and DNA-replication (Daughaday and Reeder, 1966) were subsequently shown to be dependent on some intermediary substance or substances induced by growth hormone. It was tentatively concluded that sulfation factor might be responsible for all these effects (Daughaday and Reeder, 1966; Daughaday and Kipnis, 1966).

Chapter two

STUDIES ON SOMATOMEDIN

A. *Partial characterization*

1. *Partial characterization of sulfation and thymidine factors in acromegalic plasma.*

This study was done in collaboration with J.J. Van Wyk, R.P. Weaver and H.E. Mayberry. It was undertaken to characterize the growth hormone dependent plasma factors which stimulate sulfate uptake (PSF) and thymidine incorporation (PTF) in cartilage segments from hypophysectomized rats.

An assay system utilizing labeling with $^{35}SO_4$ and ^{3}H-methyl-thymidine was devised to measure both types of activity simultaneously and to determine whether these activities run in parallel during chemical fractionation procedures.

Acromegalic plasma stimulated sulfate and thymidine uptake as much as 18x and 30x respectively above the uptake of the basal medium. Activity measurements were not rigidly quantitative due to high variances, especially in PTF measurements.

Both activities in acromegalic plasma were thermo-labile at 80°C for 30 minutes. PSF was stable from pH 2 to 10. PTF was possibly partially inactivated at pH 2. Digestion with pronase destroyed PSF and probably PTF. Acromegalic plasma was fractionated by graded ethanol precipitation, ion exchange chromatography, starch gel electrophoresis, and molecular sieving through Sephadex (G-100, G-200). PSF and PTF activities run in parallel throughout these procedures. These findings suggest that the active material is associated with a peptide with a molecular weight between 9500 and 35000, which is more positively charged than many other plasma proteins.

Experimental details are described in paper 1, which is reprinted in the addendum.

2. *Further purification and characterization of sulfation factor and thymidine factor from acromegalic plasma.*

In a collaborative effort with J.J. Van Wyk, K. Hall and R.P. Weaver, sulfation factor (PSF) and thymidine factor (PTF) were further chemically characterized in whole acromegalic plasma and were also studied in acid ethanol (AE) extracts of plasma. In whole plasma PSF and PTF are excluded by the XM-100 ultrafiltration membrane and behave as large proteins. After extraction with acid ethanol the molecular weight

appeared to be $>3{,}900 \leqslant 12{,}400$, suggesting that in native plasma PSF and PTF are either aggregated or bound to a larger carrier protein.

Two species of peptides with PSF and PTF activity were found. Chromatography of whole plasma on DEAE-cellulose revealed a major peak of biologic activity which was not adsorbed at pH 8.5 and a smaller component which was adsorbed at pH 8.5 but eluted at pH 5.5. Electrofocusing of AE extracts revealed a sharp minor peak at pH 5.2 and a broader major peak at pH 6.6-6.7. The possibility that the more acidic component may be due to insulin cannot be excluded. HV electrophoresis of a purified AE extract revealed activity only in the neutral zone. Chromatography of an AE extract on CMC-cellulose at pH 5.6 in a linear ionic gradient between 0.01 and 0.40 M resulted in the recovery of PSF and PTF in a small protein peak well separated from the bulk of other proteins.

Rechromatography of the CMC eluate on Sephadex G-50 yielded a preparation with an increase in specific activity over native plasma of 6,200x for PSF and 15,000x for PTF. This discrepancy probably reflects the removal of inhibitors having a greater effect on thymidine incorporation, since no separation of PSF from PTF was observed in any system. If a provisional molecular weight of 8,000 is assumed and no allowance is made for residual contaminants, the purest preparation is highly active at 3.1×10^{-8} molar concentration.

Paper 2 in the addendum describes the experimental details.

B. *Early localization of ^{125}I-labeled human growth hormone in adrenals and other organs of immature hypophysectomized rats.*

These studies were done in collaboration with H.E.Mayberry,J.J.VanWyk and W.J.Waddell. Since labeled hormones tend to concentrate very quickly in their respective target tissues after administration, human growth hormone (HGH) labeled with ^{125}I was injected intravenously into hypophysectomized, immature rats. It was hoped that early binding of the hormone might reveal possible sources of somatomedin production. One rat was frozen 6 min and another 20 min after injection by immersion in hexane cooled with dry ice. Whole-body sections were made of the frozen rats and autoradiograms were prepared by placing these sections on x-ray film. The autoradiograms revealed that, at both time intervals after injection, there was a high concentration of radioactive material in the kidney, liver and adrenal cortex. The only other tissues found to have a higher radioactive density than that of blood were the submandibular glands, nasal mucosa, gastric mucosa, bone and follicles of vibrissae. The radioactivity in the adrenal cortex was slightly higher in the zona glomerulosa than in the zona fasciculata and it was lowest in the zona reticularis. The concentration in the epiphyseal plates was no higher than that in bone or soft tissues. The radioactivity in the submandibular gland had the electrophoretic mobility of growth hormone and not free iodide. The distribution in 8 organs of ^{131}I-albumin was compared with that of ^{125}I-HGH at 6 and 20 min by removal of the organs and direct isotope counting. The distributions substantiated the specific localizations of HGH seen in the autoradiograms. Pretreatment of the rats with HGH 6 min before administe-

ring the ^{125}I-HGH diminished the concentration of ^{125}I-HGH in liver, adrenals and to a minor extent in the submandibular glands, suggesting specific binding. It was concluded that these organs should be focused on in subsequent studies aimed at disclosing the source of somatomedin.
The experimental protocol, results and a detailed discussion are given in paper 3 of the addendum.

C. *Somatomedin: Proposed designation for sulfation factor*

Since the operational terms sulfation factor or thymidine factor did not adequately cover its apparent role, 'Somatomedin' was suggested by W.H. Daughaday, K. Hall, M.S. Raben, W.D. Salmon, J.J. Van Wyk and myself as an alternative designation of the growth hormone dependent substance mediating its effect (for detailed motivation, see paper 4 of the addendum).

D. *An improved technique for measuring somatomedin activity in vitro**

All current techniques for the measurement of somatomedin are based on its stimulatory effect on the incorporation of ^{35}S-sulfate into chondroitine-sulfate and/or ^{3}H-methyl Thymidine into DNA of cartilage. They differ mainly by the origin of the tissue used and by the incubation schedules. As a consequence of this, each of the techniques has somewhat different characteristics which are summarized in Table 1 (Salmon & Daughaday 1957; Daughaday & Reeder 1966; Almqvist 1961; Yde 1968; Fujisawa 1964 and Hall 1970).
The development of the technique to be described was aimed at improving the precision and at diminishing laboriousness which are the two main disadvantages of the techniques currently in use.

METHOD

1. *Procedure*

a. *Preparation of the tissue*
The tissue is obtained from approximately one hundred day old pigs. A small portion of the thoracic cage containing the cartilagenous section of the lowest sternal rib is removed in the slaughterhouse shortly after the animal has been killed. The tissue is placed in ice-chilled isotonic saline solution and processed within two hours. After removal of most of the muscle, the cartilagenous portion of a single rib is sliced in transverse sections of 2 mm thickness using a commercial meatslicer. Cylindrical tissue fragments are punched

* These studies were done in collaboration with M.V.L. Du Caju and will appear in Acta endocrinologica, Kbh.

TABLE 1

Survey of the current somatomedin assay techniques

Authors	Animal	Organ	Isotope	Precision	Range*
Salmon and Daughaday (1957)	hypophysect. rat	costal cartil.	^{35}S (SO_4)	± 0.26	2.5-17%
Daughaday and Reeder (1966)	„	„	^{3}H (Thym.)	–	2-17%
Almqvist (1961)	„	„	^{35}S (SO_4)	0.14**	5-20%
Yde (1968)	normal rat	„	„	0.20	5-15%
Fujisawa (1964)	hypophysect. puppies	„	„	–	5-40%
Hall (1970)	chick embryo	pelvic rudiments	„	0.20	5-60%
Van den Brande and Du Caju	pig	costal cartil.	^{35}S (SO_4) ^{3}H (Thym.)	0.15(n=57) 0.19(n=43)	5.6 or 11.3-45.2% 5.6-45.2%

* Range: Concentrations (v/v) of normal human plasma yielding the linear portion of the dose response curve.

** Obtained after rejecting otherwise non-valid assays.

using either small drills or a specially designed apparatus (Fig. 1). Care is taken to keep a constant distance from the border of the slice (Fig. 2).

b. *Preincubation in saline*

The cartilage cylinders are placed in a beaker containing enriched Krebs-phosphosaline buffer (KPS) as described by Daughaday and Reeder (1966), and are incubated in an oven at 37°C for 24 hours without shaking.

c. *Incubation in test solution* is done in a rack consisting of a Teflon ® (Dupont)-block with 90 holes (6 rows of 15) in which either glass tubes with tightly fitting punctured flat Teflon bottomplates or Teflon tubes with a similar punctured bottom, are lowered. The top rim of the tubes rests in a perspex plate which allows for simultaneous removal and processing of the tissue containing tubes. The whole rack is covered with a perspex roof (Fig. 3). After the tubes have been inserted into the holes, 1.0 ml of each of a series of dilutions of standard or unknown with KPS is dispensed in three adjacent tubes. Two tissue fragments are placed in each tube. The rack is covered with a perspex roof (Fig. 3) and is put into an incubator at 37°C for 46 hours.

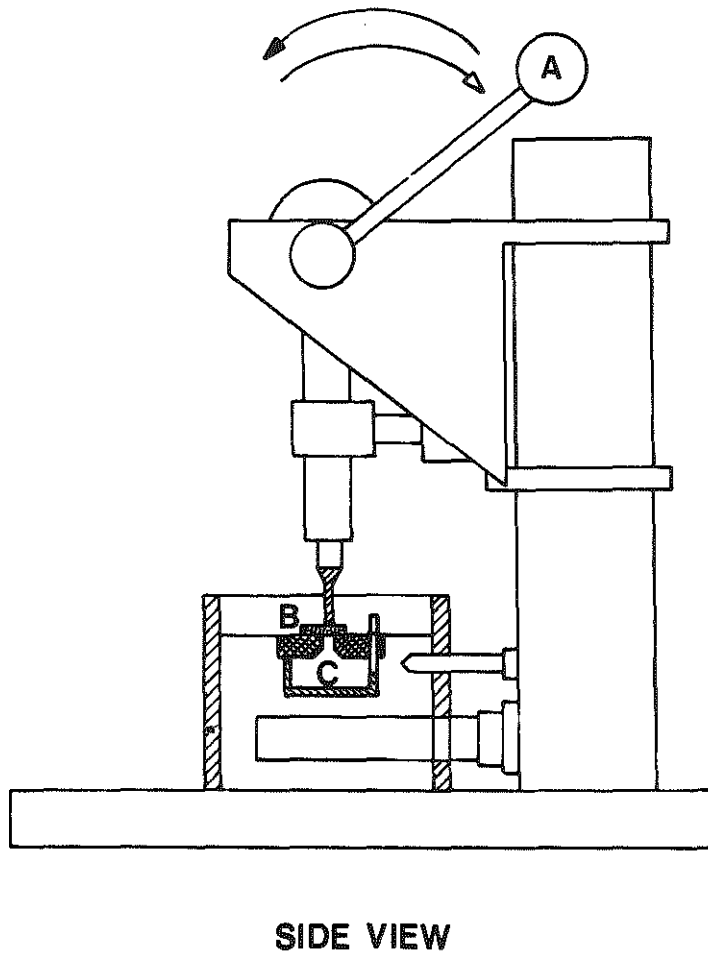


Fig. 1
This apparatus punches cylindrical tissue fragments from a transverse section of the rib cartilage. Rotation of the top lever (A), pushes a sharp metal cylinder downwards through the tissue (B). While the lever is returned, an inner shaft remains down pushing the tissue outside of the cylinder into a small removable container (C). The whole procedure is done in normal saline solution.

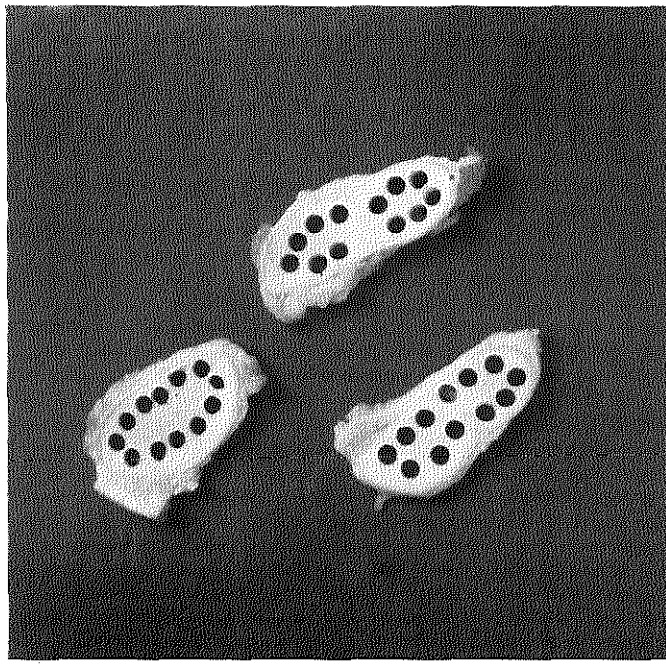


Fig. 2
This figure shows some transverse sections of the rib after the cylinders have been punched out. It demonstrates the attempt to keep a rather constant distance from the perichondrium.

d. *Postincubation in isotopes*
The perspex cover plate with the tubes, containing the cartilage, is next removed from the block. It is lowered into a waterbath such that the lower half of the tubes is immersed in order to remove most of the testsolution. Next it is placed in a flat container filled with enriched Krebs-phosphosaline buffer containing 2 μCi/ml of ^{35}S-sulfate (carrier free) and ^{3}H-methyl thymidine (specific activity $>$ 10 Ci/mMol) obtained from Amersham Ltd., England. Again only the lower portion of the tubes is below the fluid level, exposing the cartilage to the isotopes. The incubation is then continued for another three hours at 37°C.

e. *Processing the cartilage for counting*
The plate with tubes is next transferred to a container filled with water at 80°C. After 5 minutes, washing with running tap water is started. The level of the water is forced into an up and down movement by a sifoning device (Fig. 4). The tissue fragments are then transferred into counting vials to which 0.5 ml of formic acid is added.
They are covered with marbles and placed in a waterbath at 75°C for 30 minutes.

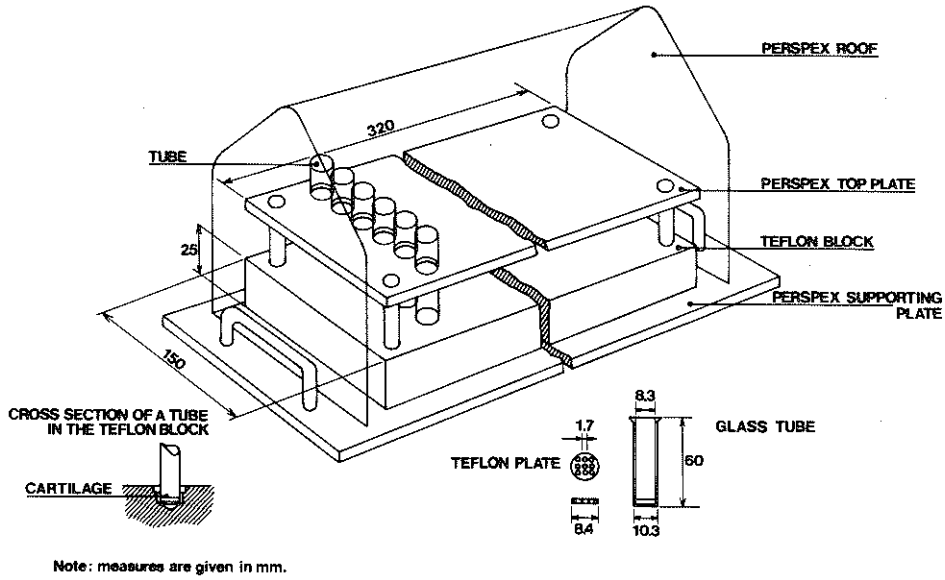


Fig. 3
Technical drawing of the incubation apparatus.

Thereafter, 10 ml of Instagel (Packard) is added and the radioactivity is counted in a liquidscintillation counter.

f. *Computation of the results* is done on a Hewlett-Packard 9100B desk calculator. After the necessary log-transformations (see below) each dose response curve is plotted, the linear portion is visually selected and statistically checked for validity. Regression analysis is done and the index of precision ($^{s}/b$) is computed. The standard and the unknown are analyzed with respect to linearity, preparation differences, regression, paralellism and heteroscedasticity. Finney's g is calculated and finally the potency ratio with 95 or 97.5% fiducial limits is obtained. The mathematical formulae are according to Finney (1952).

2. *Discussion of some procedural aspects*

a. *Choice of the tissue*

Before the pig cartilage was selected, other tissues had been tried. Monkey rib cartilage was satisfactory and was utilized in some studies on purification (same chapter, section-A) and some in clinical studies (same chapter, section G). When this tissue was used, the technique was somewhat different since total transverse sections through the ribs were incubated, preincubation was not yet done, and the radioactive isotopes were added during the last 24 hours of the incubation in plasma in stead of during a postincubation

as described earlier. The best results, however, were obtained with cartilage from pig ribs. Moreover, since this tissue has the advantage of being available at no cost in large quantities and since dissection is easy, it was selected for further use.

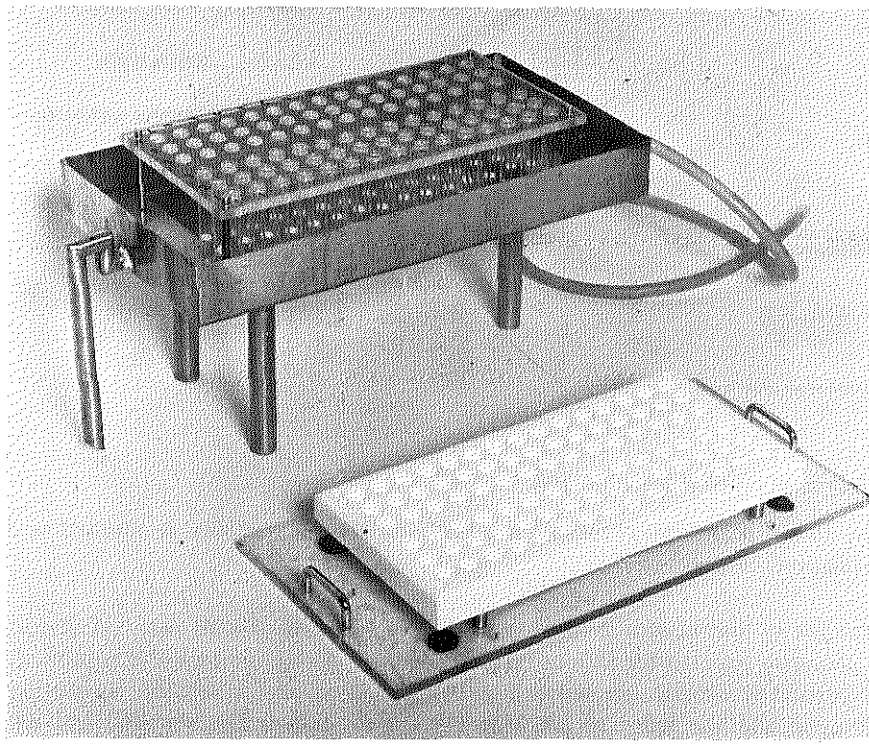


Fig. 4
Container for boiling and washing of the tissue after finishing the incubation. The container has a sifoning outlet so as to force the water into an up and down movement.

b. *Incubation schedule*
Studies by Daughaday et al.(1968) on normal rat rib cartilage have shown that preincubation for 24 hours lowers the endogenous chondroitine-sulfate synthesis to the level obtained with cartilage from hypophysectomized animals. Since responses to stimulation with active serum might be increased by this procedure, different preincubation periods with pig rib cartilage were tried. The best results were obtained with 24 hours of preincubation. In these experiments the preincubation was followed by 48 hours of incubation in the presence of plasma, the last 24 hours of which with addition of radioisotopes. This scheme has been followed until recently. On a suggestion by Dr.

W.D. Salmon, the postincubation schedule has since been introduced. From the many schedules tried, incubation in the testsubstance for 46 hours followed by a 3 hours exposure to radioisotopes, after removal of the test substance, yielded the best results. This diminishes the laboriousness of the procedure. The shallowness of the slope is adequately compensated for by the decrease of the variance, as can be concluded from the unchanged index of precision (Fig. 5).

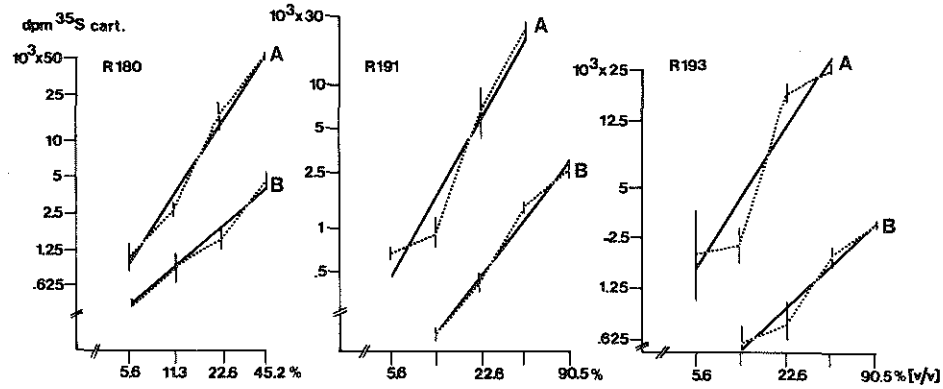


Fig. 5
In this experiment the standard curves obtained for ^{35}S-uptake after different incubation schedules are shown.
A: 24 hrs. preincubation in KPS, 48 hrs. incubation with the testsubstance, the last 24 hrs. of which in the presence of the isotopes.
B: 24 hrs. preincubation in KPS, 46 hrs. of incubation in the testsubstance and 3 hrs. postincubation in KPS + isotopes.

Indices of precision for both ^{35}S-uptake and ^{3}H-Thymidine incorporation:

	^{35}S		^{3}H	
	A	B	A	B
R 180	0.09	0.14	0.17	0.13
R 191	0.13	0.08	0.17	0.11
R 193	0.22	0.14	0.13	0.21

c. *Washing*
Washing the tissue with running tap water efficiently removes non incorporated $Na_2{}^{35}SO_4$ and ^{3}H-Thymidine. After only a few minutes, residual counts are reduced to a minimal and constant level. 30 Minutes was selected as a suitable washing period.

d. *Drying and weighing*
Before the dissection of the cartilage had been rigorously standardized, it was necessary to express the results as a function of tissue mass. It was found that after drying by exposure to ambient air, a stable weight was obtained after a few hours. This technique has been used until the incubation rack was introduced. Since drying appeared not to be

fast enough when the tissue was left in the tubes, an additional one hour of drying in the oven at 60°C was added which gave satisfactory results.
Introduction of the punching apparatus, however, has reduced the variation of the tissue fragments to an extent where the omission of weighing no longer importantly affects the index of precision (average $^{S}/_{b}$, weighing versus not weighing, ^{35}S-uptake: 0.167 vs 0.161; n = 16, ^{3}H-uptake: 0.169 vs 0.166; n = 17).
This results in a significant reduction of the amount of work involved since not only drying and weighing can be omitted but the punch-taped counting data can be directly read into the calculator.

e. *Standard*
Heparinized plasma from 5 healthy adult volunteers was pooled and separated in 5 ml aliquots in order to avoid repeated freezing and thawing. The samples are stored at −70°C.

3. *Sulfate pool*

The sulfate concentration in the incubation medium is 1.162 mMol/L. In plasma it is 0.303 mMol/L on the average (Sturm & Pothmann 1940). Since a wide range of concentrations is being used this results in important changes of the sulfate pool. When a constant amount of ^{35}S is added to this mixture, as is customary in all current techniques and as we also did until postincubation was introduced, one can anticipate a simple

TABLE 2

Log dpm ^{35}S as a function of log SO_4-pool at different plasmaconcentrations.

Plasma concentration (%)	Slope Exp. 1	2	3	average	μMol sulfate/ml usually present in the assay	Correction factor towards constant SO_4-pool (0.303 μMol/ml)
2.8	0.57	0.63	0.42	**0.54**	1.165	2.92
5.6	0.57	0.58	0.52	**0.56**	1.141	2.94
11.3	0.59	0.49	0.62	**0.57**	1.092	2.82
22.6	0.73	0.53	0.73	**0.66**	0.995	2.86
45.2	0.60	–	0.97	**0.78**	0.803	2.45
90.5	0.96	0.81	0.84	**0.87**	0.411	1.24

The sulfate pool of the incubation medium was varied between 0.3 and 2.4 μMol/ml for each of the current plasmaconcentrations used in the assay. A constant amount of $^{35}SO_4$ was added during the last 24 hours of a 48 hours-incubation. Regression analysis was done on the change of log dpm as a function of log SO_4 for each plasma concentration. Only curves with statistically valid criteria for linearity were included (97.5% confidence limit).

inverse relationship between sulfate-pool and ^{35}S-incorporated for any given amount of biological activity.
Experiments to document this, showed however, that the slope of log dpm as a function of log sulfate pool, was less steep than −1.00 in all instances. It varied between −0.54 and −0.87, and was well correlated with the plasma concentration (Table 2). Knowing the slope for each plasma concentration in the assay, a factor could be calculated correcting the dpm ^{35}S for differences in sulfate pool. These correction factors towards a constant pool of 0.303 μMol are represented in Table 2, and appeared to be constant up to a plasma concentration of 22.6% and deviated only slightly at 45.2%. In practice therefore, correction is not necessary up to 45% of plasma. The linearity of the standardcurve up to 45% in the postincubation experiments (Fig. 5) supports this conclusion. Although we have not done this as yet one could consider lowering the concentration of sulfate in the incubation medium to 0.3 mMol/L. When purified fractions are measured in which the sulfate concentration is totally unknown, sulfate should be removed or determined prior to testing.

RESULTS

1. *Shape of the dose-response curves. Transformations*

Since all tissue for one assay is derived from a single animal, interassay variation is intrinsic to the method. Semi-log transformation yields a linear section in the ^{3}H-Thymidine-uptake curve. For ^{35}S-sulfate-incorporation a log-log transformation is necessary.

2. *Sensitivity*

The utilizable portion of the dose-response curve starts at a 5.6 or 11.3% concentration of our normal adult plasma pool, apparently depending on variation of the sensitivity amongst animals, when ^{35}S-uptake is computed, and more regularly at 5.6% for ^{3}H-Thymidine-uptake. The curve is usually linear up to 45.2%. The sensitivity is less than with the methods using rib cartilage from hypophysectomized rats but is comparable to the other current techniques (Table 1).

3. *Precision*

The precision of the assay satisfies the criteria for a quantitative assay (in most cases $^{S}/b \leqslant 0.2$) for ^{35}S-sulfate incorporation, but remains less satisfactory for ^{3}H-Thymidine-incorporation. Average $^{S}/b$ values obtained in a series of subsequent assays on plasma, without rejection of any, except if the upper doses of the curve did not reach the lower limits of the standard, were: 0,146 (n = 57) for ^{35}S-uptake, and 0,192 (n = 43) for ^{3}H-Thymidine uptake.

4. *Specificity*

Many substances are known to influence the rate of chondroitin sulfate synthesis and DNA-replication of cartilage. As any other assay using these indices, the technique described can be anticipated not to be specific for growth hormone dependent somatomedin. However, when the amount of endogenous or exogenous growth hormone is the only variable, the change in activity of the plasma reflects accurately and only the growth hormone dependent changes. This can be concluded from the results obtained when plasma of a hypopituitary patient before treatment was mixed with that during treatment with exogenous growth hormone. Indeed, the usual incubation with increasing plasma-concentrations gave the following potency ratio's and 97.5% confidence limits. Before treatment: *0.27* (0.22 – 0.34), mixed in equal portions before and on treatment: *0.41* (0.37 – 0.53), on treatment: *0.55* (0.43 – 0.72). The data also allowed for comparison of the slopes when increasing biological activity was plotted with and without changing the plasma concentration (Fig. 6). No deviation from paralellism could be demonstrated. This suggests that only growth hormone dependent changes are reflected by the slope obtained.

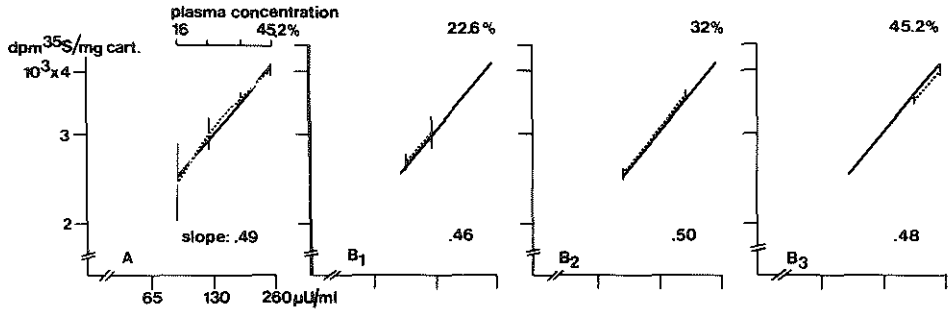


Fig. 6

A. Shows the regular curve obtained with plasma of a hypopituitary patient on treatment with human growth hormone. The data are expressed as mU by comparison with a normal standard, the content of which is 1 U/ml by definition. The solid line represents the calculated curve.

B. shows the curves obtained when less and more active plasma is mixed so as to keep the total amount of plasma constant but to increase the biological activity (dotted lines). For comparison the curve obtained in A is repeated (solid line). Here again the data are expressed as mU of biological activity. No non-paralellism can be demonstrated.

CONCLUSIONS

The assay technique described seems to have the following advantages:

1. It possesses an increased precision over the presently used methods.
2. The utilization of porcine cartilage and of special dissection and incubation apparatus has diminished laboriousness.
3. The costs have been diminished.

The disadvantages of the technique are:

1. It is less sensitive than the hypophysectomized rat assay.
2. The assay takes three days before the results are known. This is one day longer than the chick-embryo assay.

The method has the disadvantage of all present techniques: it measures overall stimulatory activity on chondroitine sulfate synthesis and/or DNA replication and lacks specificity. This necessitates well controlled experimental conditions for conclusions with respect to growth hormone dependent somatomedin levels. The future development of a specific receptor assay and/or a radio-immuno assay should by-pass this problem and render the biological assay even more valuable in the study of non growth hormone dependent growth disturbances.

SUMMARY

A method for measuring somatomedin activity in vitro is described. Porcine costal cartilage fragments are prepared and incubated using special dissection and incubation apparatus. The schedule consists of 24 hrs preincubation, 46 hrs of incubation with the test substance and 3 hrs of postincubation in medium containing both ^{35}S-Na_2SO_4 and ^{3}H-Thymidine. The average index of precision is 0.15 for ^{35}S-uptake and 0.19 for ^{3}H-Thymidine. Although the assay is less sensitive than the hypophysectomized rat assay, it yields a considerable increase in precision, it is far less laborious to perform and is not so expensive.

E. *Studies on plasma somatomedin activity in different animal species**

Growth hormone influences the growth of cartilage (and possibly other tissues) by stimulating the production of somatomedin (SM) which in turn transmits the somatotropic effect onto the target tissue (Daughaday & Reeder 1966). In this respect, somatomedin bears the same relationship to growth hormone that thyroxine and the steroid hormones bear to their respective trophic hormones. Growth hormone possesses a much higher degree of species specificity than do the other pituitary hormones (Knobil and Greep 1959), but it is not known whether this is due to specificity in the ability to stimulate somatomedin production or to species specificity of the somatomedin produced. The present studies were undertaken to determine the nature of responses of rat, pig, monkey and human cartilage to the somatomedin-like activity in the plasma of a wide range of species.

Materials and Methods

Blood was obtained by venapuncture, collected in heparinized vessels and immediately

* This study was done in collaboration with F. Kootte, R. Tielenburg, M. van der Wilk and T. Huyser and will appear in Acta endocrinologica, Kbh.

cooled in an icewater bath. The plasma was separated by centrifugation and stored at −20°C until assayed. In the earlier experiments plasma from a single animal was tested, whereas pools from up to 5 animals were tested in later studies. All blood was obtained from adult animals, except for the rats and the dog, which prepubertal. In the text and the figures some of the common names used need further specification:
Monkey plasma was from the Rhesus Mulatta Macacca, rat plasma from Wistar rats, the turtle plasma from the Testudo Hermanni, pigeon plasma from the domesticated form of Columba Livia and the fish plasma was from the Cyprinus Carpio.
The bioassay was essentially as described by Daughaday and Reeder (1966) with modifications (Same chapter, sections A1 and D). Additional variations amongst experiments concern the origin of the cartilage and the number of tissue fragments incubated per dosage of plasma.
Monkey costal cartilage was obtained from a single male prepubertal Rhesus Mulatta Macacca per assay. The fragments incubated were transverse sections through the rib (average weight: 0.86 mg).
Human cartilage was obtained from a female newborn presenting with duplication of the lower half of the body. Surgical correction was performed on the ninth day of life. Fragments of articular cartilage (average weight: 0.76 mg) were obtained from the knees and the ankle joints.
Rat costal cartilage was obtained from normal male Wistar rats 25 days of age using the same technique as described earlier. (Average weight of the cartilage fragments: 1.64 mg).
Plasma samples were serially diluted in incubation medium to the appropriate concentrations and 1 ml was added to each of the replicate tubes.
Incubations of monkey rib, and human articular cartilage were carried out with three fragments of cartilage in each of three tubes, whereas one rib cartilage segment from each of six rats was incubated in an individual tube. After 24 hrs. of incubation in the presence of the test-substance, one μCi of both ^{3}H-methyl-Thymidine and ^{35}S-Na_2SO_4 were added to each incubationflask and the incubation was continued for another 24 hrs. Further assay conditions were left unchanged. Assays with *porcine rib cartilage* were entirely as described earlier (same chapter, section D). The variance of ^{3}H-methyl-Thymidine was too large to allow for conclusions, and hence only ^{35}S-Na_2SO_4 results will be discussed.

RESULTS

Monkey cartilage. Steep dose-response curves were obtained with plasma from monkey, horse, pig, cow and dog. The slope of the curve obtained with turtle plasma was less steep but not significantly different from the others. Within the dosage range 3-30% (v/v), neither pigeon nor fish plasma were stimulatory (Fig. 7). Because of the suggestion of activity in the pigeon plasma, another study was carried out using plasma from a different animal. Again some stimulation was observed at a very low concentration, but the plasma rapidly became inhibitory, beginning at a concentration of 1%.
Human cartilage (Fig. 8). The general pattern is similar to the one obtained with monkey

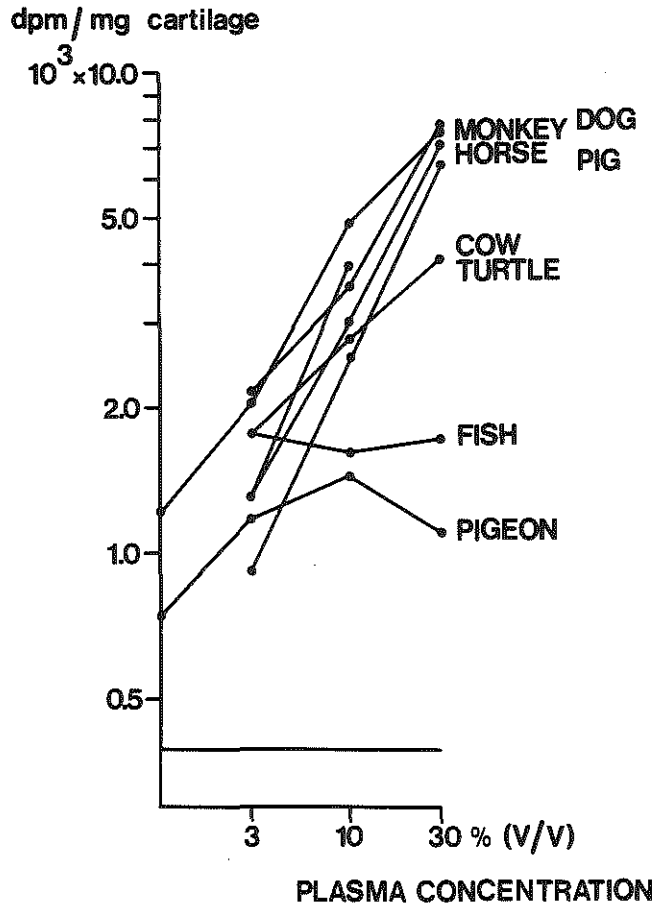


Fig. 7
Fragments of monkey costal cartilage were incubated in the presence of increasing concentrations of heparinized plasma from monkey, dog, horse, pig, cow, turtle, fish and pigeon.
Results are plotted on a log/log scale. Each point represents the mean of triplicate determinations.

cartilage. The highest average values were obtained with plasma from an acromegalic, monkey and pig. Highly significant responses were also obtained with cow and calf plasma.

Rat cartilage. This type of cartilage yielded responses with such variation amongst replicates, that only examination for a general trend was possible. Rat, horse and cow plasma gave steep dose-response curves. The turtle and fish plasma were not stimulatory, and pigeon plasma was very inhibitory.

Porcine rib cartilage. Since this assay technique possesses a greater precision, pooled plasma from man (n:5), pig (n:3) and cow (n:5) was tested in order to obtain more

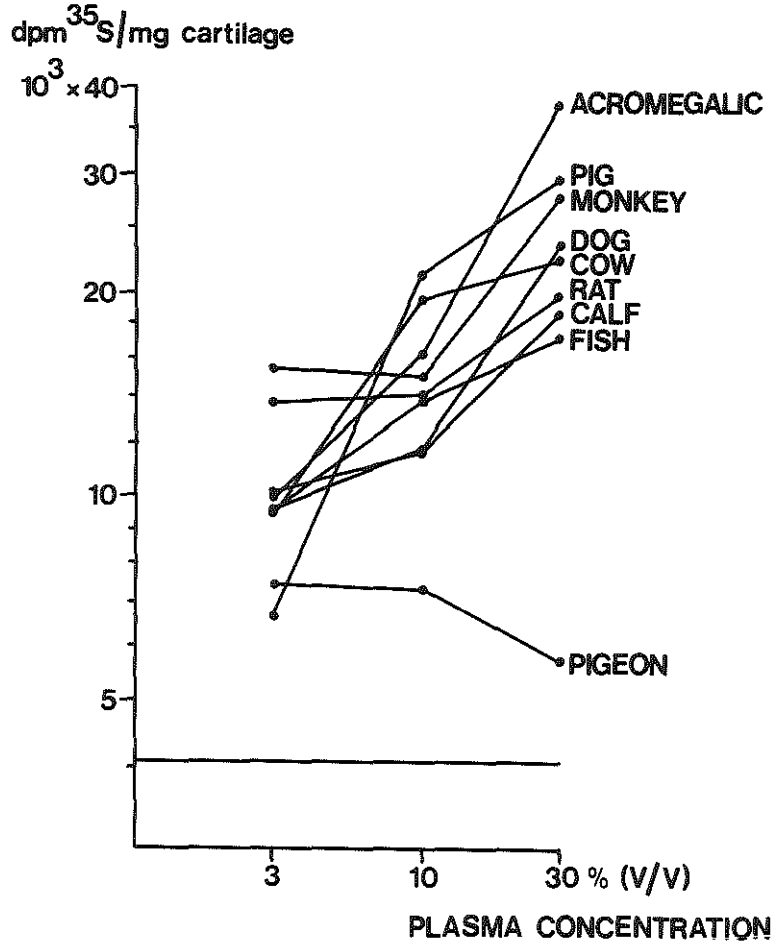


Fig. 8
Human cartilage incubated in the presence of increasing concentrations of heparinized plasma from an acromegalic, from monkey, pig, dog, cow, rat, calf, fish and pigeon.
Results are plotted on a log/log scale. Each point represents the mean of triplicate determinations.

detailed information on possible differences in slope and relative potency (Fig. 3).
No significant differences in slope were demonstrated but the relative potencies were very dissimilar.

DISCUSSION

The data presented suggest that cartilage from man, monkey, pig and rat is capable of responding to the plasma somatomedin activity present in the blood of all mammals

tested. It cannon be concluded whether the response obtained with turtle plasma differs from that obtained with mammalian plasmas. Pigeon and fish definitely distinguish themselves from mammalian plasma since they were either inactive or inhibitory. Experiments in hypophysectomized rats have shown that pretreatment with GH is necessary for any SM-activity to be demonstrable (Salmon and Daughaday, 1957). If this holds true for other species, it would be fair to conclude that the specificity with respect to the responsiveness to GH from different species should be located at the level of SM-generation and not at the level of SM-effect. Since the SM-assay is highly sensitive to inhibition by interfering substances, the possibility must be considered that differences amongst species is more an expression of differences in inhibiting substances than in the nature and amount of somatomedin-like activity.

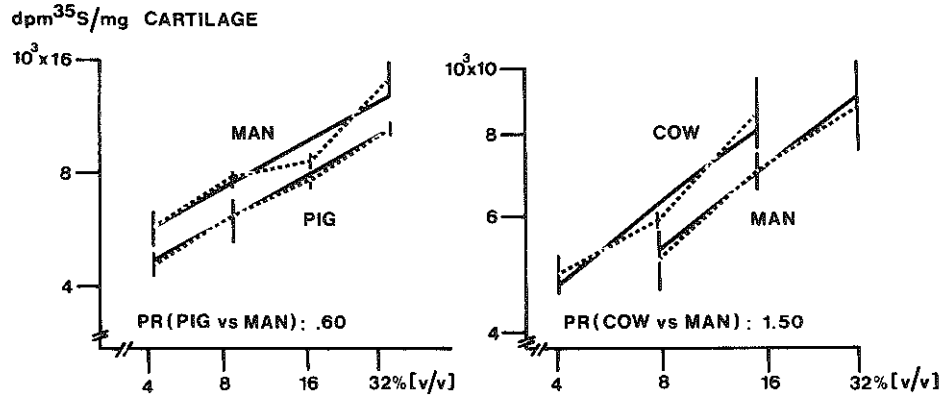


Fig. 9
Plasma from pigs (n = 3), and cows (n = 5) compared to a normal human plasma pool (n = 5) in the pig rib assay. Note the paralellism of the linear portion of the dose response curves. Vast potency differences (PR) are also apparent.

Moreover, the observation that plasma from different species induces similar responses does not eliminate the possibility of qualitative differences in the active substance. Such differences are also suggested by studies of Liberti (1970) and ourselves (same chapter, section A2), since quite different results were obtained with regards to the behaviour of bovine SM and human SM on ultrafiltration membranes.
Studies on the chemical characteristics of pig plasma with respect to SM are being conducted both to obtain more information on this problem and in order to investigate its suitability for large scale preparation of SM.

SUMMARY

Since responses to growth hormone (GH) have a well-defined species specificity, these studies were undertaken to determine whether responses to somatomedin exhibit similar specificity. Heparinized plasma from 10 different species ranging from fish to man was

accordingly assayed for somatomedin-like activity in rat, pig, monkey and human cartilage. The magnitude of $^{35}SO_4$-uptake was assessed by previously described methods and the paralellity of the curves compared.
A stimulatory effect was obtained with plasma from man, monkey, pig, horse, cow, dog, rat and possibly turtle, whereas pigeon and fish plasma had either no effect or was inhibitory. Although some species differences with respect to slope and magnitude of response were observed, it was concluded that inter-species responses to somatomedin are far less restricted than inter-species responses to growth hormone. This suggests that unless the overall somatomedin-like activity measured in this assay can be attributed to substances which are quantitatively and/or qualitatively different in the various species, the species specificity of growth hormone exists at the level of somatomedin induction rather than in specificity of the somatomedin produced.
The responsiveness of human cartilage to somatomedin from a series of other mammals opens up practical possibilities for large scale preparation of this hormone from non-human sources.

F. *Dose-response relationship between growth hormone, plasma somatomedin and total urinary hydroxyproline excretion. A pilot study**

Detailed information on the dose-response relationship between growth hormone (GH) and indices of its effects in children is not available. If the characteristics of such relationships were known, assessment of the hormone-target organ interaction in the many clinical conditions where a quantitative or qualitative abnormality of this interaction is suspected might become possible. Examples of such conditions are partial growth hormone deficiency, the large group of small children with either normal physical appearance or congenital malformations, obesity, malnutrition etc. The present pilot study was aimed at testing the possibilities of such an approach.

Patients, materials and methods

Six children were selected for the study. Their diagnostic data are summarized in Table 3. Patients D1 and D2 are siblings with isolated GH deficiency. P1 and P2 could not be classified since their plasma GH was limited but not absent on stimulation** (maximal levels: 9 and 10 μU 1st IRP/ml respectively, as compared to an average of 36.8, range 14 to 75, in twenty children with no apparent endocrine disorder). From their clinical appearance, skeletal age and normal plasma GH levels, N1 and N2 were considered normal delayed children. None of the patients had any physical abnormalities except for their short stature, and they had received no treatment prior to the study. They were hospitalized and placed on a collagenfree and calorie-constant diet. After one week of equilibration, the studies were started.

* These studies were done in collaboration with M.V.L. Du Caju, C.M. Hoogerbrugge, A.M. van Male, J.K. Schouwstra and T. Zurcher.

** Patiens P1 and P2 were kindly referred for this study by Dr. R. Steendijk and Prof. J.J. van der Werff-Ten Bosch.

In an attempt to reach a plateau of maximal response to each dose of human growth hormone (HGH) from which the patients could move to a higher level on the next dose, they were given a constant amount of HGH for consecutive periods of 5 days in P2 and 7

TABLE 3

Summary of diagnostic data

Patient	Sex	Age years^{months}	Skeletal age years^{months}	Height (SD)	Maximal plasma Growth Hormone
D1	M	12^{1}	4^{0}	− 9.1	<0.5
D2	F	6^{6}	1^{9}	− 9.5	<0.5
P1	F	14^{7}	9^{7}	− 4.3	9
P2	M	10^{1}	9^{4}	− 3.7	10
N1	M	7^{2}	5^{6}	− 3.2	31
N2	M	5^{11}	4^{0}	− 2.6	45

Skeletal age was assessed using the Greulich and Pyle atlas (1959).
Height is expressed as the number of standard deviations separating the patient from the mean. Plasma growth hormone, measured by a charcoal-dextran method (Schopman and Hackeng, 1971) is expressed in μU 1st IRP per ml. The patients were tested either by insulin induced hypoglycemia (P2), arginine infusion test (D1, D2, N1, N2) or both (P1). All had normal plasma thyroxine levels.

days in all other children. The doses used were 0, 2, 4 and 8 mg/m^2/day.* The biological potency of this GH preparation is not known, which limits the conclusions to the comparison of differences between individual patients. Growth hormone was administered I.M. at 8 p.m. Day 1 of each study period is defined as the day following the first injection. Urine was collected throughout the study. Venous blood was drawn at 8.15 a.m. before breakfast on day 4, and the plasma was stored at −20°C until assayed for somatomedin activity (SM). Creatinine was measured using a routine technique (Fawcett and Scott, 1960). Total urinary hydroxyproline was measured with the Hypronosticon-test® (Organon) (Goverde and Veenkamp, 1972). Plasma somatomedin activity was estimated as described in this chapter, section D.

RESULTS

Plasma Somatomedin

Since Daughaday et al. (1959) found maximal SM values on the 4th and 5th day of GH

* The HGH, clinical grade, was extracted by Organon N.V. and kindly supplied by the 'Nederlandse Groeistichting' (Dutch Growth Foundation).

treatment, plasma obtained on day 4 was tested. In order to correct for age differences (Du Caju and Van den Brande, 1973) results are expressed as the potency ratio to the mean normal for age (which is 1.00 by definition). The results are represented in Fig. 10. D1 and D2 start from extremely low levels (< 0.14) and reach the normal range at the highest dose (D1 : 1.13, D2 : 0.83). P1 and N1 rise from slightly subnormal to supernormal values (P1 : 0.64 to 1.83, N1 : 0.63 to 1.47). P2 and N2 increase their plasma SM to the normal on the lowest dose of HGH without exceeding it on higher dosages (P2 : 0.40 to maximally 0.86, N2 : 0.71 to maximally 0.99).

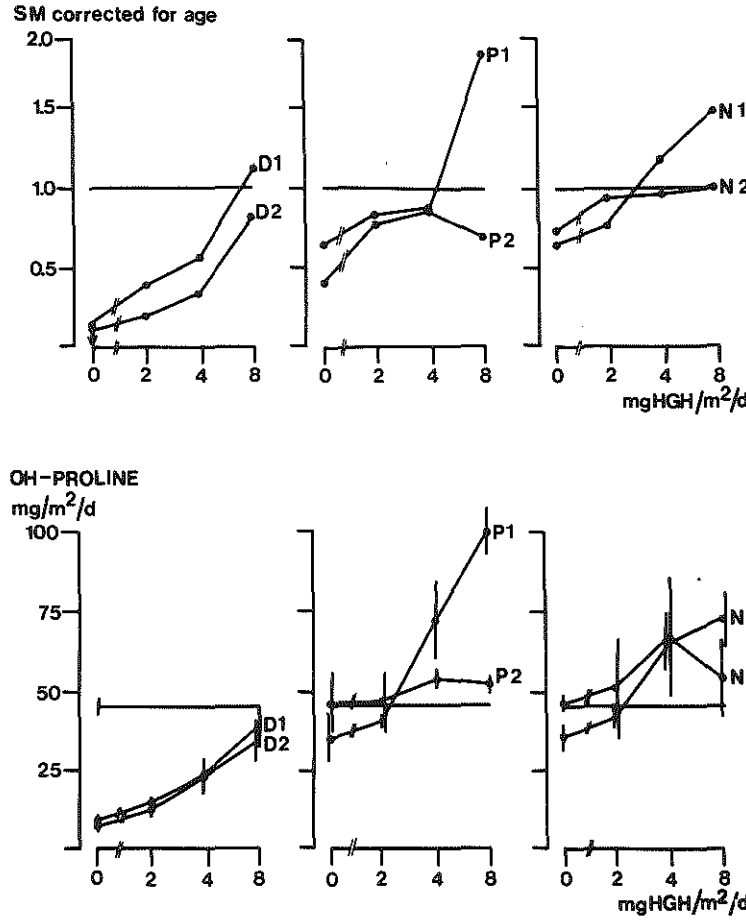


Fig. 10

Plasma somatomedin levels (SM) and average total urinary OH-proline excretion (± 1 SEM) during consecutive periods of HGH administration at increasing dosages. SM is expressed as the potency ratio of the plasma of the patient to the average obtained in plasma of children of the same age (horizontal line). OH-proline values are compared with the average ± 1 SEM obtained in 9 children of the same age range.

Note the similarity of the individual dose-response curves for both parameters and the importance of the differences between patients.

Urinary hydroxyproline excretion

In spite of the collagenfree diet the OH-proline varied considerably from day to day. Expressing the results of any given patient as the OH-proline/creatinine ratio reduces this variation (Fig. 11). However, Zorab et al.(1970) reported a good correlation with growth rate when the data are expressed in absolute excretion of OH-proline. Jasin et al. (1962) and Job et al. (1966) had previously demonstrated the importance of

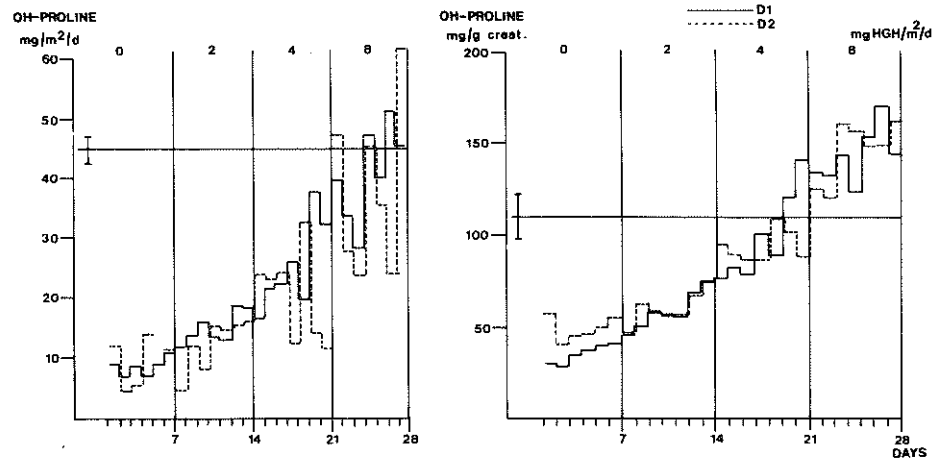


Fig. 11
Urinary total hydroxyproline excretion in two extremely GH deficient patients (D1 and D2) during 7-day periods of treatment with increasing doses of HGH. The horizontal line represents the normal mean ± SEM obtained in 9 normal children of the same age range. Note the reduction of the day-to-day variation when the data are expressed as mg/g creatinine instead of mg/m² /day.

correcting for body surface area when comparing individuals. Hence, the large variation resulting from expressing the data as mg/m² /day was accepted.
Contrary to the expectation the OH-proline excretion did not reach a plateau within each study period. To allow comparison with SM levels, results on days three to five of each study period were averaged (Fig. 10). In each patient the pattern of the dose-response curve is similar to the one obtained for plasma SM. The severely GH deficient children start from very low values (D1 : 8.6, D2 : 7.2) and gradually approach the normal range on the highest dose of HGH (D1 : 39.2, D2 : 34.3). The four remaining patients are all close to the normal range during the baseline period. On treatment P1 and N1 more than double their values (P1 : 34.5 to 100.07, N1 : 34.5 to 71.9) while the two others show only a minimal rise (P2 : 45.6 to maximally 53.1, N2 : 45.7 to maximally 66.2). The overall correlation between SM and OH-proline is visualized in Fig. 12. In spite of the fact that the data of P2 and N2 position slightly to the left of the others, a linear relationship is found with a correlation coefficient r = .89.

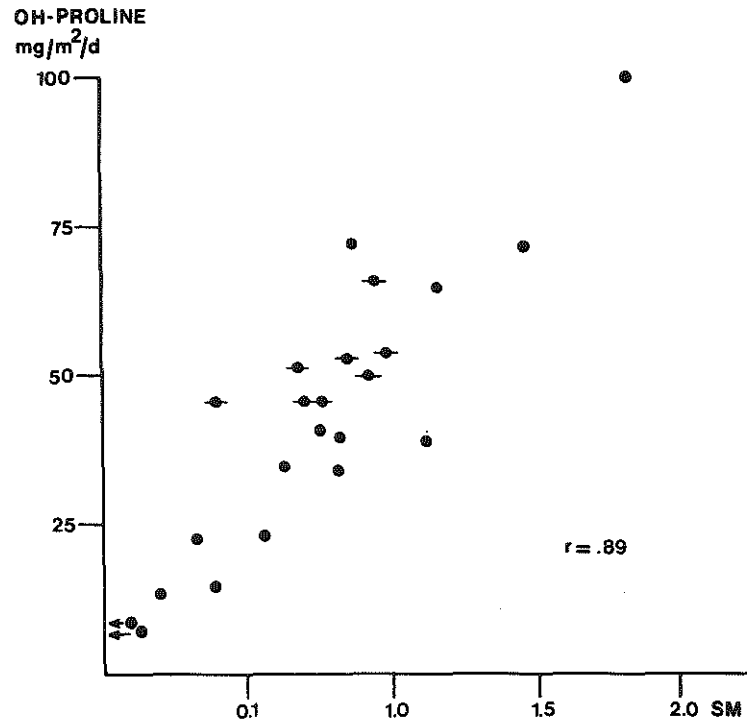


Fig. 12
OH-proline excretion as a function of plasma SM. All SM values (potency ratio to the normal for age) and the average OH-proline excretion on days three to five of each treatment period are included. The patients who responded poorly to GH administration (P2 and N2) are indicated with ⇐. Their OH-proline excretion in the presence of a given amount of SM is not grossly different. Linear regression on all points : $y = 49.7\ x + 5.3$.

DISCUSSION

Studies of the responsiveness to short term GH administration have primarily been aimed at quantitating the degree of GH deficiency in short children (Daughaday and Parker, 1963; Wright et al. 1965, Prader et al. 1968; Clayton et al. 1971 and Zachmann et al., 1972). The purpose of this pilot study was to investigate if we could elicit a similar dose-related response in hypopituitary patients with respect to somatomedin levels as had been obtained by Almqvist (1960), and how these would correlate with the changes in total urinary hydroxyproline excretion. The inclusion of patients in whom GH-secretion was either limited but not absent or apparently normal, was aimed at screening for a difference in the character of such a relationship, either in terms of SM-generation or with respect to the hydroxyproline excretion.
The measurement of somatomedin in plasma has now reached an acceptable degree of

precision (see same chapter, section D). In addition to the studies of Almqvist (1960) mentioned above, Daughaday and Parker (1963) had found an increase of SM of varying magnitude during shortterm administration of GH to short children. In general they noticed the best growth response to longterm treatment in the patients in whom the baseline SM value was low. This was confirmed by Wright et al. (1965). Hall (1972) found a good correlation of SM levels and growth velocity in GH treated children.

Urinary OH-proline excretion was chosen as an index of a, presumably SM-mediated, periferal GH effect. This parameter has amply been demonstrated to be GH dependent (Jasin et al. 1962; Job et al. 1966; Wright et al. 1965 and others). Its selection had become possible by the development of the Hypronosticon-test® (Organon), which is as accurate as previous methods and much easier to perform (Goverde and Veenkamp, 1972).

In the two children with extreme GH deficiency, who can be anticipated to have a normal responsiveness, curves are obtained which are very similar in both, in spite of differences in age and skeletal maturation. They show a close to linear relationship of both SM levels and OH-proline excretion to the logarithm of the dose of GH administered (Fig. 1). There is an excellent linear correlation between SM and OH-proline ($r = 0.95$).

Amongst the four remaining children, two responded well (P1 and N1), whereas the others barely changed their plasma SM levels or their OH-proline excretion. This difference in responsiveness was quite independent of the maximal plasma GH levels obtained during the diagnostic studies. The significance of this finding in terms of the pathogenesis of their short stature is limited since the reproducibility of these observations has not yet been investigated. In particular, the question of the prognostic value of a positive metabolic response during shortterm treatment, with respect to growth response on longterm treatment should be treated with caution. The N-retention test developped by Prader et al.(1968) segregates most GH deficient patients from normals. Nevertheless, it does not allow quantitation of the degree of the deficiency (Clayton et al. 1971). Since SM levels and OH-proline excretion are closely related to skeletal growth, they may prove to be better prognostic parameters. The studies of Daughaday and Parker (1963) suggest this. However, at present longterm treatment undoubtedly provides the best test to diagnose partial GH deficiency (Tanner et al. 1971).

The heterogeneity of the results obtained in the four patients who have no extreme GH-deficiency suggests individual differences with respect to their capacity to generate somatomedin in response to acute GH-stimulation. This perhaps represents the most promising aspect of this pilot study. The overall good correlation between plasma SM and OH-proline is also of interest since it suggests a normal SM/collagen interaction in the patients without GH deficiency. It also indicates that in GH-deficient patients, where the correlation coefficient was the highest, urinary OH-proline measurements may have the same value as the measurement of somatomedin. This cannot a priori be said for non GH-dependent growth failure. It is our intention to perform a larger number of observations, with longer study periods and higher dosages of HGH, in order to test the validity of the impressions obtained in this pilot study.

SUMMARY

Six children with short stature and varying maximal plasma growth hormone (GH) levels on stimulation were given clinical grade human growth hormone (HGH) at dosages of 0, 2, 4 and 8 mg/m^2/day in consecutive periods of 5 to 7 days duration. Plasma somatomedin (SM) levels, expressed relative to the normal for age, and total urinary OH-proline excretion (OH-P), corrected for body surface area, were monitored. Dose related response curves were obtained which were almost identical for both parameters. In the two severely GH deficient children starting values were very low (SM : < 0.14, av. OH-P : 7.8). They progressively approached the normal range on increasing doses of HGH (SM : 0.98, av. OH-P : 36.7). Of the four remaining children, two started in the subnormal range (SM : 0.63, av. OH-P : 34.5) and more than doubled their values on the highest dose of HGH. The two remaining children, starting with comparable SM, and somewhat higher OH-P values (SM : 0.55, av. OH-P : 45.6), showed only a slight increase without exceeding the normal range (SM : 0.84, av. OH-P : 52.3).

In these four patients there was no correlation between maximal plasma GH on stimulation and the response to acute GH administration. The correlation coefficient of the SM/OH-proline relationship of the pooled data of all patients was 0.89.

G. *Primary somatomedin deficiency. A case report**

Patients presenting with the clinical appearance of growth hormone deficiency, but in whom high levels of immunoreactive growth hormone were found, have first been described by Laron, Pertzelan and Mannheimer (1966). Since then, additional patients were reported by Laron, Pertzelan and Karp (1968), Merimee et al. (1968), Tanner et al. (1971), Najjar et al. (1971), Elders et al. (1971) and New et al. (1972). This paper describes a similar patient and adds evidence suggesting an overall and specific non responsiveness to growth hormone.

Patients

The propositus, BK, a white boy, was born at 37 weeks gestation. Pregnancy had been complicated by hypertension during the last weeks. Birth weight: 3250 gram (+ 1.0 SD), length: 44 cm (– 2.6 SD) (Usher and McLean, 1969).
During the first days of life he was noted to drink poorly and to vomit occasionally. Psychomotor development was normal. Growth was slow from early in life (Fig. 13). At the age of two years he had documented hypoglycemic spells for which he was given cortisone (5 mg/day). An attempt to increase his growth rate with Orgabolin®Organon 0.5 mg/day during six months failed. Cortisone was discontinued at the age of six years when Perthes-like deformaties were discovered at both hips. All treatment was withheld until he was 10 years and 3 months when he was admitted for the present studies. Family history is negative for short stature. Fathers height: 170.7 cm (– 1.1 SD), mothers height: 155.7 cm (– 1.7 SD) (Van Wieringen et al. 1968). There is no consanguinity.
PL, another white boy, 6 years and 4 months old at the time of diagnostic studies, with severe hyposomatotropic dwarfism and of similar body size as BK, was selected to serve as a control. He was born by cesarean section, 3 weeks post term after presentation in breech position. Pregnancy had been complicated by hypertension after the 36th week. Birth weight: 3500 gram (equal to the mean for gestational age), length: 50 cm (–1.5 SD) (Usher and McLean, 1969).
During the first days of life he drank poorly and vomited occasionally. He was noticed to grow slowly. (Fig. 13). His psychomotor development was quite normal. He has had no history of hypoglycemia and had received no medications before admission to the hospital. Family history is negative for short stature. The parents are full cousins.

* These studies were done in collaboration with M.V.L. Du Caju, H.K.A. Visser, W. Schopman, W.H.L. Hackeng and H.J. Degenhart and will appear in Archives of Diseases in Childhood.

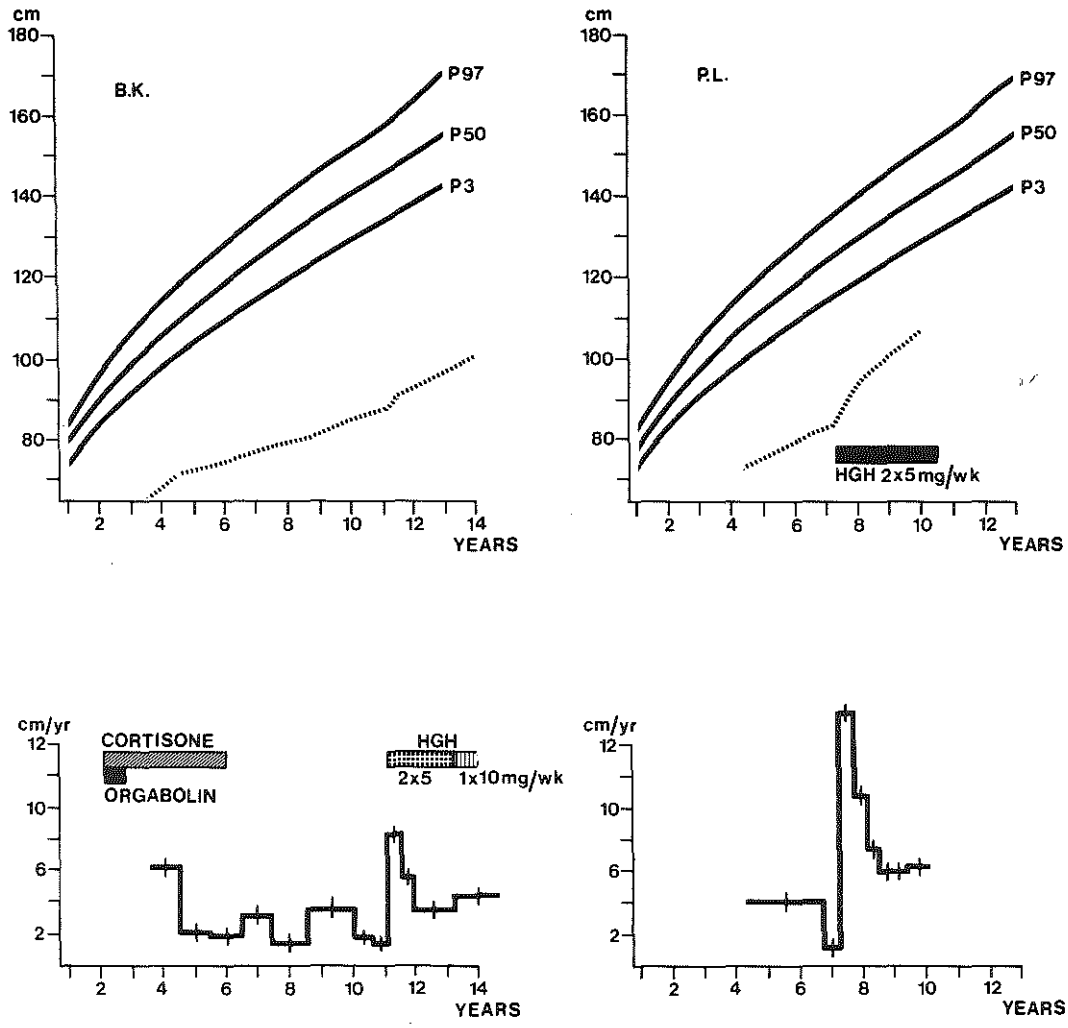


Fig. 13
Height is plotted against Dutch growth standards (Van Wieringen et al., 1968). Growth-hormone administration causes only a slight increase in growth rate in the propositus (BK). The control patients (PL) with isolated GH deficiency responds to GH-treatment with a pronounced growth spurt.

Materials and methods

Growth hormone and insulin were measured with a charcoal dextran radioimmunoassay (Schopman and Hackeng, 1971). Urinary total nitrogen, calcium, phosphorus, creatinine and 17-hydroxysteroids, as well as bloodglucose and free fatty acids were measured using routine methods (respectively Fawcett and Scott, 1960; Dunsbach, 1963; Fiske and Subbarow, 1925; De Vries and Van Daatselaar as described by Gorter and De Graaf, 1955; Degenhart, 1973; Schmidt, 1963; Ko and Royer, 1967 and Korovina et al., 1966). Total urinary hydroxyproline was determined with the Hypronosticon test® (Organon) (Goverde and Veenkamp, 1972). Plasma somatomedin was measured with a double isotope technique, using hypophysectomized rat or normal monkey rib cartilage (same chapter, section A).
For the electrofocusing experiments a LKB 8102 Ampholyne column (440 ml) was used with LKB ampholite ranging from pH 3 to 6. The procedure was as described earlier (see this chapter, section A2). Equilibration time was between 60 and 120 hrs. Fractions were tested for their growth hormone content by radioimmunoassay.

RESULTS

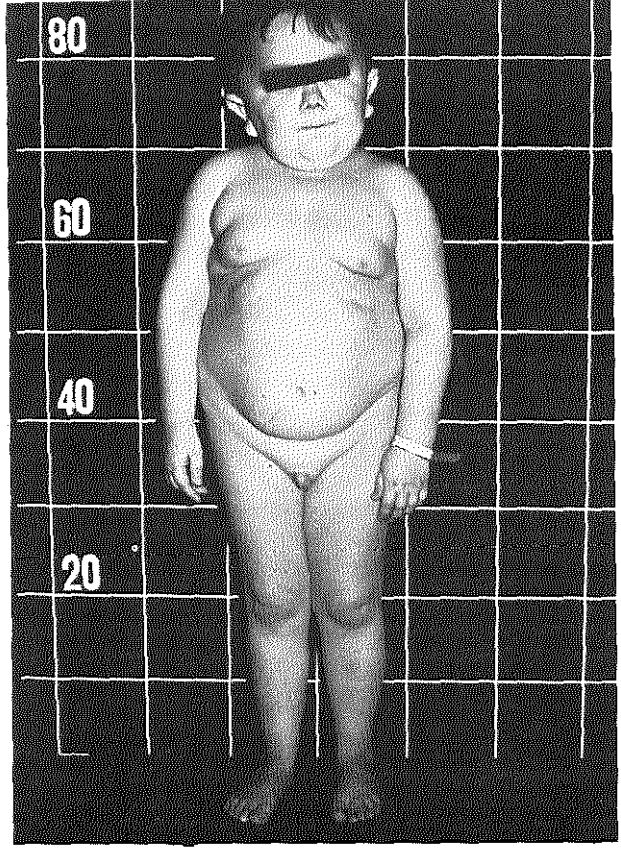
a. *Physical examination and baseline studies.*

The *physical appearance* of both boys was very similar (Fig. 14a). The abnormalities were more pronounced in BK than in PL. Both were short, obese and reasonably well proportioned. Some details are represented in Table 4. Their faces were small. Particularly BK's ears were disproportionally large. Teeth were in poor condition. Thoracic and

TABLE 4

Anthropometric data before and during treatment.

	BK				PL			
Age (years)	11.1	11.6	12.2	13.3	7.3	7.8	8.4	9.5
Time after initiating growth hormone treatment (years)	0	0.50	1.12	2.23	0	0.51	1.09	2.25
Length (cm) Supine	87.8	91.2	93.8	98.0	83.0	90.6	96.0	–
Standing	–	–	–	–	–	90.5	95.3	103.0
Span (cm)	88.0	90.2	97.0	97.0	81.5	88.5	96.0	102.0
Headcircumference (cm)	51.0	51.5	51.5	51.6	48.2	48.3	48.8	49.4
Weight (kg)	15.7	17.9	19.0	20.0	10.2	11.3	12.8	15.5
Skinfold thickness % of initial	100.0	91.1	90.5	99.6	100.0	62.3	59.3	58.4



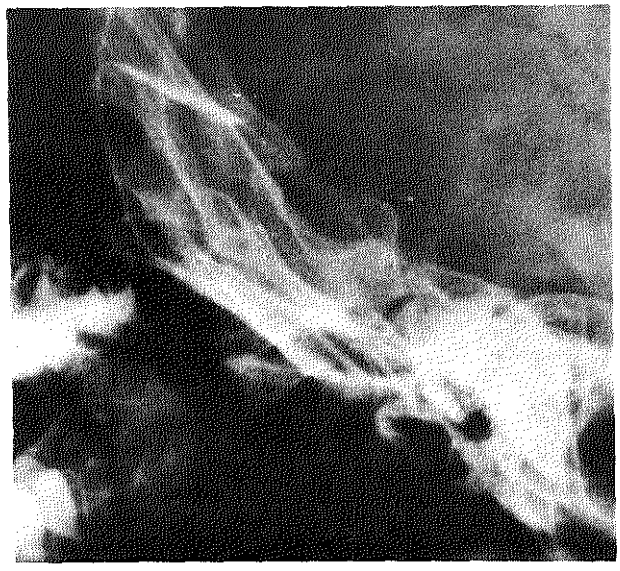
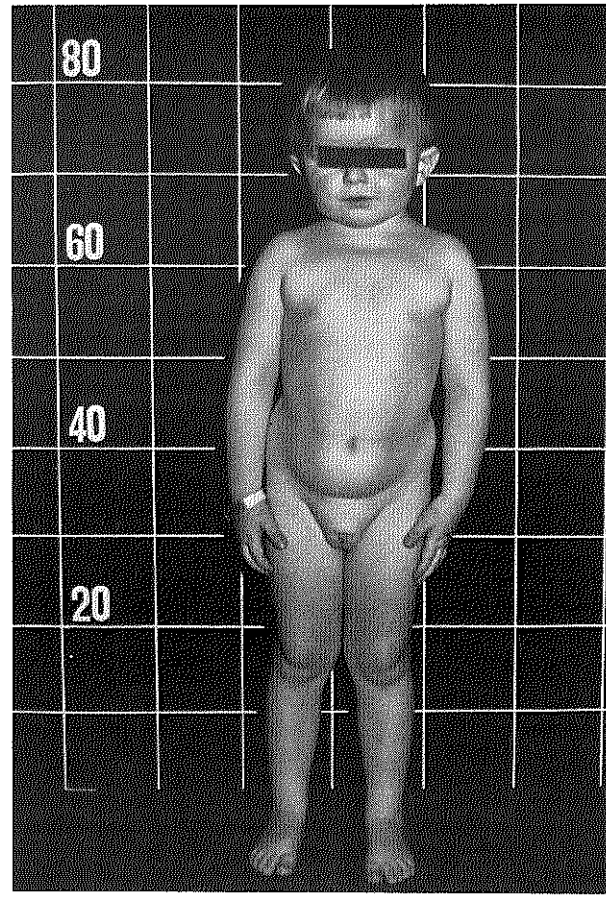
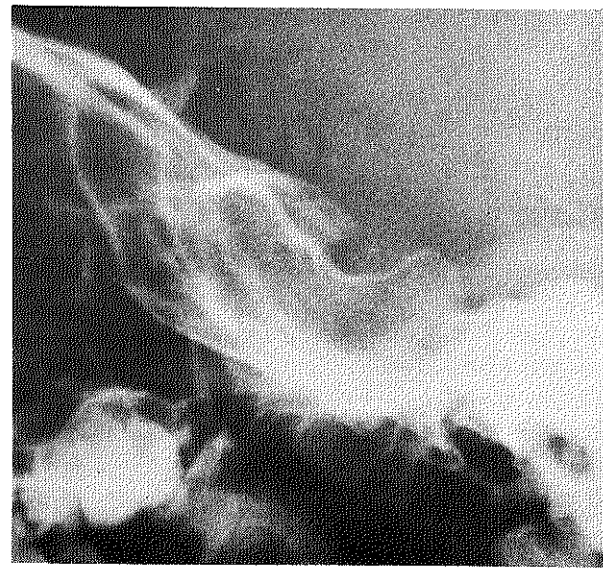


Fig. 14
a. The propositus (BK-left) and the control patient (PL-right) before treatment. Obesity was more pronounced in BK. Otherwise their appearance was very similar.





b. The sella turcica was different: BK's sella (left) showed an impression of the floor of the sella, whereas PL's (right) was small but otherwise normal.

abdominal examination was not relevant. Both children had small external genitalia with a rather short penis (BK 2.5 cm, PL 1.5 cm) a hypoplastic scrotum, the content of which could not with certainty be identified as testes. Eye grounds, visual fields and detailed neurological examination were all witin normal limits. Skeletal age was 5 yr. 0 mth (5 yrs. 2 mths. retarded) in BK, and 2 yr. 10 mths. (3 yrs. 7 mths. retarded) in PL (Greulich and Pyle, 1959). On X-ray examination, the skull bones were found to be very thin. Whereas PL's sella was small, in BK impression of the floor of the sella suggested a large pituitary gland (Fig. 14b).

Baseline laboratory examination:

Thyroid and adrenal function (BK respectively PL): PBI: 4.1 and 5.0 μg %. ^{131} I-uptake: 30 and 23,4% of the dose after 24 hrs. Metyraponetest (maximal 17-OH steroid excretion on 250 mg at 4-hourly intervals for two days): 6.9 and 5.4 mg/m^2/24 hrs. All these results are normal.

Plasma Growth Hormone was measured during intravenous infusion of arginine-monohydrochloride (0.5 g/kg I.V. infused in 30 minutes), and with insulin induced hypoglycemia (0.05 U/kg I.V.; bloodglucose fell to 51% of the initial value in BK and to 65% in PL), PL showed almost no response, reaching a maximum value of 2 μU/ml (1st IRP for Human Growth Hormone) during arginine infusion. BK in contrast had very high baseline values (average 69 μU/ml, range 60 - 76) which rose further to the extreme level of 508 μU/ml during insulin induced hypoglycemia.

Plasma Insulin levels were also measured during the arginine infusion test and were low in both cases. Although their peak values were similar (BK: 8, PL: 9 μU/ml, BK had a sustained baseline level between 4 and 6 μU/ml, whereas in PL the baseline was less than 1 μU/ml.

Plasma Somatomedin measured in the hypophysectomized rat assay was equally low for both patients as compared to two acromegalics. This low value was found as well when ^{35}S-sulfate as when the ^{3}H-Thymidine incorporation was used as an index of somatomedin activity (Fig. 15).

b. *Short term metabolic studies* (Table 5 and Fig. 16).

Human Growth Hormone (HGH), clinical grade* was administered at a dose of 8 mg/m^2/day at 8 p.m. for seven days. Results obtained from the day after the second injection on have been selected for comparison with the baseline data, except for OH-proline excretion which is known to change slowly during GH treatment (Van Gemund et al., 1969; see also chapter two, section F). For this reason only the data obtained after the fourth administration of HGH were included. During these studies the patients were hospitalized and kept on a calorie and N-constant diet.

Fasting blood glucose and free fatty acids were only slightly affected. Blood glucose increased somewhat in the control and not in BK. Fatty acids did not change significantly.

The urinary excretion of Ca and P is expressed as their ratio since this reduced the daily

* The content in immunoreactive HGH of this preparation is 1.45 1st IRP/mg.

fluctuation considerably. A slight but significant increase was noted in BK, while PL showed a very large rise.

The urinary N-creatinine ratio fell slightly in BK and drastically in PL. The drop was significant for both patients but the differences between them were large.

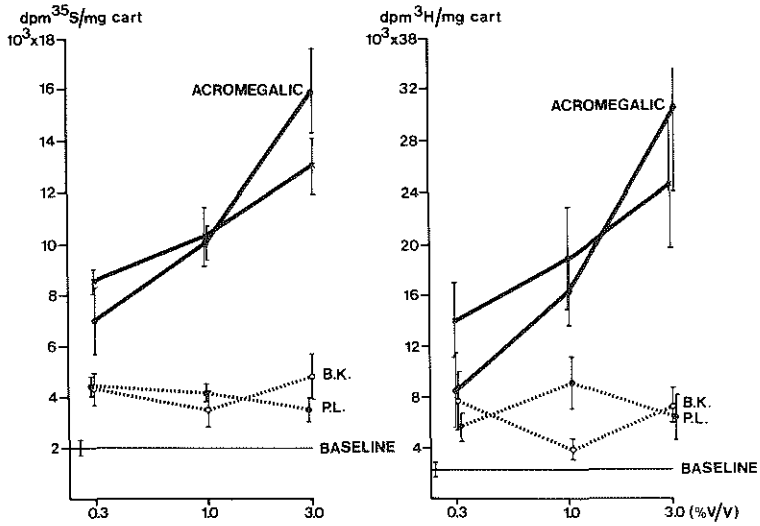


Fig. 15

Plasma somatomedin activity measured in the hypophysectomized rat assay using a dual labeling procedure. Within the limits of the concentrations used no increased incorporation of ^{35}S-sulfate or ^{3}H-Thymidine occurs with plasma form BK nor PL while the plasma from two acromegalics is very stimulatory.

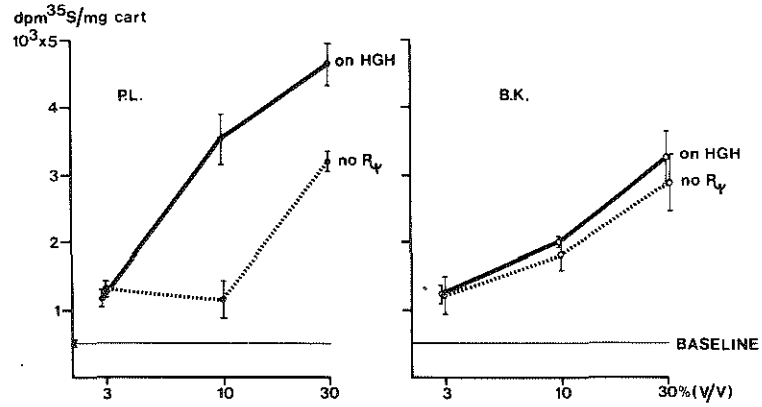


Fig. 16

Plasma somatomedin activity measured in the normal monkey assay using a dual labeling procedure. Plasma from BK and PL before starting treatment (no. R_ψ) and on the 4th – 5th day of HGH administration at 8 mg/m^2 day are compared. Note that the dose response curve obtained with plasma from BK does not change in contrast to the potency of PL's plasma which increased markedly. ^{35}S-sulfate and ^{3}H-thymidine incorporation were identical (the latter is not shown).

TABLE 5

*Effect of shortterm growth hormone administration**

		baseline mean ± SEM (n)		on treatment mean ± SEM (n)		% change	P-values** baseline versus treatment
Fasting blood glucose (mg %)	BK	76.75 ± 3.7	(4)	72.30 ± 2.0	(5)	− 5.8	n.s.
	PL	64.25 ± 5.2	(4)	81.04 ± 2.24	(5)	+ 26.1	< 0.05
Fasting plasma FFA (mEq/L)	BK	1.080 ± 0.060	(4)	1.248 ± 0.124	(5)	+ 15.6	n.s.
	PL	1.231 ± 0.270	(4)	1.375 ± 0.116	(5)	+ 11.7	n.s.
Urinary Ca/P ratio	BK	0.060 ± 0.003	(3)	0.085 ± 0.012	(5)	+ 41.7	< 0.05
	PL	0.085 ± 0.004	(3)	0.315 ± 0.038	(4)	+ 270.6	< 0.01
Urinary N/creatinine ratio	BK	22.57 ± 0.34	(3)	19.44 ± 0.54	(5)	− 13.9	< 0.005
	PL	25.73 ± 2.51	(3)	10.50 ± 0.41	(6)	− 59.2	< 0.001
Urinary OH-proline/ creatinine ratio	BK	67.60 ± 3.71	(3)	57.4 ± 2.37	(4)	− 15.1	< 0.05
	PL	104.23 ± 5.60	(3)	197.6 ± 23.25	(4)	+ 89.6	< 0.01

* dose of HGH: 8 mg/m^2/day. See text.

** by Student t-test, n.s. = not significant.

The urinary hydroxyproline/creatinine ratio decreased somewhat in BK and doubled in the control patient.

Plasma Somatomedin was measured in pretreatment plasma and on the fourth day of GH-treatment. In baseline conditions both patients are indistinguishable. On treatment the somatomedin activity of PL rose to approximately three times the baseline levels, whereas BK remained unchanged, both when the results are expressed as ^{35}S-sulfate and as ^{3}H-Thymidine incorporation.

c. *Effect of longterm GH-treatment.*

Linear growth and skinfold thickness: Over periods of two years before and on treatment the growth rate of BK increased from 3.1 to 4.6 cm/yr. as compared to a change from 3.3 to 9.2 cm/yr. in the control patient (PL). Over the same period of treatment average skinfold thickness, measured with a Harpenden caliper and computed from measurements at eight sites (above the biceps, triceps and quadriceps and in the subscapular area bilaterally), after an initial drop to 90.5% of the initial value, increased again to 99.6% in BK, while it dropped to 58.4% in PL (Table 4).

Skeletal maturation advanced 2 yrs. 9 mths. in BK and 2 yrs, 6 mths. in PL over the same two year period.
Plasma somatomedin activity. As is shown in Fig. 17 dose response curves with BK's plasma before treatment, after four days and six months of growth hormone administration are not different.
Plasma growth hormone and insuline levels. An arginine telerance test was repeated in BK after six months of treatment. The test was done 36 hrs. after a HGH-injection. The results are shown in Table 6. Treatment has not resulted in a decreased endogenous HGH nor has it stimulated insulin secretion in response to arginine infusion.

d. *Some immunological and physico-chemical characteristics of endogenous growth hormone in BK.*

To study possible differences in immunological characteristics plasma was serially diluted, measured in the growth hormone radioimmunoassay and the slopes obtained were compared. No significant deviation from paralellism could be demonstrated (Fig. 18).
In order to find possible differences in charge, plasma from BK was electrofocused and

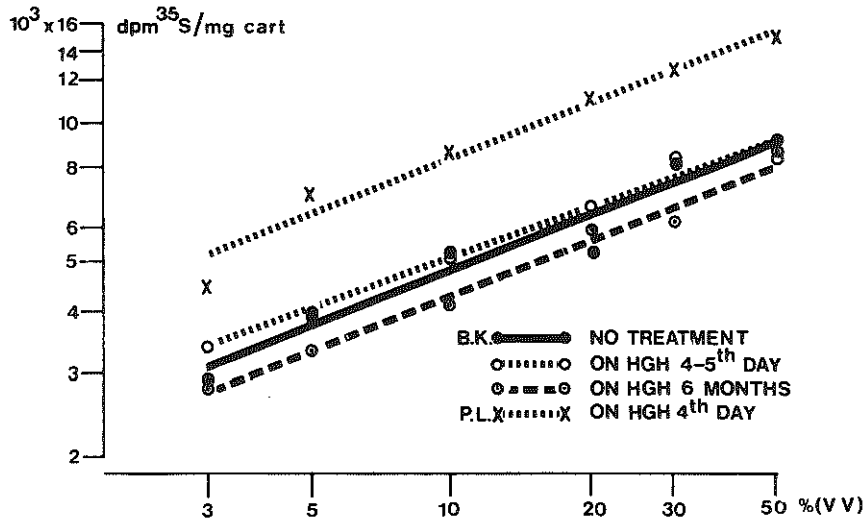


Fig. 17
Plasma was incubated over a wide range of concentrations in the normal monkey assay using ^{35}S-sulfate incorporation as index. Dose-response curves obtained with plasma before treatment, after four days of administration of HGH (8 mg/m^2/day), and after six months of treatment with HGH 5 mg bi-weekly are identical. In order to demonstrate that this assay would pick-up an active sample. PL's plasma on the 4th day of HGH treatment was also measured.

TABLE 6

Arginine infusion before and after six months of growth hormone administration.

Time	−15	−1	+15	+30	+45	+60	+75	+90	+120	+150
Plasma Growth Hormone										
before treatment	63	75	105	118	170	470	508	295	133	–
after six months										
of treatment	217	216	141	192	315	228	180	198	222	198
Plasma Insulin										
before treatment	6	4	4	6	8	6	4	4	5	–
after six months										
of treatment	4	2	2	3	4	2	2	2	2	2

Arginine hydrochloride 0.5 g/kg bodyweight I.V. over a 30 min. period after overnight fast.
Time expressed in minutes before (−) and after (+) the initiation of the infusion.
Growth Hormone is expressed as μ U 1st I.R.P./ml, insulin as μ U/ml.

compared both to plasma from an acromegalic and HGH (same preparation as used for treatment). GH was localized by radioimmunoassay.
Initially, GH in BK's plasma focused at a rather wide peak with a suggestion of two maxima (Van den Brande et al., 1971a). After improving the technique by the selection of the more appropriate pH-range of 3 - 6 instead of 5 - 8, and using the same plasma sample a sharp single peak was repeatedly found at an average pH of 5.03. In acromegalic plasma, GH focused in a single zone at an average pH of 5.01. Finally, HGH, clinical grade yielded a broad zone of immunoreactive material with a maximum at pH 4.98. Examples are shown in Fig. 19. Within the limits of the technique we find the GH from these three sources to behave indistinguishably.

DISCUSSION AND CONCLUSIONS

At first most of the patients studied by Laron et al., (1968) seemed to respond to exogenous HGH with N-retention, lipolysis and increased growth rate. More recently however, after a longer period of observation, the authors concluded that the metabolic and growth response of their patients was variable but mostly lacking (Laron et al., 1971). This is an agreement with the findings by others (Merimee et al., 1968, Tanner et al., 1971, Najjar et al., 1971, Elders et al., 1971 and New et al., 1972). Laron et al. (1971) favour the hypothesis that the growth hormone molecule in such patients might have an abnormal structure, lacking biological activity but with the preservation of its immunological characteristics. The subnormal response to exogenous HGH could be the

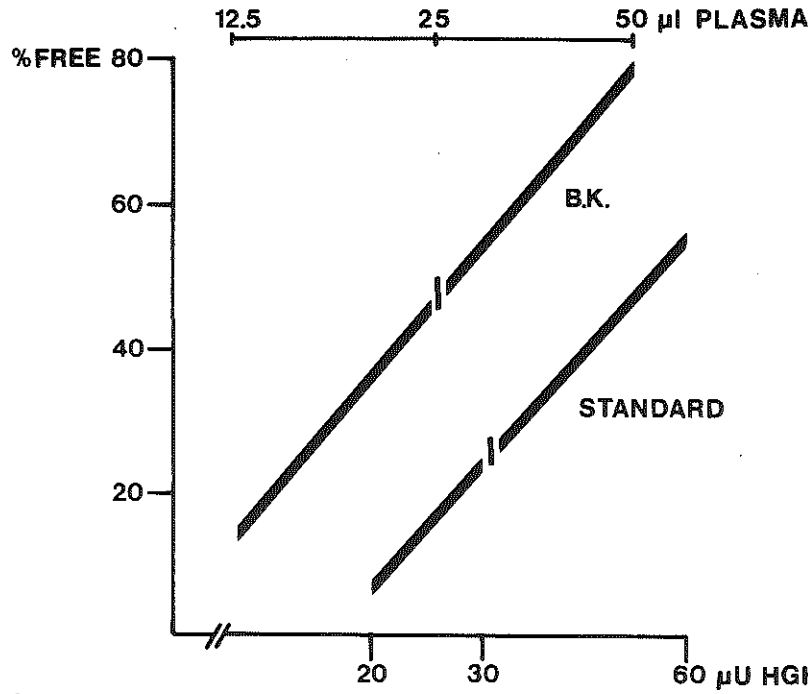


Fig. 18

Plasma from BK was serially diluted and measured in the HGH-radioimmunoassay. Each of the points on the standard curve is an average of four determinations. The plasma was measured in duplicate at each dose-level. HGH is expressed in μ U 1^{st} I.R.P. of Human Growth Hormone. No deviation from parallellism can be demonstrated (F: 4.8,– P: 0.05).

consequence of an unsuccessful competition with the endogenous molecule for binding sites. Daughaday et al., (1969) found low plasma somatomedin (SM)-levels as well before as on treatment with HGH in Laron's patients. They suggested that the lack of SM-generation may be involved in the pathogenesis of the growth failure, while the other abnormalities such as lack of lipolysis, N-retention and calciuria may be the expression of a more generalized defect in growth hormone response. New et al., (1972) came to similar conclusions. Our patient also presents with a complete picture of hyposomatotropism, which not only includes short stature and the typical appearance, but also a documented hypoglycemia, insulinopenia and low plasma somatomedin levels. Noteworthy is the low lenght at birth with slightly increased weight. Laron et al. (1968), also found low birth length in half of their patients. This suggests some GH dependency of intrauterine growth at the end of pregnancy.

The endogenous growth hormone, present at very high levels in plasma, cannot be distinguished from normal growth hormone, neither from its immunological behaviour, nor from its isoelectric point.

Our results during short term treatment with HGH, at high dosage, confirm previous reports: changes of metabolic indices of growth hormone effect such as bloodglucose,

N-retention, calciuria and hydroxyproline excretion are minimal or absent. Somatomedin is low before and on treatment. The finding of others (Laron et al., 1971, Tanner et al., 1971, Najjar et al., 1971 and New et al., 1972) that chronic treatment did not induce the

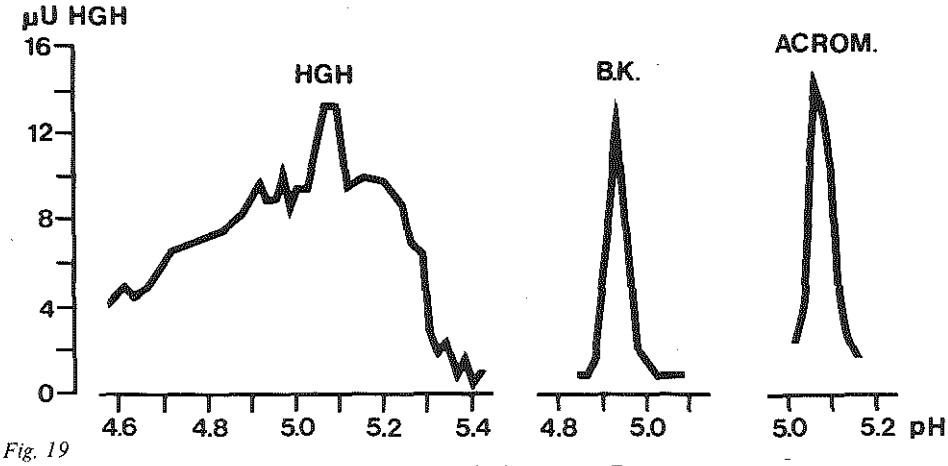


Fig. 19
Examples of electrofocusing of human growth hormone. From left to right: HGH (clinical grade, same preparation as in the metabolic studies, 0.72 mU), 2 ml of plasma respectively from patient BK and an acromegalic. In repetitive experiments the GH of all three preparations was found between the extremes of pH 4.93 and 5.14. Average peak-positions were HGH: 4.98, BK: 5.03, acromegalic: 5.01. (For technical details see text.)

expected increase in growth rate was also confirmed in our patient. In addition, similarly to the observation of Tanner et al. (1971), we found a slight temporary decrease of the skinfold thickness, measured with the Harpenden caliper, followed by an increase. By radiography however, Tanner et al. (1971) noticed little change in fat or muscle. While Elders et al. (1971) reported that in acute conditions the endogenous GH-secretion could not be suppressed by exogenous administration of the hormone, we found that prolonged treatment was not effective in this regard either. Insulinopenia and low plasma somatomedin levels, which had been shown by others to be resistant to acute GH-treatment (Laron et al., 1971, Elders et al., 1971 and New et al., 1972) were still unchanged after six months of administration of the hormone.

All these findings suggest a specific and overall impaired responsiveness to growth hormone. No evidence is present supporting the hypothesis of an abnormal endogenous GH-molecule.

While all effects of GH are diminished, its failure to induce SM seems to have the most dramatic consequence. Since SM is supposed to mediate the growth promoting effect of GH (see same chapter, section C), the extreme short stature of our patient should most likely be attributed to his SM-deficiency.

Studies of the characteristics of the GH-receptors in these patients, and evaluation of their responsiveness to exogenous SM should contribute to the elucidation of the pathogenesis of this syndrome.

SUMMARY

A child presenting with the clinical features of hyposomatotropism but with high immunoreactive plasma growth hormone is described. During shortterm administration of human growth hormone (HGH) his response with regards to fasting bloodglucose and free fatty acids, plasma somatomedin, urinary excretion of calcium, nitrogen and hydroxyproline were minimal or absent. Six months of treatment with HGH did not reduce the endogenous HGH secretion. Insulin secretion had not increased and plasma somatomedin levels remained extremely low. Over a period of two years of treatment, growth response and loss of subcutaneous fat were minimal. On serial dilution in the radioimmunoassay, his GH-molecule yielded a parallel line with the HGH-standard. In electrofocusing experiments the GH-molecule positioned at the same pH-range as GH in acromegalic plasma and the major peak of clinical grade HGH (average 5.03 versus 5.01 and 4.98).
It is concluded that an overall and specific diminished responsiveness to HGH is present in this patient. This includes a lack of generation of somatomedin, which is thought to be the cause of his short stature. No evidence suggesting an abnormality of the GH-molecule was found.

CURRENT KNOWLEDGE OF SOMATOMEDIN

Only a few articles have appeared recently reviewing the subject (Daughaday and Garland, 1972; Hall, 1972 and Grant, 1972). Since much progress has been made during the last year, it seemed of interest to summarize the current status of our knowledge of somatomedin.

1. *Somatomedin-assay*

Most present methods for estimating SM *in vitro* are derived from the technique described by Salmon and Daughaday (1957), which measures ^{35}S-sulfate incorporation into chondroitine-sulfate in rib cartilage of hypophysectomized (hypox) rats. Its imprecision and laboriousness had lead many investigators into a search for a better method. Yde (1968) used the cartilage of normal fasted rats. This simplified the technique and yielded a slightly better precision (av. $^{S}/b$: 0.20 as compared to ± 0.26 in the hypox rat-assay). Recently, Alford et al. (1972) modified the Yde-technique further. At the end of the incubation, the cartilage is digested and precipitated onto paperstrips, which facilitates the washing procedure. Fujisawa (1964), using hypophysectomized puppies, by-passed the important inter-animal variation of the other methods, since cartilage from a single animal sufficed for one assay. However, the magnitude of the response in this tissue to SM is very small.

Hall (1970) uses the pelvic rudiments of chicken embryo's. This shortens the procedure with one day but does not improve the precision and is less sensitive than the hypophysectomized rat assay.

Our own method (chapter 2, E), combines many of the advantages of these modifications since the manipulations have been reduced to a minimum and the precision of the technique has been improved considerably. ^{3}H-Thymidine and ^{35}S-sulfate incorporation are measured simultaneously. The average precision ($^{S}/b$) of the latter, which is the best parameter, is 0.15. The sensitivity is similar to all other methods except for those which use hypophysectomized animals. Garland et al. (1972) have used suspensions of chondrocytes liberated from pelvic leaflets of chicken embryo's. They showed a marked stimulation of ^{3}H-Thymidine incorporation into DNA when the cells were incubated with increasing doses of plasma. However, the correlation between the results in this system and those in the other SM-bioassays remains to be established.

A quite different approach was made by Herbai et al. (1970), who utilized the *in vivo*

incorporation of ^{35}S-sulfate into the costochondral junctions of mice as an index of sulfation activity. This method is particularly suited for studying physiological variations and *in vivo* experimentally induced changes (Herbai et al., 1970; Herbai, 1971a, 1971b and 1971c). All these techniques have the disadvantage of not being specific. Many substances other than somatomedin have been recognized to influence sulfation and DNA-replication in cartilage. The most important amongst them are insulin, cortisone and amino acids (Layton et al., 1951; Murphy et al., 1956; Salmon and Daughaday, 1951 and 1958; Salmon et al., 1968; Koumans and Daughaday, 1963). Their influence will be reviewed in detail by Du Caju in his thesis (to be published). The lack of specificity necessitates carefully controlled experimental conditions before any definitive conclusions can be drawn from the results obtained with the current assay-techniques.
A radioimmunoassay may by-pass this problem. Purification of SM is now in such a stage that its development can be expected soon. An alternative possibility is raised by the data of Hintz et al. (1972a), which suggest that somatomedin and insulin compete for the same receptor sites on chondrocytes. This phenomenon could potentially be used for the development of a radio-receptor assay with ^{125}I-insulin as the marker. At present apart from somatomedin, no other animal substances than insulin, pro-insulin and substituted insulins have been demonstrated to compete with ^{125}I-insulin for binding to receptor sites. Such a technique has the potentiality of being highly specific. The combination of measuring SM by bio-assay and either a radioimmuno- or radio-receptor assay should be particularly interesting for the study of those conditions in which the SM-values by bio-assay have been unexpectedly high or low (Du Caju and Van den Brande, 1973). Moreover, a radioreceptor assay for SM in which the cells of patients represent the variable, should be of great value to investigate these conditions where the SM/target-organ interaction is suspected to be disturbed.

2. *Characterization and purification of somatomedin*

A. Characterization

Most of the studies on the physical characteristics of SM have been on either human or rat plasma or -serum. Our studies on plasma SM in different animal species did not reveal qualitative differences in the somatomedin of a series of mammals (chapter 2. D). More recently, studying SM in porcine plasma with acid-ethanol extraction, gelfiltration, ion exchange chromatography and ultrafiltration, we have been unable to demonstrate any difference with human SM (to be published). This suggests at least a close structural similarity.
The structure of SM is not known, but many of its characteristics have been described.

1. *Identity of sulfation and DNA-replication stimulating activity.* In the course of all our experiments we have never found separation of SM into a fraction which stimulates

sulfate incorporation and another stimulating DNA-replication. This suggests that both these effects of SM may reside in the same molecule (chapter 2, A).

2. *Molecular size.* In native rat plasma, SM behaves as a large molecule. This was already apparent from early studies in Daughaday's laboratory which demonstrated that SM was not dialysable and resided in the macromolecular fraction both by ultracentrifugation and by chromatography on Sephadex G-25 (Salmon and Daughaday, 1958; Daughaday and Kipnis, 1966; Koumans and Daughaday, 1963). This was confirmed by Bala et al. (1970) and by our own studies in plasma from acromegalics (chapter 2, A).
In line with this is its retention by ultrafiltration membranes with an exclusion limit of 50,000 daltons (chapter 2, A). Somatomedin, extracted from plasma with 20% ethanol and subsequent chromatography on DEAE-cellulose was recovered on Sephadex G200 (pH 7.4) in a zone suggesting a M.W. between 9,500 and 35,000 (chapter 2, A). Acid ethanol extraction yields an active fraction of smaller size (Van Wyk et al., 1969). When such an extract is subjected to gel filtration on Sephadex G100 at pH 7.5 the apparent M.W. is between 4,000 and 12,400 (chapter 2, A). The same procedure with 1% formic acid as eluent yields an active fraction with an apparent M.W. between 4,000 and 8,000 daltons (Hall, 1972). These discrepancies suggest that SM is either bound to a larger carrier in plasma or aggregates in neutral or alkaline conditions.

3. *Charge.* The behaviour of SM on ion exchangers, starch gel, high voltage electrophoresis, and iso-electric focusing suggested that the main component responsible for SM activity in plasma is a molecule with an isoelectric point between 6.6 and 6.7. By starch gel electrophoresis, iso-electric focusing and DEAE-chromatography, a minor active fraction was recovered, which was more acid and focused at a pH of 5.2 (chapter 2, A).
No further information is at present available about other qualitative differences between these two fractions. The findings are similar irrespective of whether the preparation used was native plasma or acid-ethanol extract.

4. *Peptide nature.* We found pronase to destroy the biological activity of SM in a semipurified fraction (obtained by DEAE-cellulose chromatography) of acromegalic plasma, and concluded that SM was either a peptide or needed a peptide linkage for its expression (chapter 2, A). This was confirmed by Salmon (1972): Exposure of an active fraction, obtained by heating acidified normal rat serum, to trypsin destroyed all biological activity.

5. *Stability.* Somatomedin is relatively stable. It tolerates pH extremes (from 2 to 10) (chapter 2, A). Its activity is lost by heating to 80°C for 30 min. in an alkaline solution (chapter 2, A). In contrast, after acidification it is not destroyed even when exposed to 100°C for 15 min. (Salmon and Duvall, 1970).

B. *Isolation*

Based on these observations, different isolation schemes have been developed, either starting with acid-ethanol extract (Van Wyk et al., 1969) or with acidified and heated plasma (Salmon and Duvall, 1970). The GH-dependency of extracts obtained by heating acidified plasma has been demonstrated (Salmon and Duvall, 1970). This has not been verified for acid-ethanol extracts.

For subsequent isolation many techniques have proven to be efficient: gel filtration on Sephadex G75 (Van Wyk et al., 1972; Hall, 1972), chromatography on CM- and DEAE-cellulose or -Sephadex, fractionation on Dowex-50 (Chapter 2, A, Salmon and Duvall, 1970 a; Hall, 1972) and high voltage electrophoresis (chapter 2, A, Hall and Uthne, 1972). Liberti (1970) used sequential ultrafiltration on Diaflo-membranes of frozen and thawed cow-plasma, and recently reported the successful utilization of the binding of SM to cartilage, followed by its removal with concentrated salt solution (Liberti, 1972).

The purest SM-preparation has been obtained thus far by Kerstin Hall (1972) who claimed 1 x 10^6 purification over native plasma. It migrated as a single band on polyacrylamide. Using a somewhat different scheme of isolation, Van Wyk has obtained a preparation which is equally pure (personal communication). It can be anticipated that the amino acid composition and eventually the sequence of somatomedin will be known soon.

It should be emphasized that large losses of biologically active material occur during these isolation procedures. It cannot be exluded at this time that some molecular species, structurally different, but with similar biological characteristics are lost in the process.

3. *Source of SM*

Many attempts have been made to discover the source of SM. Realizing that early binding of a hormone frequently reflects its site of action, we studied the distribution of ^{125}I-HGH in young hypophysectomized rats 6 and 20 minutes after intravenous injection using whole body autoradiography (chapter 2, B). A high concentration of radioactivity was found in the liver, the kidney and in the zona glomerulosa of the adrenal cortex. Some accumulation was also apparent in the submandibular glands. Pretreatment with GH largely prevented accumulation in the liver and adrenal cortex and slightly in the submandibular glands, suggesting specific binding to these tissues. In contrast, the accumulation in the kidney was enhanced.

Kidney. It had been demonstrated by others that GH is accumulated in the brushborder of the tubular cells in the kidney (Collipp et al., 1966), which may be a reflection of reabsorption. Since the rate of glomerular filtration is enhanced by GH-administration (Gershberg, 1960; Beck et al., 1964), increased reabsorption could explain the influence of pretreatment. McConaghey and Dehnel (1972) demonstrated GH inducible SM-like activity in kidney perfusates of hypox rats. However, they also found kidney slices both from hypox and normal animals to release SM-like activity in large and equal amounts.

Further characterization of this material is necessary to investigate its relationship with plasma SM.

Adrenal. The binding to the adrenal is likely to be associated with aldosteron production, which is enhanced by high doses of GH (Beck et al., 1957). Since adrenalectomized and hypophysectomized animals grow well on GH-treatment (Simpson et al., 1944) the binding of HGH is unlikely to be related to SM-production. This would be in line with the findings of Boström et al. (1955a) and of Hall and Bošović (1969), who were unable to extract SM from this organ.

The submandibular gland has been shown to be essential for normal growth in many mammalian species (Narisimhan and Ganla, 1968). The possibility that this organ produces SM remains to be explored.

The liver has been the subject of many studies. Boström et al. (1953) found homogenates of normal rat livers to stimulate sulfate uptake in rat costal cartilage. A long search for the identity of this material however proved it to be glutamine (Boström et al., 1955b). Perfusion of normal rat liver and incubation of rat liver slices with bovine growth hormone stimulated release of a SM-like substance (McConaghey and Sledge, 1970; McConaghey, 1972). Rat liver slices, superfused with human growth hormone, also released SM-like activity (Hintz et al., 1972b). Fractionation of it on Sephadex G-75 revealed a major peak of activity with an apparent M.W. of 6,000 - 12,000 and a minor peak in the M.W. $<$ 2,000 range. Hall and Uthne (1971) found GH to stimulate the production of SM-like material during incubation with liver microsomes, which behaved like plasma SM on Sephadex G25.

Although the identity of the SM-like activity from liver and in plasma has not been established, the evidence strongly supports the possibility that SM is produced in the liver. In agreement with this, is the rapid fall of plasma SM-activity after partial hepatectomy, and its gradual return to normal during regeneration (Uthne and Uthne, 1972).

Muscle. Hall and Bošović (1969) extracted SM-like activity from rat muscle and Hall et al. (1970) found the characteristics of this material to be very similar to SM in plasma.

In conclusion, the data suggest the release of GH-dependent SM from the liver, muscle and perhaps the kidney. Further studies on its identity with plasma SM are necessary. It is uncertain at present if other organs are involved as well.

4. *Factors influencing plasma SM levels*

A detailed review of the subject will appear in the thesis by Du Caju. Only some important points will be mentioned here.

A. *Normal variation*

Normal plasma somatomedin increases with age in children. Chesley (1962) demonstrated

that SM in cord blood was lower than in maternal serum. Almqvist and Rune (1961) and Kogut et al. (1963) obtained data suggesting a progressive increase during early childhood. This has been confirmed by studies in our own laboratory (Du Caju and Van den Brande, 1973). We found increasing values until the age of six years. After a slight rise until puberty when adult values are reached, the plasma levels remain constant throughout life (Daughaday et al., 1959; Almqvist and Rune, 1961). In none of these studies sex differences have been apparent. Some diurnal variation, without consistent circadian rhythm was noticed by Daughaday et al. (1959).

B. *Hormonal influences*

The major importance of *growth hormone* for the induction of SM has long been recognized. In humans and in rats the time relationship between GH-administration and SM-generation have been studied in detail. Salmon and Daughaday (1951) demonstrated a significant increase of plasma SM 6 hrs. after intraperitoneal administration of bovine growth hormone to hypophysectomized rats. Hall (1971) found a rise of plasma SM three hours after intravenous injection of human growth hormone to hypopituitary patients.
Studies on the quantitative relationship between the dose of exogenous GH and plasma SM levels have been few. Almqvist (1960) obtained a dose related response in three hypopituitary children during short term HGH-administration. Daughaday and Parker (1963) administered HGH to ten short children in two different dosages and found a variable increase irrespective of the baseline levels in all but one.
We studied the dose-response relationship between exogenous HGH and plasma SM levels in children with short stature who had varying maximal plasma GH levels during stimulation tests (chapter 2, F) in an attempt to segregate normal from abnormal dose-response curves. The results indicate that such studies may be developed into a tool to study clinical conditions, where SM-generation is suspected to be impaired. An extreme example is represented by the patient described in chapter 2, G, who is similar to those first reported by Laron et al. (1966). Such patients are incapable of generating SM in spite of high endogenous GH (Daughaday et al., 1969; Laron et al., 1971). In our patient no abnormality of the GH-molecule could be demonstrated, nor immunologically, nor by electrofocusing.
The normal plasma SM levels in children with craniopharyngioma who are growing well in spite of low plasma GH, and in children with obesity, perhaps represent examples of the opposite situation (Du Caju and Van den Brande, 1973).
Other hormones have been investigated for their effect on plasma SM. *Thyroxine* stimulates sulfation in rats (Murphy et al., 1956), but the data in humans is still equivocal *Testosterone* is probably without effect (own observation, to be published). *Oestrogens* suppessed the SM-activity in plasma of acromegalics (Wiedeman and Swartz, 1971). *Insulin* seems of little influence since SM values are normal or only slightly suppressed in untreated diabetics (Chesley, 1962; Jensen et al., 1963; Yde, 1964).

C. *Nutritional status*

Some nutritional conditions have a profound influence on total SM-like activity in plasma. The low values found in malnutrition exemplify this (Van den Brande and Du Caju, 1972). Whether or not this is due to inhibitors as was demonstrated in the fasted rat by Salmon (1972), or to diminished SM-production remains to be examined.

D. *Other factors*

A series of other as yet undefined factors seem to influence plasma SM levels. This is illustrated by the low SM values which we found in morning samples of so-called dysmorphic dwarfs (children with minor or major congenital malformations and short stature), and surprisingly also in young children with cerebral gigantism, a syndrome recently identified by Sotos et al. (1964). The plasma SM lowering effect of *partial hepatectomy* has already been mentioned (Uthne and Uthne, 1971). Boström et al. (1971) described increased plasma SM after *repeated bleeding* of rats. The significance of this finding is still obscure. A curious observation is the accelerated growth of hypophysectomized rats infected with *Spirometra Mansonoides* (Mueller, 1968; Steelman et al., 1970). Garland et al. (1971) did experiments which suggest that this parasite releases an SM-inducer. Antibody formation to this inducer may be the cause of the short duration of the growth stimulation.
The many situations in which SM levels are not correlated with plasma GH levels suggest that other modulating factors besides GH control plasma SM levels. One of the still purely hypothetical possibilities is the existence of a negative feedback on SM-synthesis, operative in the absence of a metabolic effect in the SM-producing cell themselves. Many alternative mechanisms such as substances in blood influencing SM-synthesis or its effect in the bio-assay or changes in SM turnover could equally well be involved.

5. *Disappearance rate of plasma somatomedin*

Following hypophysectomy in the rat, somatomedin disappears from the plasma within 24 hrs. The half-life of disappearance was found to be approximately 3 to 4 hours (Daughaday et al., 1968). Almqvist and Falkheden (1961) reported on three patients in whom they followed plasma SM after hypophysectomy. They found half-life times of 9, 10.5 and 18 hours.

6. *Biological properties of somatomedin*

The capacity of somatomedin to stimulate chondroitin sulfate synthesis in cartilage is used as point of reference by all authors when searching for other biological effects. It should be emphasized that all such experiments have been using either native plasma or incompletely purified extracts. Moreover, as mentioned earlier, the GH-dependency of

many of these extracts has not been verified, which further limits the conclusions.
The extensive *in vitro* studies by Daughaday and Salmon and their collaborators have solidly established the stimulating effect of somatomedin on a series of specific functions of the *cartilage cell* (chondroitin sulfate synthesis, formation of protein polysaccharide complexes and conversion of proline into collagen hydroxyproline). They also established that RNA-synthesis was enhanced and demonstrated a wave of DNA-replication induced by somatomedin (Salmon and Daughaday, 1957; Salmon et al., 1968; Daughaday and Mainz, 1962; Daughaday and Reeder, 1966).
Salmon and Du Vall (1970 b) demonstrated that the activity of SM was not restricted to cartilage. They showed that partially purified fractions derived from acidified heated rat plasma stimulated protein synthesis in the diaphragms of hypophysectomized rats *in vitro* significantly more than did GH. Enhancement of transport of ^{14}C-α aminoisobutyric acid into muscle during *in vitro* incubation with acid ethanol extracts was observed by Hall and Uthne (1971).
Salmon and Hosse (1971) reported a stimulatory effect of their preparations on the growth of *HeLa cells.*
Fat pads also respond to preparations with SM-activity. Raben et al. (1971) demonstrated that there is a GH-dependent factor in serum which stimulates DNA-replication in this tissue. Hall and Uthne (1971) found acid-ethanol extracts, and preparations of higher purity derived from it, which had somatomedin activity, to stimulate ^{14}C-glucose conversion into $^{14}CO_2$. Similarly prepared fractions were found to cause inhibition of glycerol release from epinephrine-stimulated rat epidimymal fat pads by Underwood et al. (1972).
Only a few *in vivo* experiments have been reported. Hall and Uthne (1971) found a significant increase of the tibial epiphyseal width of hypophysectomized rats treated with a semipurified human SM-preparation for one week. Underwood et al., reported data suggesting a suppression of glycerol release in vitro from epididymal fat pads of hypophysectomized rats injected with partially purified human SM 4 hrs. before sacrifice.

7. *Relation between somatomedin and substances with similar biological effects*

The spectrum of biological effects of somatomedin is very similar to that of insulin. This was recognized early by Salmon et al. (1968), who did extensive comparative studies between these two substances. Since insulin and SM could not be identical as judged from the failure of insulin antibodies to prevent the effect of SM, Salmon and Du Vall (1970 b) wrote: 'One may suspect from these similarities that sulfation factor is identical with one or more plasma insulin-like factors defined in bioassay systems involving effects on muscle or adipose tissue.' Around the same time, Van Wyk et al. (1969) gave substance to this suggestion by showing that SM could be extracted from plasma by the same procedures as used for the isolation of NSILA-S (the soluble fraction of non suppressable insulin-like activity) (Burgi et al., 1966; Jakob et al., 1968; Poffenbarger et al., 1968; Van Wyk et al., 1969). Hall and Uthne (1971) carefully studied the ratio of NSILA-S to

SM-activity in the course of a series of isolation procedures and found it to be constant within the limits of the assay-techniques. The data thus far strongly suggest that both biological activities may reside in the same molecule.

Similar observations were made by Pierson and Temin (1972) with regards to a substance named MSA (mitotic stimulating agent), which is responsible for the effect of calf serum on the mitosis of fibroblasts in culture. They used extraction procedures very similar to those used for SM for isolating this substance. Again the question is raised about the possible identity of MSA and SM.

Since all these substances are insulin-like in their effects, Van Wyk and his collaborators (Hintz et al., 1972a) set out to study the binding characteristics of SM to different cell and membrane preparations. Their results suggest that SM competes with ^{125}I-insulin for the same binding sites on rat fat cells, chicken embryo chondrocytes and cell free membrane preparation from rat livers. The displacement curves obtained with insulin and SM were parallel with a ratio of 1 U SM (defined as the activity present in one ml of their normal adult plasma pool) to 50 μU of insulin for fat cells, and 1 to 200 for liver membranes. Chondrocytes behaved differently since the displacement curves with insulin and SM were dissimilar. SM was a much more effective competitor for binding sites than insulin.

In fat cells, the ratio between insulin and SM in terms of their capacity to displace ^{125}I-insulin corresponds well with the ratio of their biological effect as measured by glucose degradation. These data suggest that insulin and semi-purified SM, although similar in terms of the type of biological effects they can produce, derive their vastly discongruent profile from the greater or lesser affinity to the binding sites of different cell types.

NSILA-S and MSA, which may be identical to SM, but also other substances such as nerve growth factor, erythropoietin and epidermal growth factor, which elicit similar metabolic effects albeit on different tissues, may obtain their specificity by a similar mechanism.

With regards to insulin and SM, some evidence is available suggesting that they may exert their action through a common pathway, once binding has been established. It seems well documented now that insulin inhibits the stimulated activity of adenyl cyclase in purified membrane preparations from liver and from isolated fat cells (Cuatrecasas, 1972). The same appears now to have been demonstrated for somatomedin in the laboratories of Cuatrecasas and Van Wyk (Cuatrecasas and Tell, 1973). The inhibition by cycle 3'5' adenosine monophosphate and theophylline of the effect of normal rat serum on the ^{35}S-incorporation in chick embryo cartilage reported by Rendall et al. (1972) is compatible with Cuatrecasas' and Van Wyks' findings. It is difficult however to fit this in with the slight but significant stimulation of sulfation in the same tissue by dibutyryl adenosine 3'5' cyclic phosphate in the absence of SM reported early by Fujisawa (1964) and more recently by Adamson (1970) and Hall (1972).

The possible role of SM in glucose homeostasis and fat metabolism is uncertain. Much evidence has been presented that NSILA-S and SM may be identical. NSILA-S, although very active in terms of glucose and fat metabolism *in vitro* and *in vivo* after purification, seems to play little or no role in glucose homeostasis in the organism in its native

state (Oelz, Froesch et al., 1972). It seems very well possible that the true physiological importance of native SM, and possibly NSILA-S, under physiological conditions lies in its growth promoting effect and much less if at all in its insulin-like (i.e. primarily glucose and fat metabolism regulating) character. The latter may be uncovered only after realizing a direct contact with certain target organs *in vitro*. In addition, the effect may be further magnified by extraction, either by removing inhibitory substances or by dissociating it from its aggregated form or from a carrier. Comparison of native and purified SM with regards to their binding characteristics should resolve this issue. Somatomedin appears to be of fundamental importance and its anticipated availability in clinical practice holds great promise. However, only *in vivo* experimentation with this substance, both in its native and its purified form will provide definitive answers as to its role in physiology and pathology.

In this thesis our own and collaborative work on somatomedin is reported and related to the data from the literature. The *introduction* stresses the complexity of growth regulation and the important role which hormones play in it. Amongst them, growth hormone and the supposed mediator if its effect, somatomedin, take a central position.
The experiments which led to the discovery of 'sulfation factor' (somatomedin) by Salmon and Daughaday are described in *chapter one.*
Chapter two summarizes our studies on this substance. Attempts to characterize it (*section A*) led to the following conclusions. In native plasma it is macromolecular. Certain extraction procedures reduce its apparent size drastically, suggesting either disaggregation or release from a carrier. By electrofocusing a major peak of activity at pH 6.6 - 6.7 and a minor one at pH 5.2 were found. Separation of plasma into a fraction which stimulates sulfation and another stimulating DNA replication was never obtained. Somatomedin behaves like a peptide.
The distribution of ^{125}I-growth hormone shortly after intravenous administration to young rats, suggested the liver and possibly other organs as the source of somatomedin (*section B*).
Section C mentions the motivation for changing the name from sulfation factor to somatomedin.
A method for measuring somatomedin in vitro, using porcine rib cartilage is described in *section D.* It has the advantage of being highly precise and easy to perform. However, it is less sensitive than the hypophysectomized rat assay.
Section E reports on experiments designed to investigate possible species differences, similar to those described for growth hormone. Plasma from 7 mammalian species had a somatomedin-like effect on the cartilage of rat, pig, monkey and man, suggesting that the specificity of growth hormone exists at the level of somatomedin generation rather than in the specificity of the somatomedin produced.
Section F describes the dose-response relationship between exogenous growth hormone and plasma SM in two hypopituitary children. It also demonstrates the high degree of correlation between plasma SM and total urinary hydroxyproline excretion in these patients throughout the studies. In four children, considered to be either partially growth hormone deficient or 'normal delayed', varying responses were obtained, irrespective of the endogenous growth hormone secretion. Nevertheless, the overall correlation between SM and OH-proline remained fairly good.
A case of somatomedin deficiency is reported in *section G.* This patient has high plasma levels of endogenous immunoreactive growth hormone, which by electrofocusing and immunologically appears to be normal. Both during shortterm and chronic growth hormone administration a blunted response was found with respect to all indices of growth hormone action, including SM generation, suggesting an overall and specific

non-responsiveness to the hormone. It is thought that the deficiency of SM is the cause of his short stature.

In *Chapter three* the current knowledge of somatomedin is reviewed. It can be summarized as follows.

It seems well established now that somatomedin is the transmitter of the growth promoting effect of growth hormone. Its origin is probably the liver. Other organs such as muscle, kidney and possibly others are also suspected to produce it. It circulates in blood apparently in large molecular form. When purified under dissociating conditions it behaves like a peptide with a molecular weight between 4,000 and 8,000 daltons. In terms of its molecular characteristics it is very similar if not identical to NSILA-S. It has a longer half-life than growth hormone. Somatomedin appears to be less species specific than growth hormone.

Plasma levels can now be determined with a precision, sufficient for quantitative studies. They increase up to the age of six years and remain relatively constant thereafter. There are no sex differences. Plasma SM is low in hypopituitarism and its levels are restored by growth hormone treatment. There is a quantitative relationship between exogenous GH and plasma SM-levels on one hand and the endogenously produced SM and hydroxyproline excretion on the other. Children have been reported, presenting with the clinical picture of hyposomatotropism despite high immunoreactive plasma growth hormone levels. They have low SM-levels. It is thought that somatomedin deficiency is the cause of their short stature.

In other disorders of growth and nutrition, SM is sometimes unexpectedly low such as for example in dysmorphic dwarfism and surprisingly also in young children with cerebral gigantism. In obesity it is normal in spite of low plasma GH. In contrast, in malnutrition, where GH is normal or high, plasma SM is very low. The metabolic effects of SM, as inferred from *in vitro* experimentation with either native plasma or purified extracts is qualitatively similar to insulin: it stimulates protein and RNA synthesis, and DNA replication in muscle and cartilage. In cartilage it also stimulates sulfation of chondroitin, the synthesis of the protein moiety of the mucopolysaccharide-protein complex as well as the conversion of proline into collagen OH-proline. Semi-purified SM fractions stimulate glucose consumption in fat cells and suppress epinephrine induced lypolysis. Similar partially purified SM-preparations seem to compete with insulin for the same receptor sites on fat cells, chondrocytes and liver cell membranes. Both the biological effect of somatomedin on cartilage and its binding affinity to chondrocytes are much more important than its effects and binding to fat cells when compared to insulin. The physiological importance of somatomedin in carbohydrate and fat metabolism is uncertain. Its fundamental role in the control of growth seems much better established. Nevertheless, only *in vivo* experimentation can furnish the definitive proof.

SAMENVATTING

Dit proefschrift beschrijft ons onderzoek over somatomedine (SM) en vergelijkt de resultaten met de gegevens uit de literatuur. In de inleiding wordt de complexiteit van de regulering van groei belicht en de belangrijke rol welke hormonen hierin spelen. Groeihormoon en somatomedine, dat geacht wordt het groeistimulerende effekt van groeihormoon op de weefsels over te dragen, bezetten een centrale plaats.
De experimenten die geleid hebben tot de ontdekking van 'sulfation factor' (somatomedine) door Salmon and Daughaday worden in *hoofdstuk een* samengevat.
In *hoofdstuk twee* wordt het eigen onderzoek over somatomedine beschreven.
Pogingen om somatomedine te karakteriseren (*section A*), leidden tot de volgende conclusie. In plasma is het hoog moleculair. Sommige extractie procedures reduceren zijn moleculaire grootte in belangrijke mate. Dit maakt het waarschijnlijk dat SM in plasma in geaggregeerde vorm voorkomt of aan een dragermolecuul gehecht is. Electrofocusering splitste de aktiviteit in een grote fraktie met een pH van 6.6 - 6.7 en een kleinere met een pH van 5.2. Met geen enkele techniek kon plasma gescheiden worden in een fraktie die de sulfatering en een andere die de DNA-replikatie stimuleert. Somatomedine gedraagt zich als een peptide.
In *sectie B* wordt beschreven hoe de lokalisatie van ^{125}I-groeihormoon kort na intraveneuze toediening aan jonge ratten, de lever en mogelijke andere organen als potentiële bron van somatomedine aanwezen.
In *section C* wordt de motivering aangegeven die geleid heeft tot het veranderen van de naam 'sulfation factor' in somatomedine.
Onze methode voor het meten van somatomedine in vitro, die gebruik maakt van varkensribkraakbeen, is beschreven in *sectie D.* Deze methode is nauwkeurig en eenvoudig, maar minder gevoelig dan deze waarin het kraakbeen van hypophyseloze ratten wordt gebruikt.
Sectie E beschrijft experimenten die erop gericht waren een eventuele diersoort specificiteit, gelijkaardig aan deze van groeihormoon op te sporen. Plasma van zeven verschillende mammalia had een somatomedine-achtig effekt op het kraakbeen van de rat, het varken, de aap en de mens. Dit wijst erop dat de specificiteit van groeihormoon tot stand komt bij de inductie van somatomedine en niet in de interaktie van het geproduceerde SM met de weefsels.
Het 'dosis-response' verband tussen toegediend groeihormoon en plasma SM bij twee kinderen met hypophysaire insufficientie wordt beschreven in *sectie F.* Ongeacht de dosis groeihormoon bleek er een zeer goede korrelatie te bestaan tussen plasma SM en de uitscheiding van hydroxproline met de urine. Bij vier kinderen die hetzij als partieel groeihormoon deficient hetzij als 'normaal klein' geklassificeerd waren, werden veranderingen van het plasma SM waargenomen die niet korreleerden met de endogene groeihormoon sekretie. De korrelatie tussen SM en hydroxyproline was ook bij deze kinderen goed.
In *sectie G* wordt een te klein kind met somatomedine deficientie beschreven. Deze

jongen heeft een hoge plasma koncentratie van immunoreaktief groeihormoon, dat noch door electrofocuseren noch op immunologische grond van normaal groeihormoon kan worden onderscheiden. Langdurige behandeling met groeihormoon gaf nauwelijks enig effekt te zien. Dit gold een grote reeks indices, waaronder het plasma SM gehalte. Het gebrek aan groei van dit kind moet vermoedelijk aan zijn somatomedine deficientie worden toegeschreven.

Hoofdstuk drie geeft een overzicht van de huidige kennis over somatomedine, waaruit hieronder de belangrijkste aspekten worden gelicht.

Het blijkt nu vrijwel zeker dat somatomedine het groeistimulerende effekt van groeihormoon op de weefsels overdraagt. Het wordt waarschijnlijk in de lever gesynthetiseerd. Het is niet uitgesloten dat ook andere organen, zoals de nier en de spier deze stof zouden kunnen synthetiseren. Het circuleert in het bloed als een hoog moleculaire verbinding. Na isolatie onder dissocierende omstandigheden gedraagt het zich als een peptide met een moleculair gewicht tussen 4000 en 8000. De moleculaire eigenschappen gelijken sterk op die van NSILA-S (non suppressable insulin like activity – soluble fraction).

Somatomedine heeft een langere halfwaarde tijd in het bloed dan groeihormoon. Het is minder diersoortspecifiek dan groeihormoon.

Plasma gehaltes kunnen nu gemeten worden met voldoende nauwkeurigheid om kwantitatieve studies toe te laten. Er zijn aanzienlijke leeftijdsverschillen in het normale plasmagehalte aan somatomedine. Tot de leeftijd van zes jaar is er een stijging waarna een relatief konstant niveau behouden blijft. Er is geen verschil tussen beide sexen. Het plasma gehalte aan sematomedine is laag bij patienten met hypophysaire insufficientie en wordt tijdens behandeling met groeihormoon genormaliseerd. Er is een dosis afhankelijk verband tussen de hoeveelheid toegediend groeihormoon en plasma somatomedine bij groeihormoon deficiente kinderen. Bij deze patiënten is er een uitstekende korrelatie tussen plasma somatomedine en de totale hydroxyproline uitschreiding in de urine.

Bij sommige patiënten, die het klinische beeld van hyposomatotropisme vertonen, terwijl ze toch hoge plasma groeihormoon waarden hebben, werd een laag somatomedine gehalte gevonden. Het gebrek aan somatomedine is vermoedelijk de oorzaak van hun trage groei. In andere pathologische toestanden, zowel bij groei- als bij voedingsstoornissen zijn de somatomedine waarden soms afwijkend. Zo is het gehalte laag bij dysmorphische kinderen met korte gestalte, doch ook bij jonge kinderen met het zgn. cerebrale gigantisme. In obesitas, waar plasma groeihormoon laag is, is somatomedine normaal. Bij kinderen met ondervoeding is het laag, terwijl het plasma groeihormoon normaal of verhoogd is.

De metabole effekten van somatomedine werden tot op heden bijna uitsluitend in vitro bestudeerd met somatomedine bevattend plasma of na gedeeltelijke isolering ervan. De gevonden effekten zijn kwalitatief te vergelijken met die van insuline: het stimuleert eiwit- en RNA-synthese, alsmede DNA-replikatie in spier en kraakbeen. In kraakbeen stimuleert het de sulfatering van chondroitine, de vorming van de mucopolysaccharide--eiwit complexen en van collageen. Gedeeltelijk gezuiverde extrakten hebben eveneens effekt op het koolhydraat en vetmetabolisme: de glucose konsumptie van vetcellen wordt erdoor gestimuleerd en de lipolyse wordt geremd.

Eveneens met gebruikmaking van gezuiverde frakties is aangetoond dat somatomedine in

kompetitie is met insuline voor dezelfde bindingsplaatsen op de plasma membranen van vetcellen, chondrocyten en levercellen. In kwantitatief opzicht heeft somatomedine een veel grotere bindingsaffiniteit voor kraakbeen dan insuline. Hetzelfde geldt voor het biologische effekt van beide stoffen op dit weefsel. Ten aanzien van de vetcel liggen deze verhoudingen omgekeerd.
De fysiologische betekenis van somatomedine in de regulering van het koolhydraat- en vetmetabolisme is onzeker. Zijn fundamentele rol bij het reguleren van groeiprocessen is veel overtuigender aangetoond. Toch zijn in vivo experimenten nodig om het definitieve bewijs ervan te leveren.

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Degenen, die in de afgelopen jaren met mij hebben gedacht en gewerkt in kliniek en laboratorium en door hun inzet en tolerantie een harmonieuze en produktieve groep in stand hielden.
Aan allen mijn dank.

CURRICULUM VITAE

Na de Grieks-Latijnse humaniora te hebben doorlopen, studeerde de schrijver van dit proefschrift geneeskunde aan de Rijksuniversiteit te Gent. Tijdens deze studies was hij drie jaar student-assistent bij Prof. dr. L. Vanden Driessche (fysiologische scheikunde). Na het afleggen van het artsexamen in 1958 werkte hij gedurende een half jaar in de laboratoria van de afdeling kindergeneeskunde te Groningen (Prof. dr. J.H.P. Jonxis). Hij specialiseerde in de kindergeneeskunde van 1960 tot 1965 onder leiding van Prof. dr. C. Hooft aan de Rijksuniversiteit te Gent, en in kinderendocrinologie en metabolisme gedurende de drie daaropvolgende jaren bij Prof. dr. J.J. Van Wyk aan de University of North-Carolina, U.S.A. Sinds 1968 is hij wetenschappelijk hoofdmedewerker aan de afdeling kindergeneeskunde van de Erasmus Universiteit te Rotterdam.

ADDENDUM

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PARTIAL CHARACTERIZATION OF SULPHATION AND THYMIDINE FACTORS IN ACROMEGALIC PLASMA

By

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ABSTRACT

This study was undertaken to characterize further the growth hormone dependent plasma factors which stimulate sulphate uptake (PSF) and thymidine incorporation (PTF) in cartilage segments from hypophysectomized rats.

An assay system utilizing labelling with $^{35}SO_4$ and ^{3}H-methyl-thymidine was devised to measure both types of activity simultaneously and to determine whether these activities run in parallel during chemical fractionation procedures.

Acromegalic plasma stimulated sulphate and thymidine uptake as much as 18 × and 30 × respectively above the uptake of the basal medium. Activity measurements were not rigidly quantitative due to high variances, especially in PTF measurements.

Both activities in acromegalic plasma were thermo-labile at 80°C for 30 minutes. PSF was stable from pH 2 – pH 10. PTF was possibly partially inactivated at pH 2. Digestion with pronase destroyed PSF and probably PTF. Acromegalic plasma was fractionated by graded ethanol precipitation, ion exchange chromatography, starch gel electrophoresis, and molecular sieving through Sephadex (G-100, G-200). PSF and PTF activities run in parallel throughout these procedures. These findings

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suggest that the active material is associated with a peptide with a molecular weight between 9500 and 35 000, which is more positively charged than many other plasma proteins.

The incorporation of radioactive sulphate into the chondroitin sulphate of growing cartilage can be readily demonstrated either by administering $^{35}SO_4$ to the animal *in vivo* (*Dziewiatkowski et al.* 1949; *Dziewiatkowski* 1951) or by incubating pieces of cartilage *in vitro* (*Boström & Mansson* 1952) in a nutrient medium containing the isotope. *Ellis et al.* (1953) demonstrated that sulphate incorporation *in vivo* is at least partially growth hormone (GH) dependent, since it is reduced by hypophysectomy, and partially restored by the *in vivo* administration of GH. *Salmon & Daughaday* (1957) showed that the *in vitro* incorporation of $^{35}SO_4$ into rib cartilage from hypophysectomized (hypox) rats is stimulated by the presence in the medium of serum from normal rats or from hypophysectomized rats which had been treated with GH, but not by serum from untreated hypophysectomized rats. The addition of growth hormone to the medium in concentrations up to 50 μg/ml had strikingly little effect. The results of $^{35}SO_4$ uptake studies using plasma from acromegalics, normal human subjects, and from hypopituitary patients before and during GH-treatment have been similar to those obtained in the animal experiments (*Daughaday et al.* 1959; *Almqvist* 1960, 1961; *Almqvist et al.* 1961; *Almqvist & Falkheden* 1961).

Using similar techniques the incorporation of ^{3}H-methyl thymidine as well as the conversion of proline into hydroxyproline have likewise been shown to be partially GH dependent (*Daughaday & Mariz* 1962; *Daughaday & Reeder* 1966; *Murakawa & Raben* 1968). *In vitro* incubation of cartilage from hypox rats with normal serum increases hydroxyproline conversion and thymidine incorporation, whereas serum from hypox rats is either inactive or has an inhibitory effect. It has not been demonstrated, however, whether the thymidine incorporating activity (PTF) in the serum of hypox rats can be restored by the administration of growth hormone. Experimental evidence demonstrating the growth hormone dependence of PTF and thus completing the parallelism with plasma sulphate incorporating activity (PSF), has been collected by the present authors, and will be the subject of a separate report.

Synthesis of chondroitin sulphate and collagen are representative of the differentiated biosynthetic capacities of cartilage tissue, whereas the incorporation of thymidine into DNA is characteristic of cellular proliferation. There is reason to believe that growth is a reflection of both phases. Since these events do not occur synchronously within the cell cycle, it seemed of importance to ascertain whether the plasma factor which influences sulphate uptake by cartilage tissue (PSF) is the same as that which influences thymidine uptake (PTF).

An *in vitro* assay using a dual isotope labelling technique was developed to measure both PSF and PTF simultaneously. The assay was used to characterize both factors in acromegalic plasma, and to study their stability.

MATERIALS AND METHODS

The assay technique described by *Daughaday & Reeder* (1966) for ^{3}H-methyl-thymidine incorporation was modified by the addition of $^{35}SO_4$ to the incubation medium and by the revision of other minor procedural details.

Male Sprague-Dawley rats were hypophysectomized through the parapharyngeal route at the age of 25 days[1] and used 12–15 days later in the assay. On arrival in our laboratory, they were fed pulverized laboratory chow *ad libitum* and 5 % sucrose in their drinking water until sacrifice. The animals were killed with ether. Body and gonadal weights were then obtained. If either of these findings suggested incomplete hypophysectomy, the sella was examined under a dissecting microscope. Animals with pituitary remnants were discarded.

Using an aseptic technique the extraneous tissue was removed from the costal cartilage of the 3rd through the 9th ribs. One to three cartilage segments approximately 4 mm in length were obtained from each rib. Segments closer than 2 mm to the costochondral junction were discarded.

Each segment was incubated in a glass vial containing 0.6 ml of incubation medium and covered by loosely fitting metal or glass caps. Each plasma sample or fraction to be assayed was incubated in sextuplicate with one piece of cartilage from each of six rats. Cartilage segments from the same animal were distributed at random among the samples to be assayed. The concentration of plasma or fraction in the medium was at most 16.7 % (v/v).

The incubation medium differed from that used by *Daughaday & Reeder* (1966) only by the addition of 100 μg/ml of kanamycin. The medium was prepared from frozen stock solutions on the day of the assay and filtered through a 0.45 μ Millipore filter before.

The incubation was carried out in air in a Dubnoff metabolic shaking incubator at 37°C and at 60 r. p. m. After 24 h, 1 μCi $Na_2{}^{35}SO_4$[2] and/or ^{3}H-methyl-thymidine[2] in 50 μl of incubation medium was added to each vial, and the incubation was continued for another 24 h.

At the end of the incubation the pieces of cartilage were placed between sheets of filter paper in individual plastic tissue washing capsules and immersed in boiling water for 3–5 min. They were then washed for 4–6 h in running tap water. Control incubations with heat killed cartilage revealed that this procedure reduced non-incorporated radioactivity to negligible values.

The washed pieces of cartilage were air dried for at least 16 h and weighed on a Cahn electro-balance to the nearest microgram. It was found that the weight became constant after a few hours of exposure to ambient air. The standard error among

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[1] Hormone Assay Laboratories, Chicago, Illinois.

[2] ^{3}H-methyl-thymidine with a specific activity of $>$ 10 Ci/mM was obtained from Schwarz Bio Research Inc. Carrier free $Na_2{}^{35}SO_4$ was obtained from Cambridge Nuclear Corp.

replicates was less when the radioactive uptake was related to the dry weight than when related to the weight of protein in the cartilage.

The cartilage segments were digested in liquid scintillation vials containing 0.6 ml of 90 % formic acid. The vials were covered with glass marbles and placed in a boiling water bath for 30 to 40 min with periodic vigorous shaking. Some small threadlike particles were still present at the end of the digestion. More prolonged digestion, however, resulted in a loss of ^{3}H, possibly by exchange and evaporation.

The liquid scintillation cocktail had the following composition: Toluene/triton X-100 (3/1, v/v), 0.6 g/100 ml PPO, and 0.02 g/100 ml dimethyl-POPOP. To increase counting stability, 4 g/100 ml thixotrophic gel (Cab-O-Sil) was added. Radioactivity of the sample plus 15 ml of cocktail was measured in a Packard liquid scintillation counter. Correction for quenching was made by the sequential addition of internal standards and recounting. ^{14}C-Benzoic acid was used to correct for quenching of ^{35}S and ^{3}H-palmitic acid for the quenching correction of ^{3}H.

Reference standard and starting material for the fractionation

Heparinized or citrated plasma from two adult patients with active acromegaly was used for the standard curves and as the starting material for further fractionation. The activity of PSF and PTF in unknown samples was computed from log dose response curves of the reference plasma or a semi-purified derivative of this run simultaneously. The term plasma equivalents (pl. eq.) indicates the original volume of plasma in ml from which a given sample is derived. Protein concentrations were measured by the Lowry method (*Lowry et al.* 1951).

Fractionation of acromegalic plasma

Fractionation procedures were carried out at 0–4°C and the specimens to be assayed were stored at –20°C.

(a) *Ethanol precipitation at neutral pH.* Ethanol was added with vigorous shaking over a 2 min period to whole acromegalic plasma until a 20 % (v/v) concentration was reached. After standing overnight and centrifugation, an aliquot of the supernatant was raised to a 40 % (v/v) concentration. Finally, the ethanol concentration of an aliquot of the 40 % supernatant was raised to 80 % (v/v). The biological activity remaining in the respective supernatant fractions was assayed in aliquots which had been lyophilized and re-dissolved in water to the plasma volume from which they were originally derived.

(b) *Adsorption to DEAE and CM cellulose.* The 20 % ethanol supernatant was further fractionated with pre-swollen microgranular dietylaminoethylcellulose (Whatman DE-52) and carboxymethyl cellulose (Whatman CM-52). The cellulose was equilibrated by washing five times with 10 volumes of the selected buffer. In batch experiments, 1.0 pl. eq. of the sample was intermittently shaken with 1 g of absorbant for 1 h. After centrifugation, the supernatant was filtered over glasswool and lyophilized. The non-adsorbed material remaining in the supernatant after exposure to DEAE cellulose equilibrated with 0.01 M ammonium bicarbonate buffer pH 8.5 is referred to hereafter as »DE-material«.

For gradient elution column chromatography on DEAE, the sample was applied in 0.01 M ammonium bicarbonate buffer pH 8.5. The material retained on the column was then eluted with ammonium acetate buffer in a pH gradient from pH 6.55 to 5.0 and an ionic gradient from 0.01 to 0.1 M.

(c) *Molecular sieving.* Both acromegalic plasma and »DE-material« were fractionated

on Sephadex G-100 and G-200. The Sephadex was thoroughly washed with demineralized water (at least 10 times the bed volume). This abolished the inhibitory effect of concentrated eluates on the assay. The eluant was 10 times diluted Krebs' phosphosaline buffer pH 7.4. 2 ml fractions were collected, pooled into portions according to the elution pattern (O. D. read at 280 nm), lyophilized, and re-dissolved in demineralized water. They were then dialyzed twice for 2 h against 250 volumes of a Krebs phosphosaline buffer.

(d) *Starch gel electrophoresis.* The technique was carried out as described by *Smithies* (1959). Hydrolyzed starch (Connought Laboratories) was used at a concentration of 10.3 %. The electrode buffer was NaOH 0.06 M, H_3BO_3 0.3 M, pH 8.5. The gel buffer was NaOH 0.0104 M, H_3BO_3 0.026 M, pH 8.4. The origin was placed in the middle of the plate. One lateral slot was filled with human serum stained with bromphenol blue. The remaining slots were filled with a total of 2.0 pl. eq. of »DE-material« and electrophoresed at 4°C for 5 h (300 V with 25 mA).

After electrophoresis, the lateral mårgins of the plate were cut, stained with amidoblack, and used as a guide for the division of the remainder of the plate. The gel was frozen, thawed, and cut transversely in 9 zones. The fluid was expressed from each zone by compression in a syringe stoppered with a glasswool plug. The fluid was then lyophilized, redissolved in a small volume of water, dialyzed, and assayed at a concentration of 0.21 % (plasma equivalent in medium).

Stability of the biological activity

(a) *Exposure to heat.* 1.0 pl. eq. aliquots of DE-material in 1.5 ml of a Krebs phosphosaline buffer were exposed for 30 min in a shaking waterbath to temperatures of 40, 50, 60, 70, or 80°C. The incubation was terminated by abruptly cooling the samples in ice. 0.1 ml aliquots of the supernatants were assayed after centrifugation.

(b) *Exposure to varying pH.* Under constant pH and volume monitoring, 1.0 pl. eq. aliquots of »DE-material« in 3 ml of water were slowly titrated at 4°C to pH 2, 4, 6, 8, or 10 by the addition of 0.02 N NaOH or 0.02 N HCl. Once the desired pH was reached, the sample was allowed to stand for 6 h at 4°C. The pH was then slowly readjusted to 7.4. The samples were lyophilized and redissolved in 1.5 ml with water and 0.1 ml of this solution was added to each incubation flask in the assay.

(c) *Enzymatic hydrolysis.* To 0.5 pl. eq. of the 20 % ethanol supernatant, 0.5 ml of a solution containing 3 mg/ml Pronase[3]) was added. The sample was sequentially incubated for 24 h at 37°C, heated for 30 min at 70°C, cooled in ice, and centrifuged. To remove the Pronase the supernatant was adsorbed to DEAE-cellulose by the batch method described above. Two parallel controls were run. Control one (C_1) was incubated without Pronase to monitor losses not due to enzymatic action. In control two (C_2) the biological activity was added immediately before the assay to check the effectiveness of Pronase removal.

RESULTS

The bioassay

The standard log dose response curves for thymidine and sulphate incorporation in four consecutive assays are shown in Fig. 1. In these assays, which were

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[3]) Pronase: Streptomyces Griseus protease, B grade, lot 34045, Calbiochem, Los Angeles, 45000 P. U. K. unit/g.

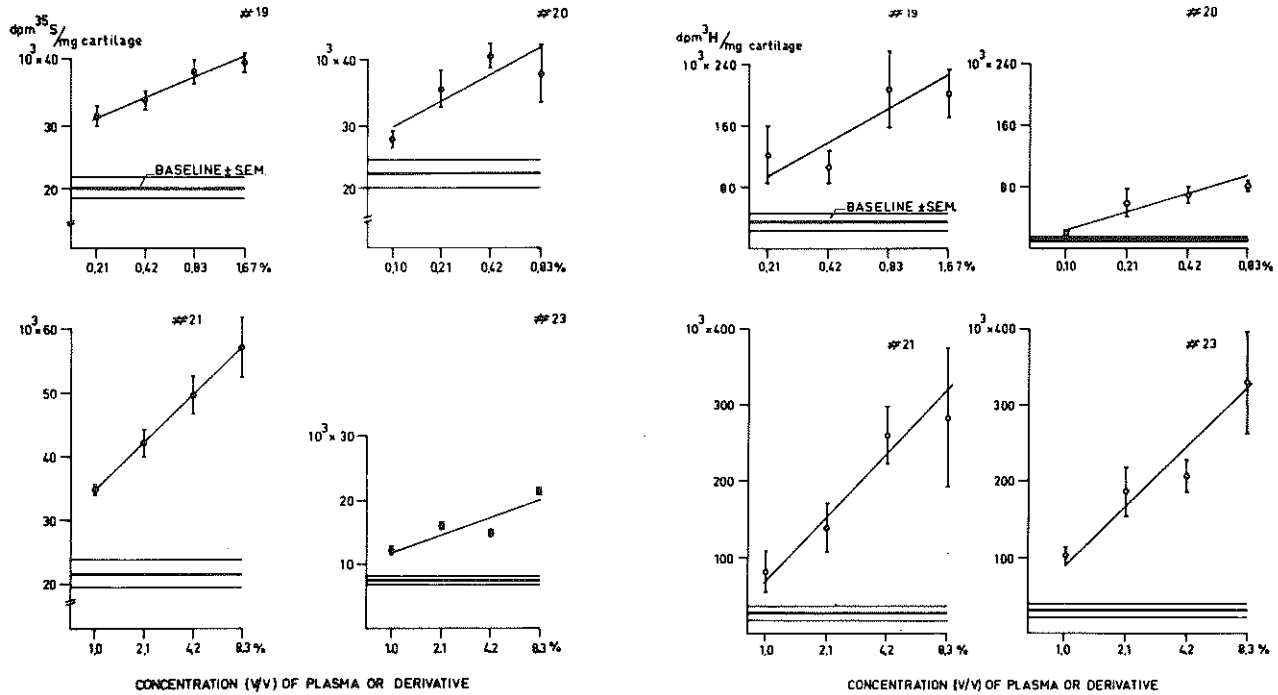


Fig. 1.

Dose-response curves of *in vitro* $^{35}SO_4$- and ^{3}H-thymidine incorporation into hypox rat costal cartilage.

Assay No. 19–20: Plasma from a patient with active acromegaly.

Assay No. 21–23: Same plasma after adsorption to DEAE-cellulose.

Doses are expressed as concentration (v/v) in the initial incubation medium.

Crossbars indicate SEM.

Values for λ No. 19 ^{35}S: 0.36, ^{3}H: 0.56.
No. 20 ^{35}S: 0.70, ^{3}H: 0.51.
No. 21 ^{35}S: 0.29, ^{3}H: 0.49.
No. 23 ^{35}S: 0.19, ^{3}H: 0.34.

typical of many others, the lambda values for sulphate uptake ranged between 0.19 and 0.70 and for thymidine uptake between 0.34 and 0.56. There were also unexplained deviations from linearity. Although rigorous attempts were made to standardize the assay procedure the variances between replicates remained distressingly large. This was particularly true for ^{3}H-thymidine incorporation, although the greater steepness of the slopes partially compensated for the larger variances. Since lambda values greater than 0.4 preclude precise quantitation, the present results should be considered as semi-quantitative estimations of potency (*Emmens* 1962).

Two way analyses of variance in numerous assays revealed that for both PTF and PSF the variance not accounted for by preparation differences was in general divided about equally between the variance between rats and the residual variance due to all other factors. An analysis of factors which might possibly contribute to the intercartilage variation was therefore carried out by multiple regression analysis of an experiment in which 2 dose levels of »DE-material« were assayed against medium. Five cartilage segments from each of 6 rats were incubated with each dose level of DE-material. The variables tested were the differences between rats, rib number, location of cartilage segment within the rib, length/weight ratio, curvature, completeness of cleaning extraneous tissue, smoothness of the cartilage surface (reflecting trauma to the perichondrial membrane), and completeness of digestion in formic acid. These variables were graded by arbitrary codes.

The factors contributing significantly to the intercartilage variance are in parallel for $^{35}SO_4$ and ^{3}H-thymidine incorporation (Table 1). Apart from the variance due to differences between the preparations, the result was also influenced by the length/weight ratio, the smoothness of the surface and the curvature of the cartilage fragment. The possible meaning of these findings will be discussed below.

Fractionation of acromegalic plasma

(a) *Ethanol precipitation at neutral pH (Fig. 2).* Starting with the 20 % ethanol concentration, a progressive loss of both PSF and PTF activity occurred in the supernatant fractions with increasing ethanol concentration. No biological activity was detectable in the supernatant fractions after precipitation with 80 % ethanol.

(b) *Adsorption to ion exchangers.* In a preliminary experiment using only $^{35}SO_4$ uptake as a measure of activity, whole plasma was exposed in batch to CM 52 and DE 52 at different conditions of pH and ionic strength. After centrifugation, the degree of adsorption was determined by comparing the biological activity remaining in the supernatant fluid with that in the original plasma. Biological activity was recovered in the supernatant fraction from DE-52 only with ammonium bicarbonate 0.01 M, pH 8.5 (Table 2).

Table 1.
Multiple regression analysis of factors contributing to the intercartilage variation.

	Code	$^{35}SO_4$	^{3}H-Thymidine
Length:weight ratio		7.63	1.59
Smoothness of the surface	1	1.65	0.44
of the cartilage fragment	2	3.28	0.87
(damage to perichondrial membrane)	3	4.92	1.31
Curvature	1	–0.91	–0.37
	2	–1.82	–0.73
	3	–2.73	–1.10
Incubation medium with			
no addition		–2.62	–0.80
0.5 % human plasma (FP)		–0.19	–0.16
2.0 % „ „		2.82	0.96
Mean		4.60	2.07
Intercept		–4.10	0.38

Data are expressed as log DPM/mg cartilage.
length/weight: Mean: 3.47.
Damage: code 1 to 3 corresponds to increasing raggedness of the surface of the tissue.
Curvature: The fragments of cartilage most curved after drying obtained the highest code.

The low total protein concentration remaining in this fraction suggests that the biologically active material is positively charged relative to many other plasma proteins. This interpretation is consistent with the findings after adsorption to carboxymethyl cellulose at pH 5.0. Under these conditions most or all of the biological activity was adsorbed, whereas there was relatively less adsorption of other plasma proteins.

These findings were further explored by applying a 20 % ethanol supernatant preparation to a DE 52 column and eluting with 0.01 M ammonium bicarbonate buffer at pH 8.5 (Fig. 3). A large amount of biological activity, both PSF and PTF, was recovered with the single protein peak eluted. Another DEAE column eluted with a decreasing pH and an increasing ionic gradient showed that most, if not all, of the biological activity was present in the first protein peak (Fig. 4). Attempts to recover the biological activity adsorbed on CM-52 were unsuccessful.

(c) *Molecular sieving.* From preliminary experiments utilizing Sephadex

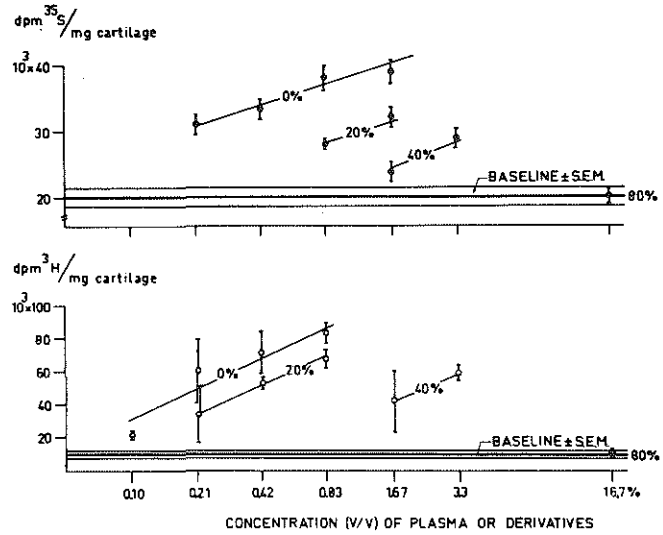


Fig. 2.

Ethanol precipitation of human plasma. Biological activity of the supernatants at 20, 40, and 80 % ethanol concentration.

Experimental details: see material and methods.

Protein concentrations of the fractions:

Starting plasma:	62.6	mg/ml pl. eq.
20 % ethanol-supernatant:	48.0	,,
40 % ,,	38.0	,,
80 % ,,	0.16	,,

G-100 the biological activity in whole plasma was shown to be associated with the macromolecular fraction. After fractionation of »DE-material« on a large column of Sephadex G-200 (Fig. 5) biological activity was recovered in a fraction with K_{av}-values between 0.58 and 0.85. The total protein content of this fraction was 0.62 mg as compared to 21.6 mg of the sample.

(d) *Vertical starch gel electrophoresis.* PSF and PTF were recovered from both the fractions comprising the origin and the adjacent anodal area (Fig. 6). In addition some PSF-activity was also present in the fraction closest to the anode.

Stability of the biological activity

(a) *Thermal and pH stability.* Fig. 7 illustrates that all biological activity was abolished after exposure to 80°C. Exposure to a pH range from 2 to 10 did not affect the PSF activity, whereas PTF was diminished by exposure to a pH of 2 (Fig. 8).

(b) *Stability to enzymatic proteolysis.* Many attempts to perform enzymatic

Table 2.

Recovery of PSF activity from acromegalic plasma after batch adsorption by DEAE cellulose pH 8.5, 0.01 M and CM-celluose pH 5.0, 0.01 M. The residual biological activity remaining in the respective supernatant fractions after adsorption was assayed at a concentration of 16.7 (pl. eq.) %.

	10^3 DPM ^{35}S/mg ± SEM	protein concentration in incubation medium mg/ml
Incubation medium	16 ± 7	0
Plasma (McBr) 1.67 %	48 ± 12	1.2
,, 3.3 %	65 ± 16	2.4
,, 6.7 %	106 ± 28	4.8
Conditions of adsorption:		
CM-52 NH_4Ac. 0.01 M. pH 5	18 ± 4	6.7
DE-52 NH_4HCO_3 0.01 M, pH 8.5	75 ± 16	0.7

proteolysis failed because of the difficulty in separating the enzyme from the biological activity. Although both PTF and PSF activities were regularly lost after exposure to the enzyme, activity was retained in both controls only when the previously described method was used for removing the Pronase from the active fraction before assay. The results conclusively demonstrate that PSF activity is destroyed by enzymatic proteolysis (Fig. 9). This is probably also true for PTF activity, but the high variance encountered in one of the controls makes this conclusion less certain.

DISCUSSION

An assay system which permits the simultaneous measurement of sulphation and thymidine incorporating activities, affords many advantages in comparing the influence of substances affecting skeletal growth. The present technique for measuring sulphation factor differs from previously described systems in that a 24 h period of pre-incubation preceded the addition of $^{35}SO_4$. This pre-incubation is required for assessing stimulation of thymidine incorporation (*Daughaday & Reeder* 1966) but may adversely affect the precision in measuring sulphation factor activity.

The great variability between costal cartilage segments is for the most part intrinsic to the use of this tissue as the test object. Metabolic activity at the costochondral end is much higher than elsewhere (*Yde* 1968). This can be circumvented by discarding the distal portions. When the remainder of the

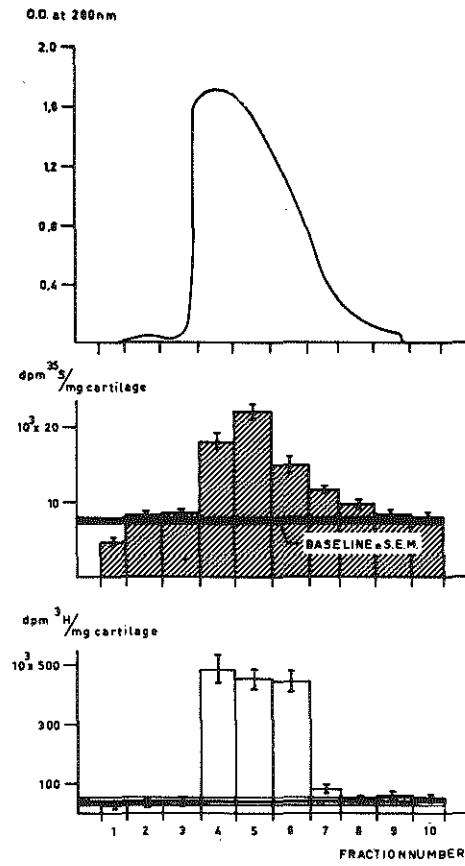


Fig. 3.

Chromatography on DEAE-cellulose. Elution with NH_4HCO_3 0.01 M at pH 8.5. Starting material: 2 pl. eq. of human plasma derivate (20 % ethanol supernatant). Column specifications:

dimensions : 23.5 × 0.6 cm
flow rate : 3.18 ml/h
fraction volume: 1.9 ml

Assay: without further processing 5.3 % of total fraction volume per incubation flask.

rib was divided, we found no systematic variation between the lateral and medial segments. However, the striking influence of the length to weight ratio (Table 1) reflects a lack of uniformity in response between thick and thin segments. Radioautography of cartilage segments after incubation reveals that cell division occurs primarily beneath the perichondrium and in the peripheral zones rather than in the inner shaft. The responses should therefore more appropriately be related to surface area (were this measurable) than to weight.

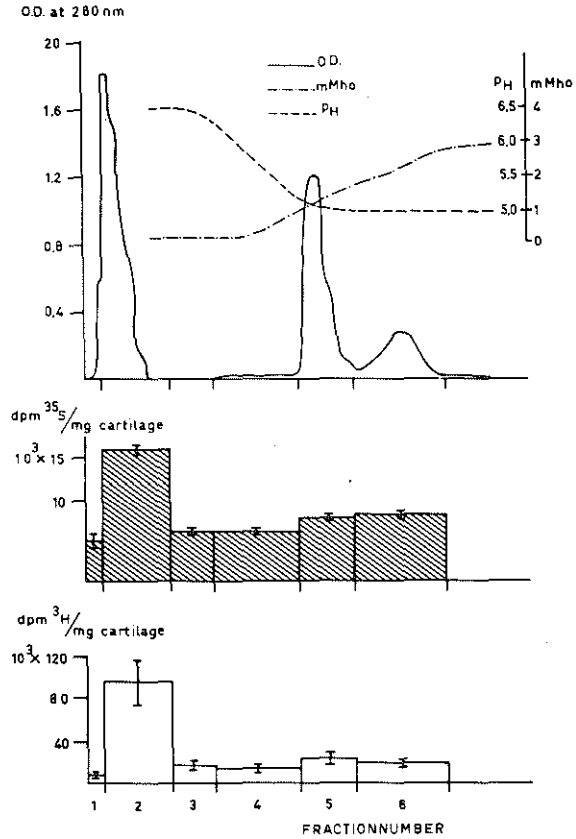


Fig. 4.

Gradient elution chromatography on DEAE-cellulose.

Starting material: 2 pl. eq. of human plasma derivative (20 % ethanol supernatant).

Column specifications:

dimensions : 18.7 × 0.6 cm

flow rate : 3.4 ml/h

fraction volume: 0.67 ml, pooled according to elution pattern.

Assay: Pooled fractions were concentrated on UM-3 ultrafilter (Diaflow) and washed twice with 5 volumes of demineralized water.

Amount assayed: Fractions 1, 4, 5, and 6: 5 %, fraction 2: 2 %, and fraction 3: 10 % of the total pooled fraction volume per incubation flask.

Fraction 1 is the basal uptake with medium alone.

The influence of curvature is less well explained. The effect of raggedness of the surface of the cartilage tissue might be considered as an attempt towards repair by the release of tissue »chalones« (*Bullough* 1967). Although the net effect of these influences precludes strict quantitation of assay results, the activities observed were sufficiently large to obtain useful information.

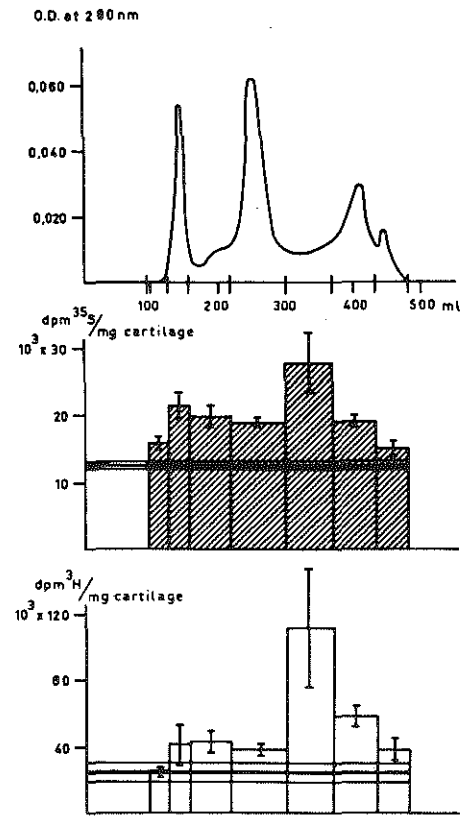


Fig. 5.

Molecular sieving on Sephadex G-200.

Starting material: 3 pl. eq. of human plasma derivate (»DE-material«).
Column dimensions: Vt: 410 ml, Vo: 144 ml
Flow rate : 11.5 ml/h, reversed flow.
Eluent : 1/10 Krebs-phosphosaline-buffer.
Fractions of 2.3 ml were pooled according to elution pattern.
Assay: 1/10 of each pooled fraction per incubation flask.

Progress in isolating and characterizing the GH dependent plasma factors which stimulate cartilage growth has been slow. *Koumans & Daughaday* (1963) demonstrated PSF activity in the macromolecular fraction eluted from Sephadex G-25, although it was not established whether the active material was a peptide. *Ferguson et al.* (1968) subjected plasma from acromegalic subjects to gel filtration and starch gel electrophoresis and found »a number of acidic proteins with growth promoting activity«.

The present studies were undertaken to define further the chemical nature

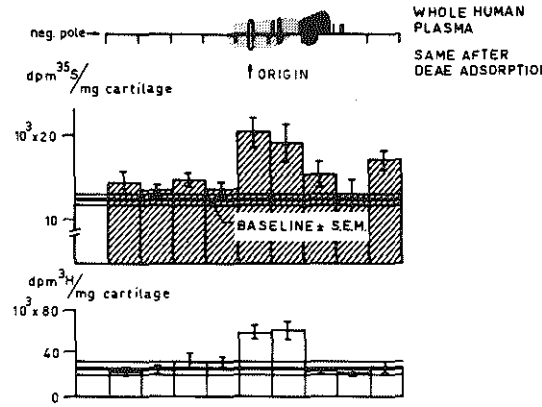


Fig. 6.
Starch gel electrophoresis of »DE-material«.
Experimental details: see text.

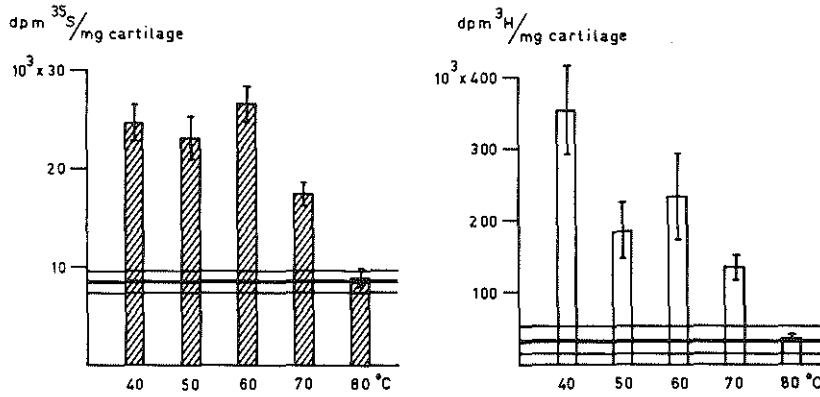


Fig. 7.
Heat stability of the biological activity.
Starting material: human plasma after adsorption to DEAE-cellulose.
Experimental details: see text.

of the active substances, using as starting material plasma from patients with active acromegaly. Incubation of active fractions with Pronase destroyed PSF activity and probably PTF activity, thus suggesting that a molecule with peptide linkage is necessary for the biological effect. The thermal inactivation curve is consistent with this conclusion.

The lack of binding to DEAE-cellulose at pH 8.5 and at low buffer ionic

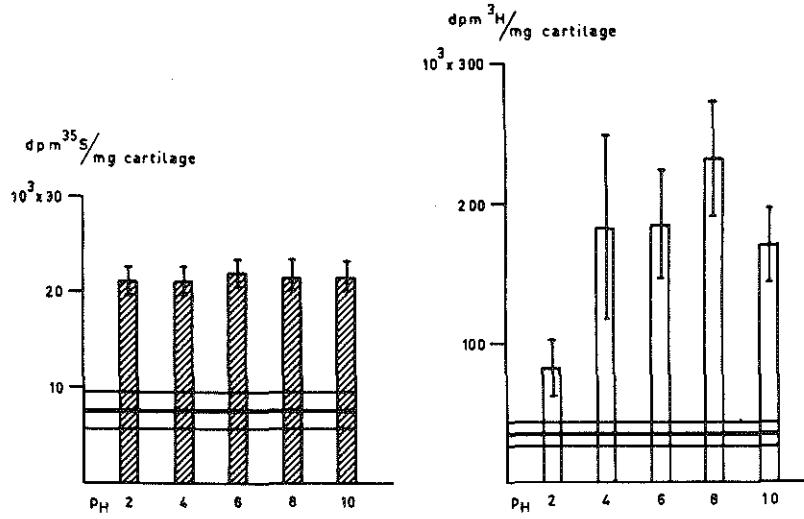
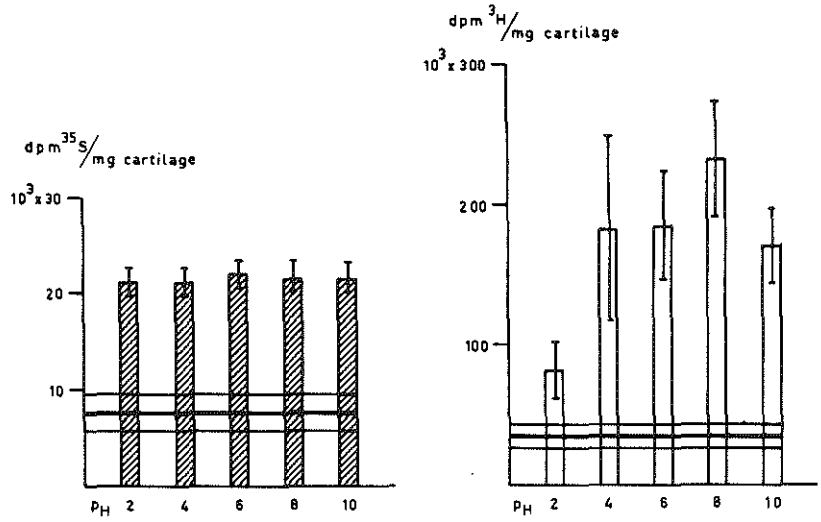


Fig. 8.
pH-stability of the biological activity.
Starting material: human plasma after adsorption of DEAE-cellulose.
Experimental details: see text.

strength and the minimal movement in starch gel toward the anode at pH 8.4, suggest that the active molecule is slightly basic relative to other plasma proteins. This conclusion is consistent with the somewhat selective adsorption of PSF to CMC cellulose at pH 5.0.

The progressive loss of biological activity with increasing concentration of ethanol at neutral pH suggests that the active material is of large molecular weight. Chromatography on Sephadex G-200 suggests a molecular weight in excess of 9500 and less than 35 000 (*Andrews* 1966), although the fractions were not cut finely enough to allow a more precise estimation. The experiments reported here do not reveal whether the active material itself has a large molecular weight or whether it is a smaller molecule bound to a protein carrier.

PSF and PTF run in parallel throughout the procedures except with regard to their stability at pH 2.0. However, subsequent studies have revealed that both PSF and PTF can be extracted from acromegalic plasma by acid ethanol extraction (*Van Wyk et al.* 1969). Whether or not these activities represent the same or different molecular species must await further steps in purification. Such studies are in progress in our laboratories. Progress in the isolation and characterization of these materials, however, will remain slow until a simpler and more accurate assay system is developed.



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Further Purification and Characterization of Sulfation Factor and Thymidine Factor from Acromegalic Plasma

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ABSTRACT. The chemical characteristics of sulfation factor (PSF) and thymidine factor (PTF) have been studied in whole acromegalic plasma and in acid ethanol (AE) extracts of plasma. In whole plasma PSF and PTF are excluded by the XM-100 ultrafiltration membrane and behave as large proteins. After extraction with acid ethanol the mol wt appeared to be $>3900 \leqq 12{,}400$, suggesting that in native plasma PSF and PTF are either aggregated or bound to a larger carrier protein. Two species of peptides with PSF and PTF activity were found. Chromatography of whole plasma on DEAE-cellulose revealed a major peak of biologic activity which was not adsorbed at pH 8.5 and a smaller component which was adsorbed at pH 8.5 but eluted at pH 5.5. Electrofocusing of AE extracts revealed a sharp minor peak at pH 5.2 and a broader major peak at pH 6.6–6.7. The possibility that the more acidic component may be due to insulin cannot be excluded. HV electrophoresis of a purified AE extract revealed activity only in the neutral zone. Chromatography of an AE extract on CMC-cellulose at pH 5.6 in a linear ionic gradient between .01 and 0.40M resulted in the recovery of PSF and PTF in a small protein peak well separated from the bulk of other proteins. Rechromatography of the CMC eluate on Sephadex G-50 yielded a preparation with an increase in specific activity over native plasma of $6200\times$ for PSF and $15{,}000\times$ for PTF. This discrepancy probably reflects the removal of inhibitors having a greater effect on thymidine incorporation, since no separation of PSF from PTF was observed in any system. If a provisional molecular weight of 8000 is assumed and no allowance is made for residual contaminants, the purest preparation is highly active at 3.1×10^{-8} molar concentration.

PREVIOUS studies have suggested that the plasma sulfation factor (PSF) and plasma thymidine factor (PTF) in human plasma from normal or acromegalic subjects are either proteins or bound to a protein carrier (1–4). Similar inferences have been made for the sulfation factor in normal beef and rat plasma, and in plasma from hypophysectomized rats after treatment with growth hormone (5, 6). In previous publications we have reported on some characteristics of this substance (or substances) in acromegalic plasma and have demonstrated that PSF and PTF can be extracted in good yield with an acid ethanol reagent (4, 3). The present paper provides further details of the physical characteristics of this material in native plasma and in acid ethanol extracts as revealed by ion exchange chromatography, gel chromatography, ultrafiltration, high voltage paper electrophoresis and isoelectric focusing. By using a combination of these techniques, two components of PSF and PTF activity have been demonstrated and the major component partially purified.

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Materials and Methods

Blood was removed from fasting acromegalic patients, the plasma separated and stored at −20 C until use. A pool of plasma from several untreated acromegalic patients was heated in a Dubnoff shaker at 56 C for 30 min, stored for 15 hr at 4 C, and then centrifuged at 2500 rpm. This procedure did not alter the biologic activity and preserved the linearity of responses at higher dosage levels. The supernatant was lyophilized, except for small aliquots which were stored at −20 C for use as reference standards in the biologic assay. In the subsequent purification procedures and biologic assays the term "plasma equivalents" (pl.eq.) is used to indicate the original volume of plasma in ml from which each preparation was derived. Dosage in the assays is expressed as pl.eq. %; *e.g.*, 75 pl.eq. % means that an aliquot ultimately derived from 0.75 ml of original plasma was represented in 1.0 ml of incubation medium.

Acid ethanol extraction. The lyophilized plasma was suspended and homogenized at 4 C in 1/4 to 1/6 of the original plasma volume of 0.1M NH_4 acetate (pH 6.8). The homogenates were vigorously shaken for 30 min at 4 C with 3 vol of acid ethanol reagent (48 ml ethanol:1 ml concentrated HCl) and then centrifuged at 10,000 $\times g$ for 30 min (7). The precipitate was resuspended once in NH_4 acetate, re-extracted with the acid ethanol reagent, washed twice in water, and re-extracted with acid ethanol. The residual precipitate was discarded.

The supernatants were combined, brought to pH 8.0–8.4 with NaOH and filtered on a Büchner funnel through Whatman #1 filter paper. The filtrate was cooled to −10 C and added in a fine stream with stirring to 4 vol of prechilled acetone-ethanol mixture (5:3 v/v). After 12–24 hr at −10 C the precipitate was collected by aspirating most of the supernatant and centrifuging the remainder. The clear yellow supernatant was discarded. The white precipitate was resuspended in a small amount of cold acetone-ethanol (5:3 v/v), and then centrifuged at 4000 $\times g$. The liquid phase was decanted and the precipitate was air dried. Thereafter the precipitate was dissolved in .05M NH_4HCO_3, pH 8.5. The insoluble material remaining after centrifugation was washed twice more with NH_4HCO_3. The supernatants were combined and the insoluble material discarded.

Ultrafiltration. Sixty ml of frozen plasma was thawed, filtered through a Millipore filter, and added to the chamber of a model 52 ultrafiltration cell (Amicon Corp., Lexington, Mass.) containing a UM 05 membrane. When the volume was reduced to about 1/3, the original volume was restored with glass distilled water and the filtration repeated. This process was repeated 4 times. The total filtrate was lyophilized and the retentate removed by repeated washing and then passed successively in similar fashion over XM-50 and XM-100 filters. Aliquots for assay were removed at each step. Other studies were performed in a similar fashion starting with XM-100 filter and passing the respective filtrates through successively smaller pore filters. All operations were carried out at 5 C using nitrogen as the pressure source.

Gel chromatography. Ascending gel chromatography was performed at +4 C through columns of Sephadex G-100, Sephadex G-50 and Sephadex G-25 (fine) equilibrated with 0.01–0.06M NH_4HCO_3 (pH 8.5). The columns were calibrated with dextran blue (2×10^6 av. mol wt), bovine serum albumin (69,000 mol wt), egg albumin (45,000 mol wt), chymotrypsinogen (25,000 mol wt), cytochrome C (12,400 mol wt), B chain of insulin (3900 mol wt) and NaCl (located by conductivity measurements). The plasma fractions and marker samples were applied in NH_4HCO_3 (.01–.05M) and eluted with the same solution. After determining the UV absorbance, the individual fractions were pooled into larger fractions and lyophilized prior to assay.

Anion exchange chromatography. A chromatographic column 20 $\times$2 cm was packed with preswollen microgranular diethylamino cellulose (Whatman DE-52) which had been equilibrated with 0.01M NH_4HCO_3, pH 8.3. To this was applied 10 ml of plasma. The column was developed by stepwise elution with 0.01M NH_4HCO_3, pH 8.53, 0.05M NH_4 acetate, pH 6.2, and 0.1M NH_4 acetate, pH 5.0.

Cation exhange chromatography

Dowex 50. Chromatography on Dowex 50 WX2 (200–400 mesh)H^+ was carried out on a column with bed volume of 50 ml at a flow rate of 2 ml/min. The resin was washed with 0.06M acetic acid, pH 3, until the pH of the effluent was equal to that applied. Samples were dissolved in $\frac{1}{2}$ bed volume of 0.6N acetic acid. Elution was carried out successively with 3 bed volumes of .06N acetic acid, demineralized water, 0.15M NaCl and .05M NH_4OH. This was followed by 9 column volumes of 0.2M NH_4OH.

Carboxymethyl cellulose. For chromatography on carboxymethyl cellulose, a column measur-

ing 1.2×13 cm was packed with pre-swollen microgranular carboxymethyl cellulose (CM-52) which had been equilibrated and extensively washed with 0.01M NH_4 acetate, pH 5.6. The sample to be applied was diluted in NH_4 acetate, pH 5.6, and further diluted with water until the conductivity matched that of .01M NH_4 acetate (0.60 mho). The total volume was pumped through the column and the column then washed with an equal volume of .01M NH_4 acetate, pH 5.6. The column was then eluted with 500 ml NH_4 acetate, pH 5.6, in a linear molar gradient between .01 and 0.40 molar.

Electrofocusing. An LKB 8102 ampholyne column (440 ml) was used. LKB ampholyte with a pH range from 5 to 8 was used at a final concentration of 1%. Sucrose gradients were made either by hand or with an LKB gradient mixer. The 2 solutions for the gradient were composed as follows: A) 100 g sucrose, 3.75 ml 40% ampholyte pH 5–8, and 150 ml water; B) 1.25 ml 40% ampholyte and 215 ml water. Acid ethanol extracts, 8 pl. eq. in one experiment and 20 pl.eq. in another, were proportioned between the 2 gradient solutions. The anode buffer consisted of 48 g sucrose in 56.0 ml 0.33N H_2SO_4 and the cathode buffer consisted of 40 ml of 2.5N NaOH. Approximately 300 V was applied until the current was stable for 12 hr. The total duration of equilibration was 94 hr. The column was drained at a rate of 12 ml/hr into a fraction collector and the optical density read at 280 mμ. The pH was monitored in every fifth fraction. The fractions were stored at −20 C and then diluted in incubation medium for biological assay. A control column without added biologic material was run in identical fashion.

High voltage electrophoresis. The lyophilized sample was dissolved in a minimum amount of acetate buffer, pH 6.5, and applied as a band to a sheet of Whatman 3 MM paper with about 1.0 mg of dried sample/cm. High voltage electrophoresis was then performed in pyridine acetate buffer, pH 6.5, at 60 V/cm for 50 min by the procedure of Ryle *et al.* (8) in a modified apparatus of Michl (9). A guide strip of the dried paper was cut and stained with ninhydrin-cadmium acetate. Ninhydrin positive bands were cut from the paper, eluted by 0.05N NH_4OH, and lyophilized. An aliquot was saved for assay and the remainder applied to a second strip for electrophoresis at pH 2.0.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out in glass tubes (6 mm i.d.) at 5 C according to the method of Davis (10) in gel concentrations of 7.5% pH 8.9. The proteins were fixed in 7% acetic acid and stained with amido black or fixed in 12½% trichloroacetic acid and stained with Coomassie blue.

Protein determination. Protein measurements were made by one or more of the following methods: Lowry's modification of the Folin Ciocalteau method (11), ninhydrin determination after alkaline hydrolysis (12), and by the difference in absorbance at 215 and 225 mμ (13).

Studies with [^{131}I] insulin and [^{131}I] HGH. Human growth hormone (HGH) and insulin were iodinated with ^{131}I according to the method of Hunter and Greenwood (14) and purified by vertical starch gel electrophoresis (growth hormone) or Sephadex G-75 (insulin). The specific activities were approximately 540 mCi/mg (HGH) and 435 mCi/mg (insulin). Purity was checked by chromatoelectrophoresis in the presence and absence of antibody excess.

Approximately 1×10^6 cpm of each of the labeled hormones was added to separate aliquots of 20 ml of normal human plasma after the plasma had been lyophilized and resuspended in 0.1N NH_4 acetate. Acid ethanol extracts were prepared which were then passed over Dowex 50 H^+ and eluted as described above. The recovery of radioactivity was determined by counting aliquots in the well of a gamma spectrometer.

Immunoassay of insulin and growth hormone. The growth hormone and insulin content of starting plasma and certain plasma fractions were determined by radioimmunoassay by a modification of the double antibody technique (15).[1]

Bioassay procedure. Biologic activity was determined *in vitro* by 3 different systems. Most preparations were assayed by a system using costal cartilage segments from hypophysectomized male rats and dual labeling with $^{35}SO_4^=$ and [^{3}H-methyl] thymidine to assess simultaneously the incorporation of sulfate and thymidine. This method is a modification of the technique described by Daughaday and Reeder (16) and has been previously described (4). Some preparations were assayed for sulfation factor activity by the embryonic chick pelvic leaflet method described by Hall (17). The fractions derived from the electrofocusing column were assayed for PSF and PTF activities by a method using

[1] Human growth hormone was Wilhelmi HS 968 C furnished by the National Pituitary Agency. Purified pork insulin assaying at 25.9 mU/mg, Lot 818194, was a gift of the Eli Lilly Co.

ostal cartilage segments from a single normal prepubertal rhesus monkey (to be published). This assay technique is approximately 1/10 as sensitive as the assay utilizing cartilage from hypophysectomized rats but is advantageous in terms of simplicity.

In scanning a large number of fractions for biologic activity, results are expressed as % increase in uptake of the respective isotopes above the basal uptake by cartilage segments when incubated in medium alone. Since the slope and sensitivity varied from assay to assay and was different for thymidine and sulfate uptake, such results provide inadequate estimates of potencies. Quantitative estimates of recovery and potency, where given, were determined on an IBM 1130 computer by a program which applies tests of validity and assesses potency ratios and 95% fiducial limits from symmetrical four- or six-point bioassay designs (18, 19).[2] Results were discarded if deviations from parallelism or linearity were significant ($p > .05$). The precision of the assays expressed as λ varied between 0.20 and 0.75.

Bioassay of PSF and PTF with anti-insulin and anti-human growth hormone antibodies. To determine whether any portion of the biologic activity assayed as PSF and PTF could be accounted for by growth hormone or insulin, an acid ethanol extract of acromegalic plasma was assayed at 3 dosage levels in the presence of both anti-HGH and anti-insulin antisera, respectively. The antisera were each present in a concentration of 1:5000.[3]

[2] This computer program accepts sample weights, specimen dosages and raw counting data from perforated paper tape. Efficiency corrections are made by either the internal or external standard method. The calculated dpm/mg for both isotopes is stored in a magnetic disc to be recalled later for comparisons between any 2 preparations in the same assay. Variances are determined by 2-way and 1-way analyses of variance in the rat and chick embryo assays, respectively. In 4-point assays contrasts between preparations are assessed for significant differences between activities in the assay and significant deviations from parallelism. In 6-point assays, significant deviations from linearity and significant opposite curvature are also assessed. The statistical treatment of results is based on formulae for orthogonal coordinates described by Finney (18) and Borth (19). This program is available on request (J.J.V.W.).

[3] In a 1:1,000,000 dilution, the HGH antisera bound 50% of growth hormone when present in a concentration of 0.2 ng/ml. In a dilution of 1:85,000, the insulin antiserum bound 50% of the insulin when the latter was present in a concentration of 5 μU/ml. These determinations of antibody potency were carried out by incubating in veronal buffer at pH 8.6 for 6 days at 5 C, whereas the assays for PTF and PSF in the presence of the respective antisera were carried out for 48 hr at 37 C in Krebs phosphosaline buffer, pH 7.4.

Results

I. *Properties of PSF and PTF in native acromegalic plasma*

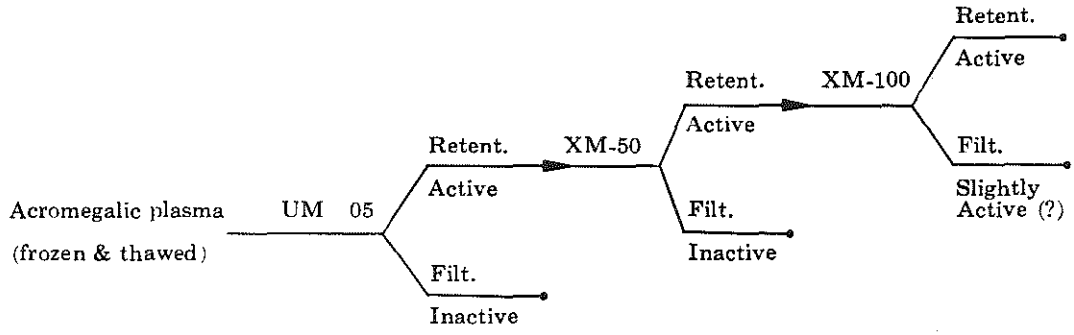
Ultrafiltration. When native acromegalic plasma was passed successively through the UM-05 and XM-50 filters, the biologic activity was quantitatively recovered in the respective retentates, whereas the filtrates were totally inactive (Table 1). When the XM-50 retentate was passed through the XM-100 membrane, most of the PSF and PTF activity was again found in the retentate. Stimulation of thymidine incorporation by the filtrate was not significant, although the filtrate was possibly slightly active in stimulating sulfate incorporation ($p = .05$). Similar results were obtained with single passage of whole plasma through filters of differing porosity.

According to the manufacturer (Amicon Publication #403), 95% of bovine serum albumin (mol wt 67,000) in dilute solution is fully retained by the XM-50 membrane, whereas only 45% of this substance is retained by the XM-100 membrane. It is possible, however, that in the presence of whole plasma the effective pore size may have been reduced below these putative limits.

Chromatography on Sephadex. When acromegalic plasma was applied to Sephadex G-25, 62% of the PSF activity was recovered in the peak of excluded protein (Fig. 1). Succeeding fractions caused slight stimulation of sulfate uptake in the chick assay but in the rat assay no activity was discernible after the major protein peak. In similar studies with more precise quantitation, the recovery of both PSF and PTF in the major protein peak regularly exceeded 75% of the activity in native plasma.

Anion exchange chromatography. In accordance with previous findings (4), when

TABLE 1. Ultrafiltration of acromegalic plasma



Preparation	Concentration % (pl. eq.) in medium	dpm/mg % of Uptake in Medium (±SEM)	
		Thymidine	Sulfate
Medium	—	100 ± 13	100 ± 7
Plasma (K.W.)	0.75	464 ± 53	223 ±11
	1.50	508 ± 56	277 ±18
	3.00	574 ± 43	264 ±10
XM-100 Retentate	1.5	495 ± 65	232 ±13
	3.0	561 ±103	251 ±10
XM-100 Filtrate	3.0	115 ± 34	123 ± 9
	12.0	111 ± 21	136 ± 7

whole plasma was applied to DEAE-cellulose in .01M NH_4HCO_3, pH 8.5, most of the activity recovered appeared in the fall-through volume (43% for PTF and 12% for PSF) (Fig. 2). Unlike the earlier study, however, a small but significant amount of biologic activity was present in two adjacent fractions eluted with ammonium acetate 0.1M, pH 5.0. The recovery of PTF in these fractions was 1.5 and 3.1%. For PSF the respective recovery figures were 2.1 and 1.1%. The material eluted at acidic pH has not been further studied. In similar DEAE columns insulin has been quantitatively recovered in this region.

II. *Properties of PSF and PTF in the acid ethanol extract.* Acid ethanol extraction of lyophilized acromegalic plasma has now been performed many times with recoveries of both PSF and PTF activities in the range of 30–40%. The reason for this loss of activity is not clear, but it may be due to physical trapping or protein:protein interactions in the bulky precipitate. Dose response curves for acid ethanol extract parallel those of whole acromegalic plasma (Fig. 3). The protein content (11) of these extracts has varied between 0.1 and 0.3 mg per plasma equivalent. The biologic activity per mg of protein has varied between 60 and 225 times greater than in native plasma.

Chromatography of acid ethanol extract on Sephadex G-100. When an acid ethanol extract containing 100 pl.eq. was applied to a column of Sephadex G-100, both PSF and PTF activities were recovered in the fractions preceding the elution volume for NaCl and well separated from the bulk of proteins in the ethanolic extract (Fig. 4). The peak was recovered at a molecular weight corresponding to that of cytochrome C (mol wt 12,400), but some biologic activity was also eluted in the lower molecular weight fractions which followed. By summing the biologic potencies in the

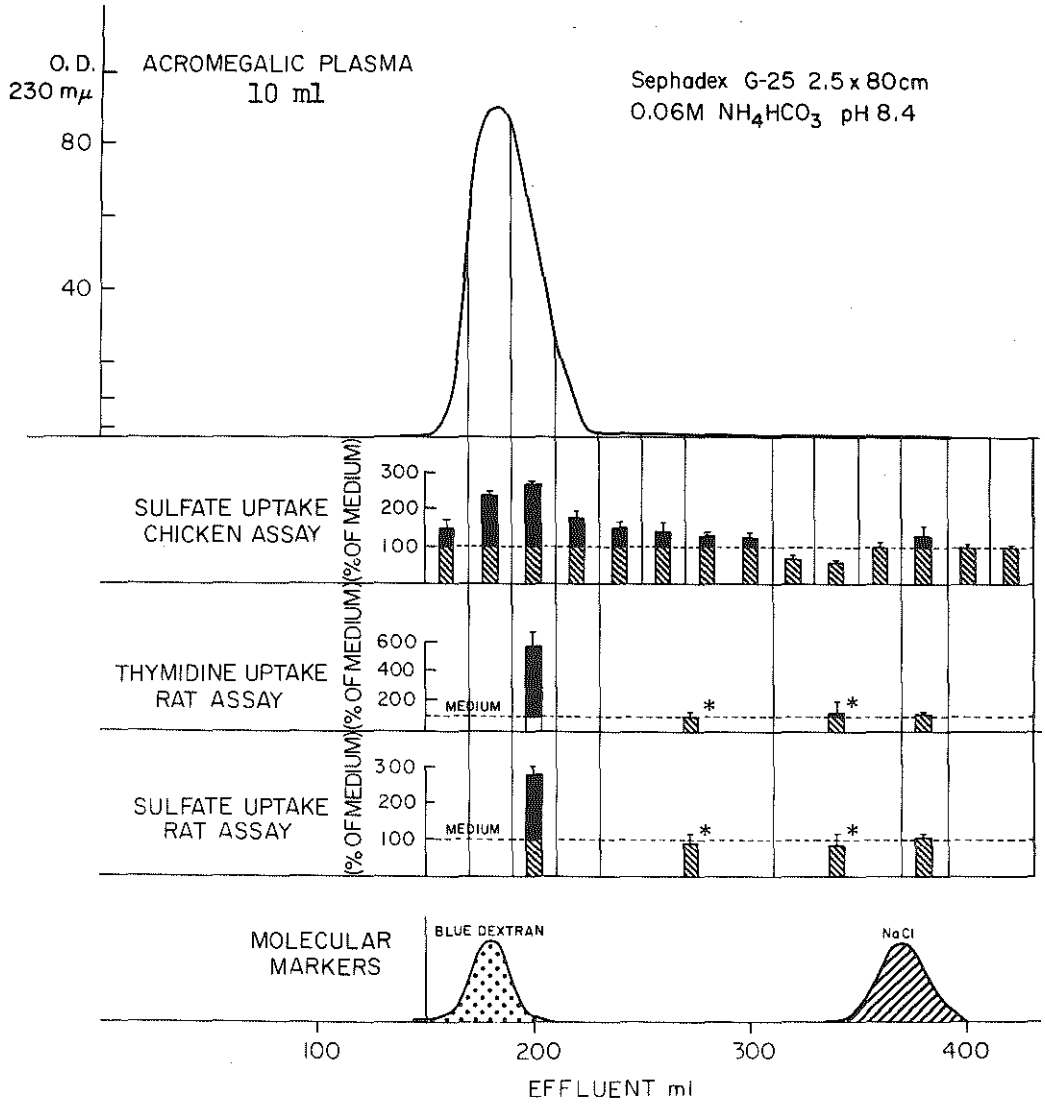


FIG. 1. Ascending gel chromatography on Sephadex G-25 in 0.06M NH_4HCO_3, pH 8.4. Ten ml acromegalic plasma was applied to the column (2.5 × 80 cm). Absorbance was measured at 230 mμ. Before biological assay, individual fractions were combined into pools as indicated by vertical lines. The unstarred fractions were obtained from the same column and assayed at a concentration of 12% (0.12 pl.eq./ml). The starred fractions were obtained from another column and assayed at a concentration of 6%. Bars represent mean uptake of $^{35}SO_4^{=}$ and [^{3}H-methyl]thymidine expressed as percentages relative to the mean uptake of cartilages incubated in basal medium alone. The mean and SEM were derived from 6 replicate determinations.

active fractions, over-all recovery values from native plasma were estimated as 21% for PTF and 11% for PSF. Accurate protein measurements and a more precise bioassay of the combined active fractions could not be carried out since some of the protein became insoluble after the second lyophilization.

Cation exchange chromatography on carboxymethyl cellulose. Five hundred pl.eq. of a pool of active fractions derived from three acromegalic donors was applied to a

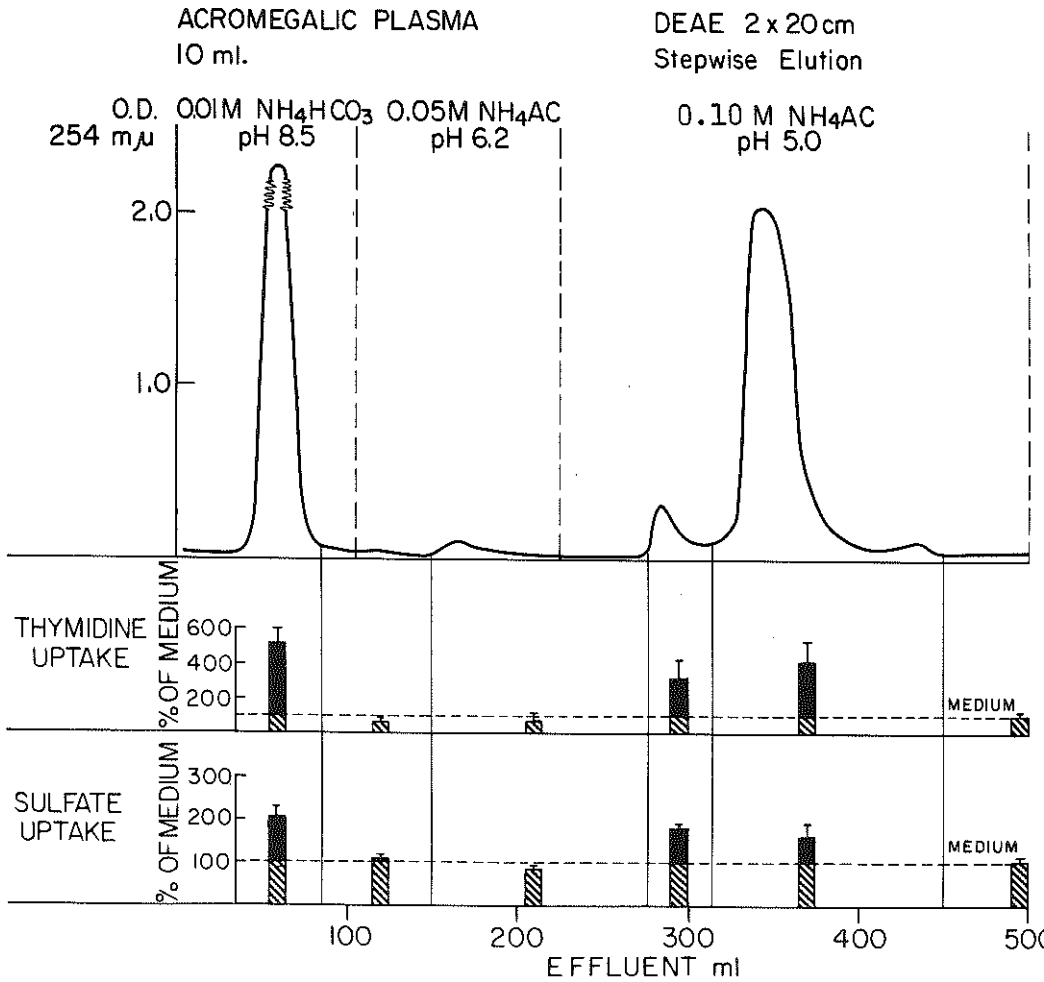


FIG. 2. Chromatography on DEAE-cellulose. Ten ml acromegalic plasma was applied to a column (2 × 20 cm) in 0.01M NH_4HCO_3, pH 8.5, and thereafter eluted stepwise as shown with 0.01M NH_4HCO_3, pH 8.5, 0.05M NH_4 acetate, pH 6.2, and 0.1M NH_4 acetate, pH 5.0. Fractions of 4 ml were collected. After measuring the absorbance at 254 mμ the fractions were combined into pools as indicated by vertical lines. The conventions described under Fig. 1 were used in plotting data. The uptakes shown for the first peak were obtained at 2% concentration (pl.eq. in medium), whereas those in the second peak were obtained at a 16% concentration.

CMC column. Some of these fractions were crude acid ethanol extracts, whereas others had been further purified on Dowex 50 and Sephadex G-100. After being dissolved in NH_4 acetate, pH 5.6, and diluted with water until the conductivity matched that of .01M NH_4 acetate (0.60 mho), the total volume was 420 ml. This volume was pumped through the column and the column then washed with another 420 ml of NH_4 acetate, pH 5.6. The column was further developed in a linear ionic gradient between .01 and 0.40 molar. Under these conditions, most of the proteinaceous material passed through the column without adsorption and was biologically inactive. Biologic activity was recovered only in a small protein peak which was eluted between .025 and .15M concentrations (Fig. 5). The two most active fractions together contained 10 μg protein/pl.eq. (11, 13) and accounted for 57% of the PTF ac-

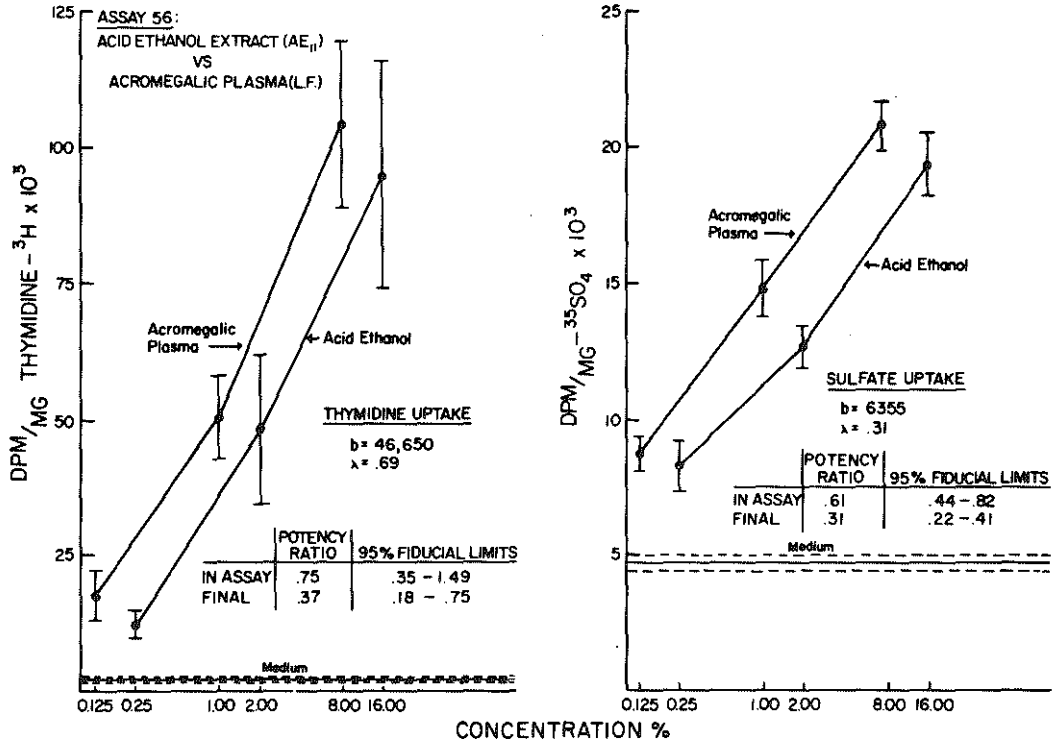


FIG. 3. Dose response curves obtained with an acid ethanol extract compared with the acromegalic plasma from which it was derived. Uptakes of $^{35}SO_4^=$ and [^{3}H-methyl]thymidine are expressed in dpm/mg dry cartilage. Each point represents the mean of 6 cartilages (1 from each of 6 rats) and the brackets indicate ±SEM. Potency ratio "In Assay" refers to relative responses in the assay irrespective of dose. "Final" potency ratios reflect the relative activities of the 2 preparations adjusted for the dosages used in the assay.

tivity applied to the column. The over-all recovery of PTF in these fractions, when assayed against acromegalic reference plasma, was about 10%. The assays for PSF were roughly similar, but did not meet statistical criteria. Since the pool applied to the column was composed in part of acid ethanol extracts which had previously been purified by other methods, the sources of loss cannot be accurately identified. Adsorption to glass surfaces and incomplete recovery from chromatographic columns are common occurrences in peptide fractionation, however, and may account for important losses during PSF and PTF purification.

The electrophoretic pattern of the active CMC fractions in acrylamide gel was contrasted with that of starting plasma and an acid ethanol extract (Fig. 6, C). A major peptide band migrating with the front and at least three fainter bands of more retarded protein are visible in the CMC preparation. Larger quantities of protein would undoubtedly have revealed even greater heterogeneity.

Chromatography of CMC fractions on Sephadex G-50. The three fractions with biologic activity from the carboxymethyl cellulose column were pooled, lyophilized, and an aliquot equivalent to 175 pl.eq. was applied to a column of Sephadex G-50. The elution pattern of proteins disclosed considerable heterogeneity (Fig. 7). The biologic activity was eluted between the cytochrome C marker (mol wt 12,400) and the insulin B chain marker (mol wt 3900). The protein content of the most active

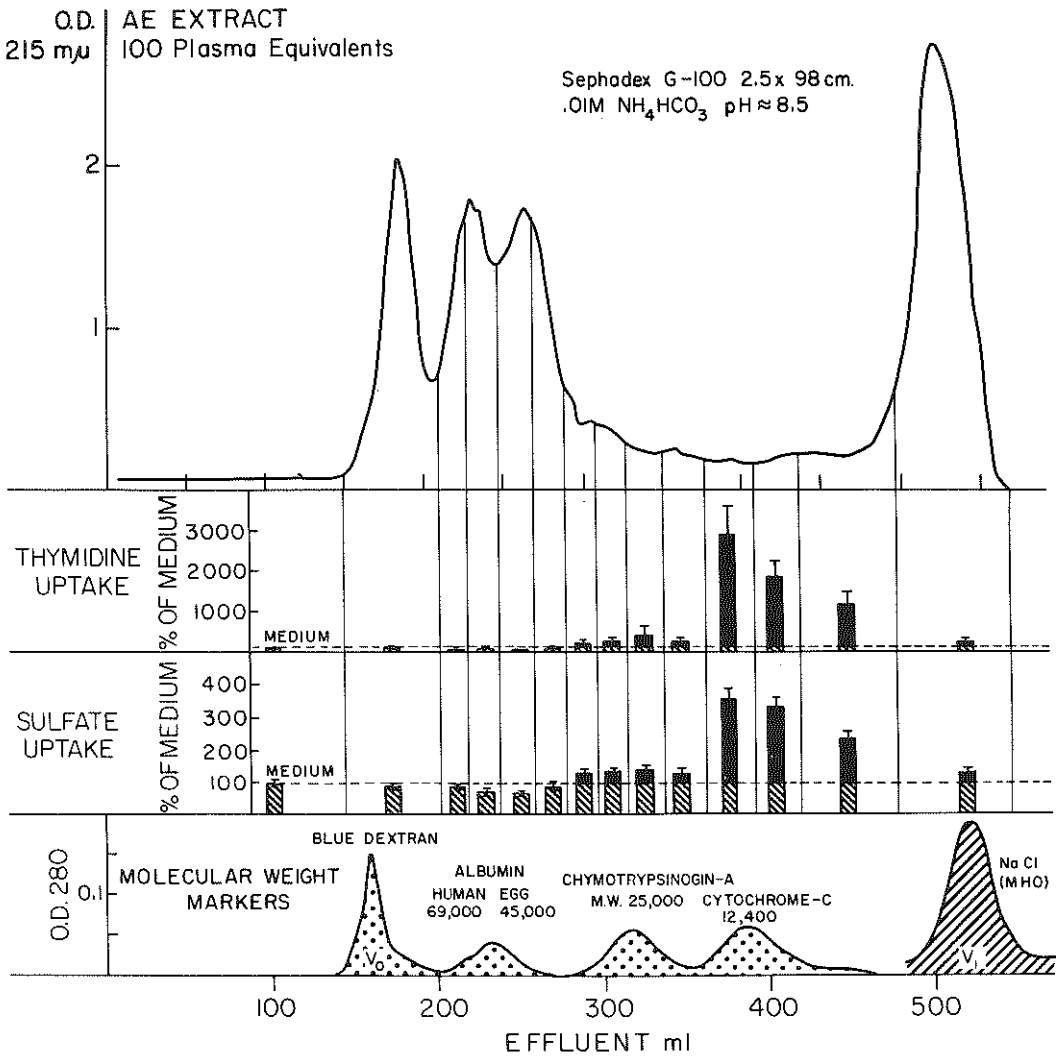


FIG. 4. Ascending gel chromatography on Sephadex G-100 in 0.01M NH_4HCO_3, pH 8.5. One hundred pl. eq. of acid ethanol extract in a volume of 5 ml was applied to the column (2.5 × 98 cm). Fractions of 4.8 ml were collected at a flow rate of 30 ml/hr. After measuring the absorbance at 215 mμ the fractions were pooled for assay as indicated by the vertical lines. The conventions used in plotting data are given under Fig. 1.

fraction was 0.26 μg/pl.eq. (12, 13). This fraction contained 5.3% of the original PTF activity in native plasma and 2.2% of the original PSF activity, yielding an over-all purification of about 15,000× for PTF and 6200× for PSF.

Cation exchange chromatography on Dowex 50 H^+. Studies of the chromatographic behavior of AE extracts on Dowex 50 were undertaken because this resin had been successfully used to separate insulin from nonsuppressible insulin-like activity in plasma (20, 21). Using the procedure described in Materials and Methods, no biologic activity was recovered until 1½ column volumes of 0.2N NH_4OH had passed through the column. The biologic activity was then eluted in a broad band with no separation from insulin and very little separation from other proteins. Although

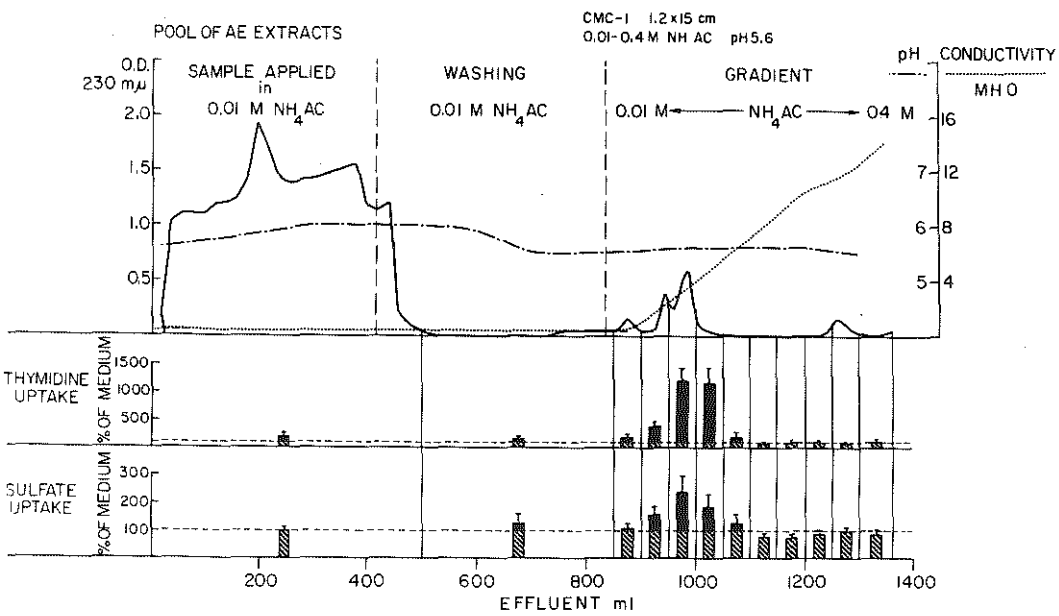


FIG. 5. Chromatography on CMC-cellulose. Five hundred pl.eq. of acid ethanol extract was applied to the column (1.2 × 13 cm) in 0.01M NH_4 acetate, pH 5.6, and washed extensively with the same buffer. Thereafter, the column was eluted in a linear gradient between 0.01 and 0.4M NH_4 acetate, pH 5.6. Fractions of 10 ml were collected at a flow rate of 40 ml/hr and absorbancies were measured at 215 mμ. The fractions were pooled as indicated by the vertical lines and assayed for biologic activity. The conventions used for plotting results are described under Fig. 1.

the recoveries in several experiments were almost quantitative, only a 2-fold purification was achieved.

Electrofocusing. Two different acid ethanol extracts were applied to an electrofocusing column. In the first extract there was a sharp peak at pI 5.2 and a larger more diffuse peak at pI 6.6–6.8 (Fig. 8). In these studies, column eluates were dissolved 1:1 (v/v) in incubation medium. In all fractions the sulfate uptake was depressed below control values although the relative values for sulfate incorporation in general paralleled the values for thymidine uptake. For this reason, dose response curves of two fractions (pH 5.0 and 6.7) were compared with corresponding fractions from a control column run in an identical manner, but without added biologic activity. In these studies (Fig. 9), it became apparent that the concentration of eluate in the medium had little effect on thymidine incorporation, but the incorporation of $^{35}SO_4^=$ was strongly affected, possibly due to isotopic dilution with inorganic sulfate.

A second acid ethanol extract from the same patient but differing in protein content was applied to an electrofocusing column and the eluate assayed at a lower concentration in incubation medium. A sharp peak of both PSF and PTF activity was again seen at pH 5.2 and a broader peak with maximum at pH 6.7.

High voltage electrophoresis. The active eluate from a Dowex 50 column was passed over Sephadex G-25. Biologic activity was found in a broad zone following the excluded protein peak. These active fractions were pooled and subjected to high voltage electrophoresis (HVE) at pH 6.5. The five ninhydrin positive bands were eluted, lyophilized, and tested for PSF activity in the chick assay. PSF activity in the chick assay was recovered only in the neutral band (Fig. 10). When this band was resubjected to HVE at pH 2.0, there was further

resolution into four ninhydrin positive bands, but there was insufficient material for assay. No ninhydrin positive material was identified in the zone where the muscle sulfation factor described by Hall (22) migrates under similar conditions.

III. *Studies with insulin and human growth hormone*

Immunoreactive growth hormone and insulin levels. The immunoreactive growth hormone levels of the acromegalic plasma specimens used in these studies ranged between 20 and 30 ng/ml. The insulin content by radioimmunoassay ranged between 38 and 62 μU/ml. The recovery of immunoreactive insulin in several acid ethanol extracts was less than 50%. Levels comparable to those in acid ethanol extracts were recovered in the 0.2N NH_4OH eluate of Dowex 50 H^+ and in a fraction eluted from DEAE at an acid pH.

Studies with [^{131}I] insulin and [^{131}I] HGH. The recovery of [^{131}I] insulin and [^{131}I] HGH in acid ethanol extracts was 73.8 and 54.3%, respectively. When incubated in the presence of an antibody excess of their respective antisera, 74.6% of the radioactivity from [^{131}I] insulin was precipitated and 79.5% of the radioactivity from [^{131}I] HGH. When these extracts were passed over a Dowex 50 H^+ column and the column developed as described previously, [^{131}I] HGH was eluted slightly earlier than [^{131}I] insulin, but neither peak was separated from the PTF and PSF activity.

Assay of acid ethanol extracts in presence of anti-insulin and anti-HGH antisera. When an acid ethanol extract was assayed in antibody excess of anti-insulin and anti-HGH antisera, respectively, there was no obvious inhibition of either PSF or PTF activity. In this assay, however, inhibition less than 20% would not have been detectable.

Discussion

Molecular size of PSF and PTF in whole plasma and in acid ethanol extracts. These ultrafiltration and gel chromatography studies of whole acromegalic plasma suggest that PTF and PSF are either large molecules themselves or are associated with a large protein. This conclusion has also been reached by other workers (1, 2, 5). Bala *et al.* found that most of the PSF activity in normal and acromegalic plasma is eluted from Sephadex G-100 in a molecular weight range similar to pituitary growth hormone (2). These results are at

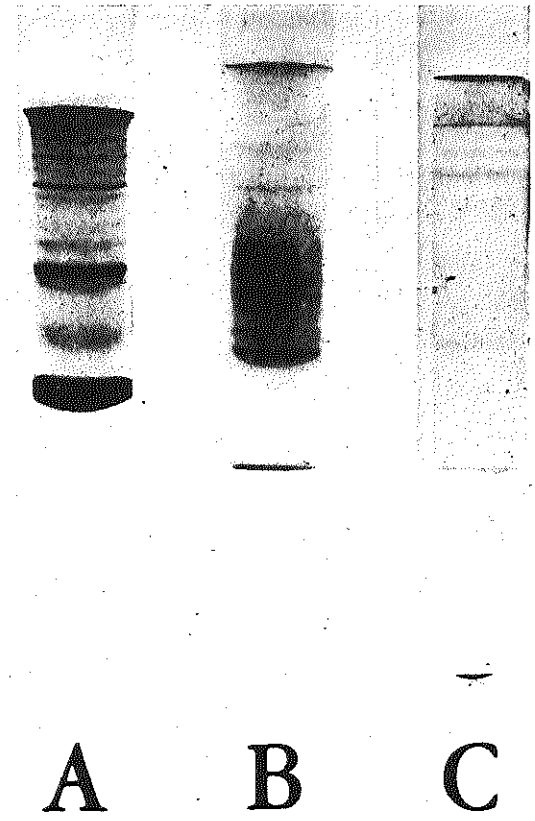


FIG. 6. Polyacrylamide gel patterns of A) native plasma (.0025 ml), B) acid ethanol extract (0.5 pl.eq.), C) a pool of the 2 most active fractions from the CMC column (5.0 pl.eq.) (Fig. 8). All were run at pH 8.9 in a gel concentration of 7.5%. The amount of protein applied to each column was, respectively, A) 187.5 μg, B) 147 μg, C) 50 μg. A and B were fixed in 7% acetic acid and stained with amido black. C was fixed in 12½% trichloroacetic acid and stained with Coomassie blue.

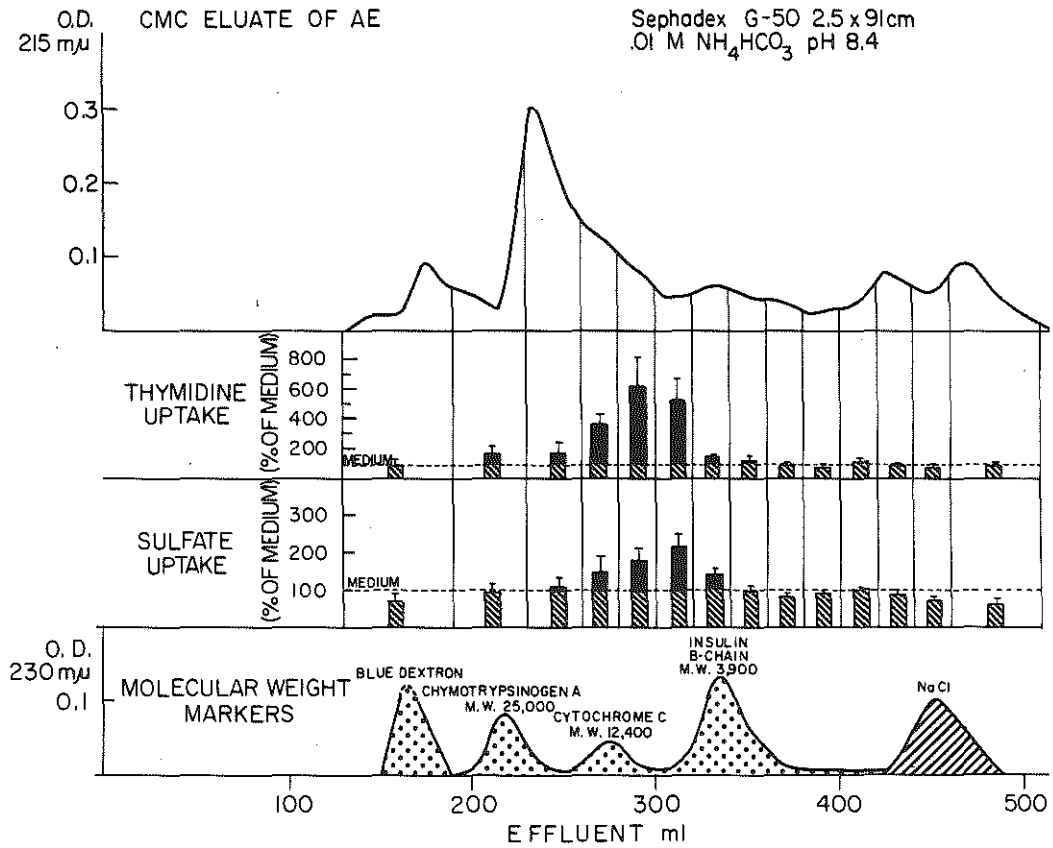


FIG. 7. Ascending gel chromatography on Sephadex G-50 in 0.01M NH_4HCO_3, pH 8.5. One hundred seventy-five pl.eq. (2.0 mg protein) of the 3 pooled and lyophilized biologically active fractions from CMC (Fig. 8) was applied to the column (2.5 ×91 cm) in a volume of 2 ml. Fractions of 10 ml were collected at a flow rate of 30 ml/hr and absorbance was measured at 215 mμ. The fractions were pooled as the vertical lines indicate and assayed for biologic activity. The conventions used for plotting results are described under Fig. 1.

variance, however, with the findings of Liberti (6) based on the ultrafiltration of bovine plasma. He found that sulfation factor activity in native bovine plasma behaves like a small molecule with a molecular weight on Sephadex G-15 ≦ 5000. This discrepancy can be most reasonably ascribed to species differences.

The procedure of acid ethanol extraction was suggested to us by the successful employment of this technique to extract from organs and blood such biologically active peptides as insulin (23), nonsuppressible insulin-like activity (7, 20, 21), prolactin (24) and parathormone (25). After extraction of whole plasma with acid ethanol and chromatography on Sephadex G-50, PTF and PSF activity were recovered in fractions corresponding to a molecular weight range >3900 ≦ 12,400. The slope obtained with this material in the bioassay was identical with that obtained with native plasma, suggesting that in whole plasma it might be either aggregated or bound to a larger carrier protein.

Purification of PSF and PTF by chromatography on CMC-cellulose and Sephadex. Both Sephadex G-100 and CMC-cellulose proved highly efficient in separating PSF and PTF activities from the large mass of protein in ethanolic extracts. With the

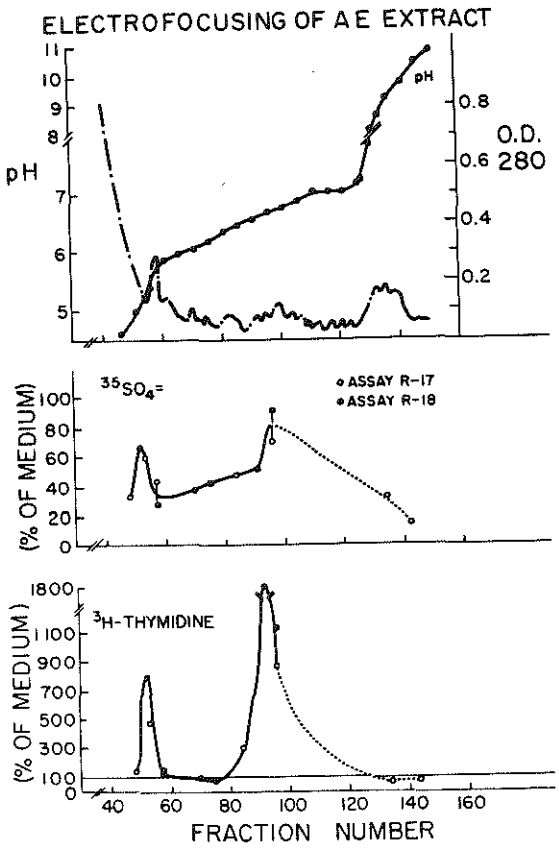


FIG. 8. Electrofocusing on an LKB-8102 ampholyne column (440 ml) at pH 5–8, equilibrated for 9.5 hr. Eight pl.eq. of acid ethanol extract was applied to the column. Fractions of 2.1 were collected, and absorbance measured at 280 mμ. The eluates were diluted in an equal volume of medium for assay. Each specimen was assayed in triplicate in an assay method utilizing cartilage segments from a single immature male rhesus monkey.

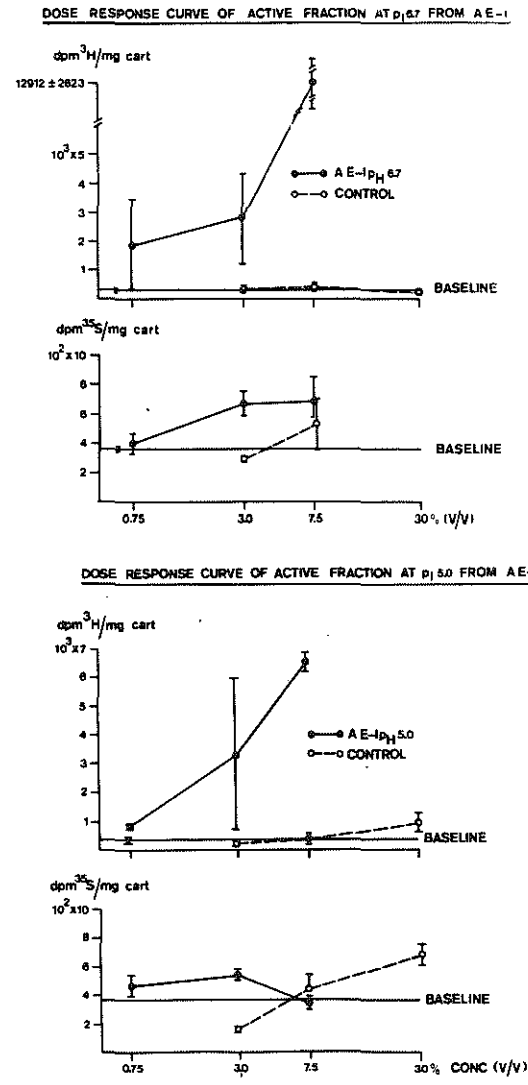


FIG. 9. Dose response curves of fractions eluted from electrofocusing column at pH 5.0 and 6.7 compared with dose response curves obtained with corresponding fractions from a control column. The dosages on the abscissa represent the concentration of eluate in medium (v/v). Eight pl.eq. of an acid ethanol extract was applied to the column.

combined procedure of acid ethanol extraction, cation exchange chromatography and gel chromatography we have purified PSF and PTF 6200× and 15,000×, respectively, over their activities in native plasma. When present in a concentration of 250 ng protein/ml, this preparation stimulated a 5.17 (±1.4)-fold increase in thymidine incorporation and a 2.17 (± 0.27)-fold increase in $^{35}SO_4^=$ incorporation. Assuming a provisional molecular weight of 8000 and making no allowance for residual contaminants, the concentration in this incubation calculates out to 3.1×10^{-8} molar. This activity compares favorably with other highly potent hormones.

Relationship of PTF to PSF. In none of the fractionation procedures was it possible to separate PSF from PTF activity. The achievement of an apparently greater

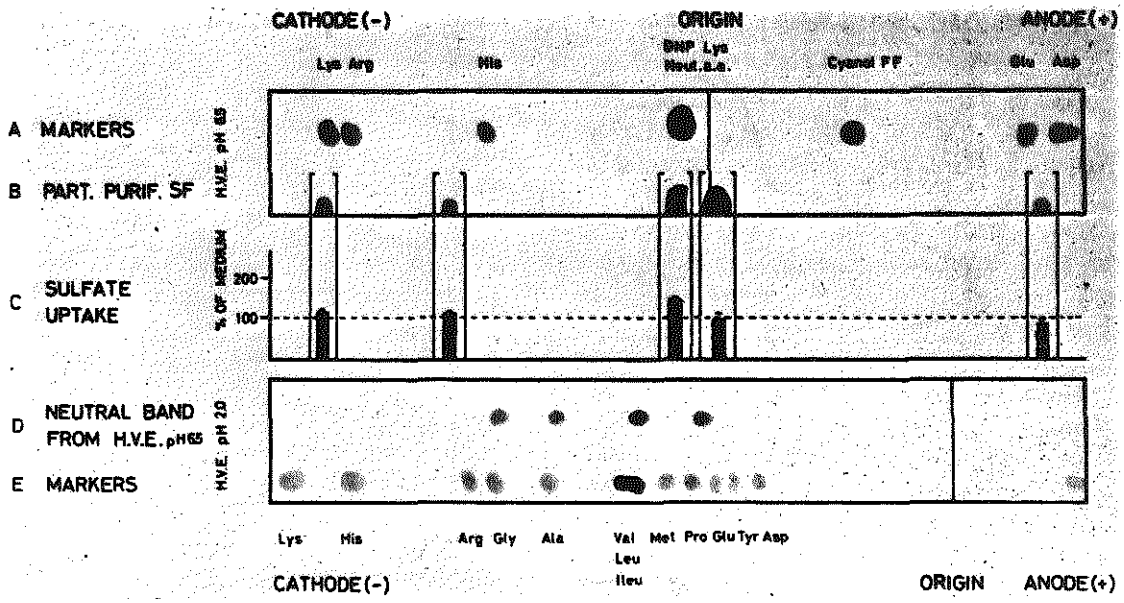


FIG. 10. A. Amino acid markers after high voltage electrophoresis at pH 6.5. B. Ninhydrin staining spots after high voltage electrophoresis at pH 6.5 of an acid ethanol extract of acromegalic plasma after further purification on Dowex 50 and Sephadex G-25 (see text for details). C. Sulfation factor activity as measured in embryonic chick pelvic leaflets, in fractions eluted as shown with the vertical lines. D. The active neutral zone was eluted and applied to the origin of another paper which was then subjected to high voltage electrophoresis at pH 2.0. Four ninhydrin positive spots were identified. Insufficient material was available for bioassay. E. Amino acid markers after high voltage electrophoresis at pH 2.0.

purification of PTF, and the recovery from some columns of more activity than was applied, is most probably attributable to the removal of inhibitory substances. We have found such substances to be normally present in plasma and that they affect thymidine uptake more than sulfate uptake (3). Salmon *et al.* (26) also point out that whole serum from normal and hypophysectomized rats contains material which is "toxic" for thymidine uptake in cartilage. Liberti observed that materials in bovine plasma which are inhibitory to sulfate incorporation can be removed by fractionation through ultrafiltration membranes (6).

Evidence for at least two molecular species. Our results on anion exchange chromatography of whole plasma and on electrofocusing of acid ethanol extracts reveal that there is more than one active molecular species. Although only one of these species was identified on HVE, its mobility at both pH 6.5 and 2.0 suggests similar charge properties to the predominant peptide (pI 6.6–6.8) found on electrofocusing. The behavior of the predominant component on both anion and cation exchange resins is likewise consistent with a neutral peptide.

The smaller peak of activity eluted from DEAE and from the electrofocusing column at pH 5.2 could conceivably have been due to the insulin content (pI 5.3) of the particular plasmas studied. Failure to detect this component after HVE might thus be explained since the chick assay is insensitive to insulin up to 8 mU/ml. Salmon has demonstrated that insulin stimulates both sulfate and thymidine uptake by rat cartilage, although the dose response curve is more shallow and maximal values are less than those obtained with acromegalic plasma (5, 26). Unfortunately, these plasma samples are

no longer available for assay in the presence of anti-insulin serum. The lesser peak is slightly more basic than is human growth hormone. One of us (J.L.VdB.) has found that after electrofocusing HGH the peak found by radioimmunoassay appears at pH 4.9–5.0.

It should be emphasized that our purest preparation is still heterogeneous and that yet other serum components with PSF and PTF activity may account for the losses of biologic activity sustained in the various purification steps. Nevertheless, since we have encountered no suggestion of biologic activity resident in protein fractions differing in size or charge properties from the two peptides or families of peptides described in this paper, we are inclined to believe that they represent most of the PSF and PTF activity found in human serum. Further details of the molecular structure, origin and biologic actions of these substances are under study.

Acknowledgments

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Early Localization of ^{125}I-Labeled Human Growth Hormone in Adrenals and Other Organs of Immature Hypophysectomized Rats

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ABSTRACT. Human growth hormone (HGH) labeled with ^{125}I was injected intravenously into hypophysectomized, immature rats. One rat was frozen 6 min and another 20 min after injection by immersion in hexane cooled with dry ice. Whole-body sections were made of the frozen rats and autoradiograms were prepared by placing these sections on x-ray film. The autoradiograms revealed that, at both time intervals after injection, there was a high concentration of radioactive material in the kidney, liver and adrenal cortex. The only other tissues found to have a higher radioactive density than that of blood were the submandibular glands, nasal mucosa, gastric mucosa, bone and follicles of vibrissae. The radioactivity in the adrenal cortex was slightly higher in the zona glomerulosa than in the zona fasciculata and it was lowest in the zona reticularis. The concentration in the epiphyseal plates was no higher than that in bone or soft tissues. The radioactivity in the submandibular gland had the electrophoretic mobility of growth hormone and not free iodide. The distribution in 8 organs of ^{131}I-albumin was compared with that of ^{125}I-HGH at 6 and 20 min by removal of the organs and direct isotope counting. The distributions substantiated the specific localizations of HGH seen in the autoradiograms. Pretreatment of the rats with ACTH or HGH 6 min before administering the ^{125}I-HGH altered the concentration of ^{125}I-HGH in kidney, liver and adrenals. (*Endocrinology* **88:** 1309, 1971)

STUDIES of the distribution of tritiated and radioiodinated growth hormone (GH) in rabbits and guinea pigs and in hypophysectomized (hypox) and intact rats have revealed significant concentration of radioactivity in several organs, but in none of these studies was there significant localization of labeled material in epiphyseal cartilage (1–6). Concentration of radioactivity in the kidney and liver has been a constant finding, while varying results have been obtained concerning the concentration of radioactivity in muscle, fat, bone, pancreas, thymus, adrenals and spleen.

Evidence is accumulating that GH does not act directly on epiphyseal growth, but rather stimulates the formation of secondary growth factors (sulfation factor and thymidine factor), which in turn stimulate the proliferation of epiphyseal chondrocytes and synthesis of cartilage matrix (7, 8). The chemical identity and sites of production of these secondary growth factors are unknown. In an attempt to obtain information regarding possible sites where skeletal growth factors are produced, the distribution of labeled GH was restudied with whole-body autoradiography in immature, hypox rats. This technique was used so that all possible sites with increased affinity for growth hormone could be evaluated. These results were compared with results obtained by counting excised organs after the administration of ^{125}I-human growth hormone and ^{131}I-human serum albumin.

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Materials and Methods

Injected materials. ^{125}I-labeled human growth hormone (^{125}I-HGH)[2] was obtained from the Iso-Serve division of the Cambridge Nuclear Corporation (lot 1088; SA 122 mCi/mg) and

[2] Originally obtained from Dr. A. E. Wilhelmi.

Abbott Laboratories (lot GH-080; SA 96 mCi/mg). Purity was checked by electrophoresis on Whatman 3 MC paper utilizing veronal buffer 0.05M, pH 8.6, with 600 V for 20 min at 4 C. After drying the strips at 90 C, the distribution of radioactivity was measured on a Baird Atomic strip scanner. Planimetric tracings were made to determine the percentages of radioactivity in the zones migrating as protein and free iodide (9). ^{131}I-human serum albumin (HSA) was obtained from Squibb (lot 52XM-M18-500; SA 25 μCi/mg). Unlabeled human growth hormone was obtained from the NIH hormone distribution program (lot HS1182B).[2] ACTH was from Parke, Davis and Company (lot JF217). All solutions were injected in a tail vein.

Animals. Male Sprague-Dawley rats[3] were hypophysectomized at 25 days of age and used in the experiments 2–3 weeks later. In the studies in which the organs were removed for direct counting of radioactivity, completeness of hypophysectomy was determined by examination of the sella turcica and by the weights of the adrenals and gonads. Examination of the sella turcica and weights of the adrenals and gonads could not be used to determine completeness of hypophysectomy in animals used for whole-body autoradiography. Extensive experience in our laboratory with similar rats, however, has shown that body weight correlates well with completeness of hypophysectomy; the present rats weighed no more than those of the same age in which hypophysectomy was proven to be complete.

Whole-body autoradiography. Two rats were injected with 10 μCi of ^{125}I-HGH dissolved in 0.5 ml of 0.9% NaCl. The rats were anesthetized with ether and frozen by immersion in a hexane-dry ice bath. One of these rats weighed 54 g and was frozen 6 min after injection; the second weighed 62 g and was frozen 20 min after injection.

Whole-body sagittal sections, 20 and 40 μ thick, of the frozen animals were taken on No. 800 Scotch tape at −15 C. The sections were dried and processed for autoradiography by a modification (10) of the technique described by Ullberg (11) which does not remove or translocate any compounds from the sections. The sections were placed against Kodak industrial type AA x-ray film in individual metal presses. After development of the autoradiograms, some of the sections were stained with hematoxylin and eosin for histological verification of the sites where radioactivity was concentrated. The figures shown were prepared by using the autoradiograms as negatives to produce enlarged prints. Consequently, white areas on the prints correspond to radioactivity. The concentration of radioactivity in the injected tissues was directly proportional to the degree of whiteness in the prints. Comparisons were usually made with blood in the heart since that represents the concentration of radioactivity in whole blood.

Electrophoretic mobility of radioactive materials in serum and submandibular glands. To determine how much of the radioactive material seen on the autoradiograms might represent degraded free iodide, 2 rats, weighing 63 and 58 g, were injected iv with 2 and 3 μCi, respectively, of the same labeled HGH preparation. They were then anesthetized with ether and sacrificed by cardiac exsanguination at 6 and 20 min, respectively, after the injection.

The serum was subjected to paper electrophoresis as described above except for the measurement of the radioactivity, which was performed in a Packard gamma spectrometer after the paper strips were cut into 1 cm segments. The amount of radioactivity migrating as protein and as free iodide was compared with a similar electrophoretogram in which the labeled HGH was added *in vitro* to plasma from a control hypox rat.

The radioactivity appearing in the submandibular glands was studied by cutting out the area containing the submandibular glands from five 40 μ sections taken from the rat killed 6 min following its injection. These tissues were applied to the origin of a paper strip and subjected to electrophoresis and evaluated in the manner described.

Accumulation of ^{125}I-HGH and ^{131}I-HSA in organs and effect of pretreatment with ACTH and HGH. For direct counting of radioactivity, the pancreas, spleen, liver, kidney, adrenals, bone (distal end of femur), thymus and submandibular glands were excised, carefully trimmed of excess tissues, and weighed on a torsion balance. The adrenals, pancreas, right kidney, bone, thymus and submandibular glands were counted *in toto*, whereas a weighed representative portion of the liver was counted. All counting was done in the well of a Packard gamma spectrometer. Four groups, each containing 2 rats, were used. One member of each group was killed 6 min after receiving the radiolabeled compound and the other member was killed at 20 min. The groups were treated as follows:

[3] Obtained from Hormone Assay Laboratories, Chicago, Ill.

Group 1, 2.5 μCi ^{131}I-HSA; Group 2, 7 μCi ^{125}I-HGH; Group 3, 0.5 U ACTH followed 6 min later by 7 μCi ^{125}I-HGH; and Group 4, 56 μg HGH 6 min before 7 μCi ^{125}I-HGH. The distribution of radioactivity among the respective organs at each time interval was calculated as the ratio of radioactivity found per mg of tissue to injected dose of radioactivity per mg of whole rat. In these studies the accumulation of ^{131}I-HSA was used as a reference standard to which the accumulation of ^{125}I-HGH was compared. The ratio of the accumulation of ^{125}I-HGH to the accumulation of ^{131}I-HSA was calculated at both time intervals for each organ.

Results

The ^{125}I-HGH preparations used were of acceptable purity. No more than 3% of the radioactivity migrated as free iodide and this was not significantly increased (4%) when added *in vitro* to hypox rat serum. Six minutes after the ^{125}I-HGH was injected, free iodide made up 8.6% of the total plasma radioactivity and by 20 minutes this had increased to 22.5%. Because of this rapid deiodination, more value should be assigned to rats with the shortest interval between injection and sacrifice. In the submandibular glands, 10.5% of the total radioactivity was present in the form of free iodide at six minutes.

Autoradiographic distribution of ^{125}I-HGH. The general patterns of distribution of radioactivity in the whole-body sections of the two rats were quite similar. In the rat frozen six minutes after injection, the liver, adrenal cortex, submandibular gland, kidney, nasal mucosa and follicles of vibrissae were found to have a higher concentration of radioactive material than was present in the blood (Fig. 1 and 2). Concentration of radioactive material in the pancreas (Fig. 2) was approximately equal to that of blood. In both rats, concentration of radioactivity in the thymus was much lower than that in blood. In bone, the highest concentration of radioactive material was found in the cancellous bone in the epiphyses and metaphyses, while a somewhat lower concentration was present in the compact bone of the diaphyses (Fig. 3). There was no visible evidence of accumulation of radioactive material along the epiphyseal plates or in articular cartilage.

Fig. 4 is a print of an autoradiogram from the rat which was frozen 20 minutes after injection. The pattern of distribution was the same except for an increased concentration of radioactivity within the contents of the stomach. No radioactivity could be seen along the epiphyseal plate of the humerus (Fig. 5). The lack of accumulation of radioactivity in the pancreas and the higher concentrations in the liver, kidney and adrenal cortex in these sections are apparent (Fig. 5).

Fig. 6 is an enlarged print from another autoradiogram of the rat frozen 20 minutes after injection. This enlargement shows in greater detail the radioactivity in the adrenal cortex and kidney. Comparison of this autoradiogram with the stained section from which it was produced revealed that the highest concentration of radioactivity was in the zona glomerulosa, with decreasing concentrations in the zona fasciculata and zona reticularis. Due to the thickness of the sections and the tissue damage from freezing, it could not be verified histologically whether the intense radioactivity in the kidney was concentrated in the glomeruli or proximal tubules.

Direct counting of excised organs. The ratios of the distribution of radioactivity from ^{125}I-HGH to that of ^{131}I-HSA for the eight excised organs counted are shown in Table 1. At both 6 and 20 minutes after injection the accumulation of radioactivity from ^{125}I-HGH was greater than that from ^{131}I-HSA in adrenals, liver, kidney and submandibular glands. Radioactivity in the pancreas from ^{125}I-HGH was equal to that from ^{131}I-HSA at six minutes and higher than that from ^{131}I-HSA at 20 minutes. The values for ^{125}I-HGH in spleen, thymus and bone were lower than those for ^{131}I-HSA at both time intervals. Pretreatment

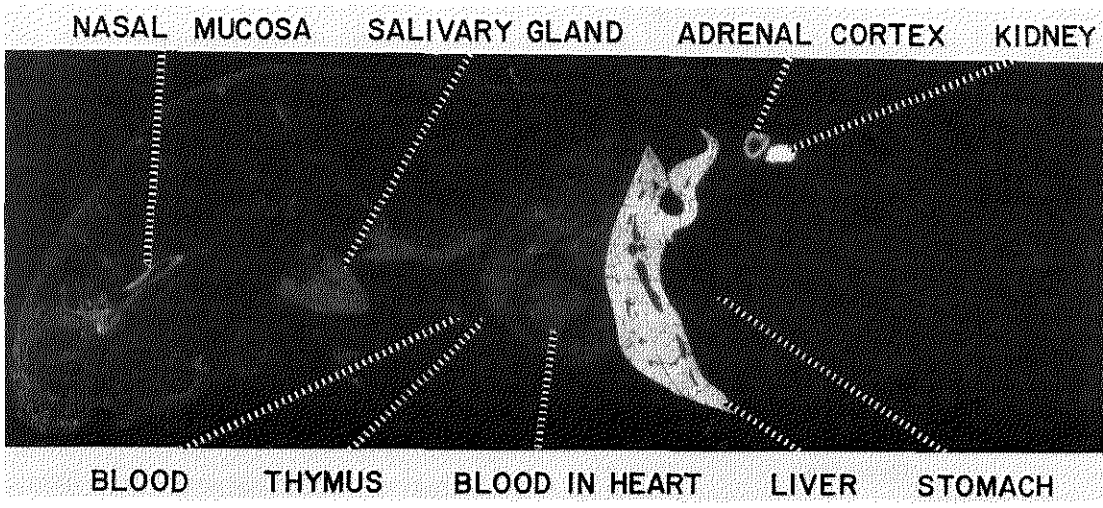

FIG. 1. Whole-body autoradiogram of rat killed 6 min following 10 μCi of ^{125}I-HGH iv. White areas correspond to radioactivity. Compare the concentration in the blood in the heart with that in the submandibular gland, liver, kidney and adrenal cortex.

with ACTH increased the accumulation of radioactivity from ^{125}I-HGH over that from ^{131}I-HSA in adrenals at both time intervals, while these ratios were unchanged or slightly lowered in the other organs counted. At both 6 and 20 minutes, pretreatment with unlabeled HGH greatly decreased the ratio of accumulation of radioactivity from ^{125}I-HGH to that from ^{131}I-HSA in the liver and adrenals, while this ratio was substantially increased in kidney and only slightly altered elsewhere.

Discussion

In previous studies the distribution of iodinated or tritiated GH was determined by measuring the radioactivity in whole or homogenized organs which were removed at various times after injection (1–3, 5). With such techniques, high concentrations in small portions of an organ may be obscured. In other reports selected organs were placed in fixing solutions, dehydrated, and subjected to routine histological techniques. Autoradiograms were then prepared in order to locate the radioactivity within the organ studied (2, 4–6). In this study, whole-body autoradiograms made from freeze-dried, but otherwise untreated, tissues were used to determine whether important concentrations of radioactivity had been overlooked previously. In addition to preventing the removal or translocation of labeled compounds within tissues, this technique reveals gradients of concentrations within organs and tissues.

Earlier studies (1, 2, 12) have suggested that labeled hormones tend to concentrate very quickly in their respective target tissues after administration. Therefore, time intervals of 6 and 20 minutes between administration of the labeled hormone and death of the animals were chosen in this study.

By both the autoradiographic technique and the technique of counting excised organs directly, the distribution of radioactivity was found to be similar in rats frozen 6 and 20 minutes after receiving ^{125}I-HGH. The consistent findings in this and in all earlier reports (1–6) are higher concentrations of radioactivity in the liver and kidney and the absence of significant concentrations of radioactivity in epiphyseal cartilages. A striking finding in this study was the early concentration of large amounts of radioactivity in the adrenal cortices. Sonenberg *et al.* (1, 2) found increased levels of radioactivity in the adrenals of rats killed ten minutes following intracar-

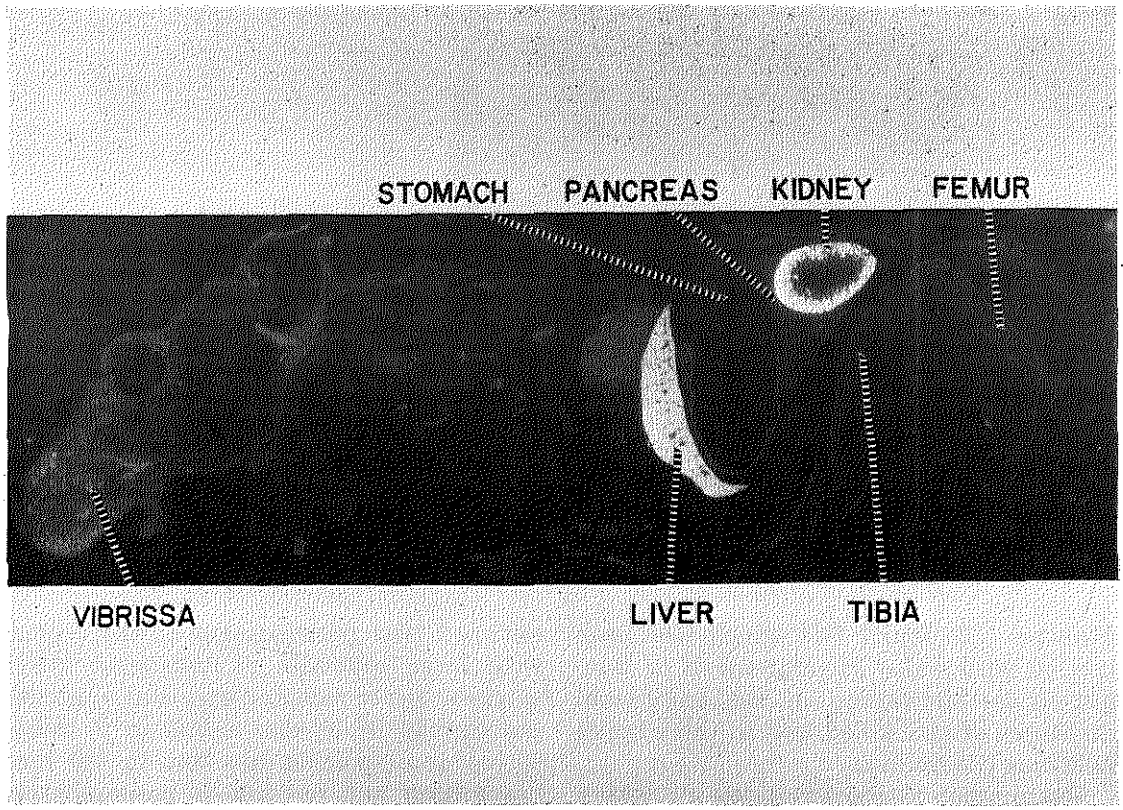

FIG. 2. Same rat as in Fig. 1. Very little radioactive material is present in the pancreas. Epiphyseal cartilages do not concentrate ^{125}I-HGH. Concentrations in epiphysis and diaphysis of femur are slightly higher than that of blood. Note activity in follicles of vibrissae.

dial injections of ^{131}I-bovine GH. Since autoradiography was not employed, the distribution of radioactivity within the gland was not discerned. Radioactivity was found to be concentrated primarily in the zona reticularis of the adrenal cortices of rats, 20 days of age, following ip injections of ^{125}I-HGH (6). No activity was demonstrable in the adrenal cortices of similar rats injected with ^{125}I-labeled human luteinizing hormone, bovine serum albumin, or ^{125}I-NaI. Autoradiograms from rats injected with ^{131}I-ACTH or histological preparations from rats injected with ACTH conjugated with fluorescent dyes revealed labeled materials concentrated primarily in the inner two layers of the adrenal cortex (12, 13). In the present study, the concentration of radioactivity was greatest in the zona glomerulosa.

In addition to the adrenals, liver and kidney, the autoradiograms studied here revealed localizations of radioactive material in the submandibular glands, nasal mucosa, gastric mucosa, bone and follicles of vibrissae, as judged by autoradiographic densities. No other organ or tissue showed a higher concentration of radioactivity than that present in the blood. The high concentrations of radioactivity seen in the stomach, nasal mucosa and vibrissae were probably due to iodide which had been removed from the growth hormone. High concentrations in all these areas are seen in whole-body autoradiograms of mice and rats injected with ^{125}I-NaI (14).

Several studies on the distribution of serum protein labeled with either radioiodine or fluorescent dyes (6, 12, 15) have not revealed specific localization in either the adrenal cortex or the submandibular gland with any of the substances injected.

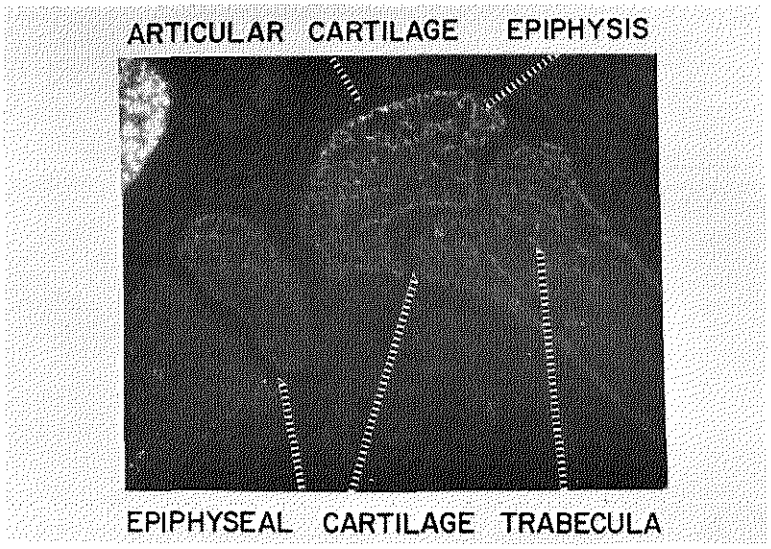

FIG. 3. Enlargement of area containing femur and tibia from Fig. 2. The kidney is in the upper left corner. Radioactive materials are restricted to bone. There is no detectable activity in articular and epiphyseal cartilages.

Mancini (12) found GH which had been labeled with either Rhodamine B or fluorescein isothiocyanate in the epiphyseal cartilages of rats killed two to three hours following iv injection.

Quantitative determinations of radioactivity in excised organs were done to determine the specificity of the localization of ^{125}I-HGH. It was considered that the distribution of ^{131}I-HSA would serve as a nonspecific protein for comparison. It was assumed that pretreatment of the animal with large amounts of unlabeled HGH would saturate receptor sites and minimize the uptake of labeled hormone if radioactive accumulation was due to the hormone itself and not due to contaminants or degradation products. Similarly, pretreatment with ACTH was carried out to eliminate the possibility that the radioactive preparation was contaminated with ACTH.

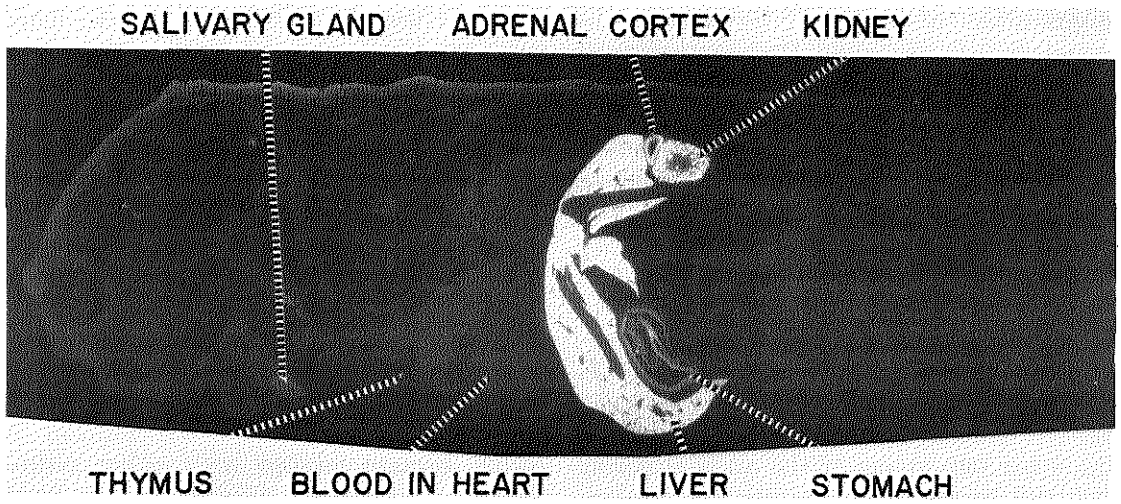

FIGURE 4

FIG. 4 and 5. Whole-body autoradiograms from rat killed 20 min following 10 μCi ^{125}I-HGH iv. Except for concentration of activity in gastric mucosa and in contents of stomach, the distribution is similar to that of rat killed 6 min following the injection.

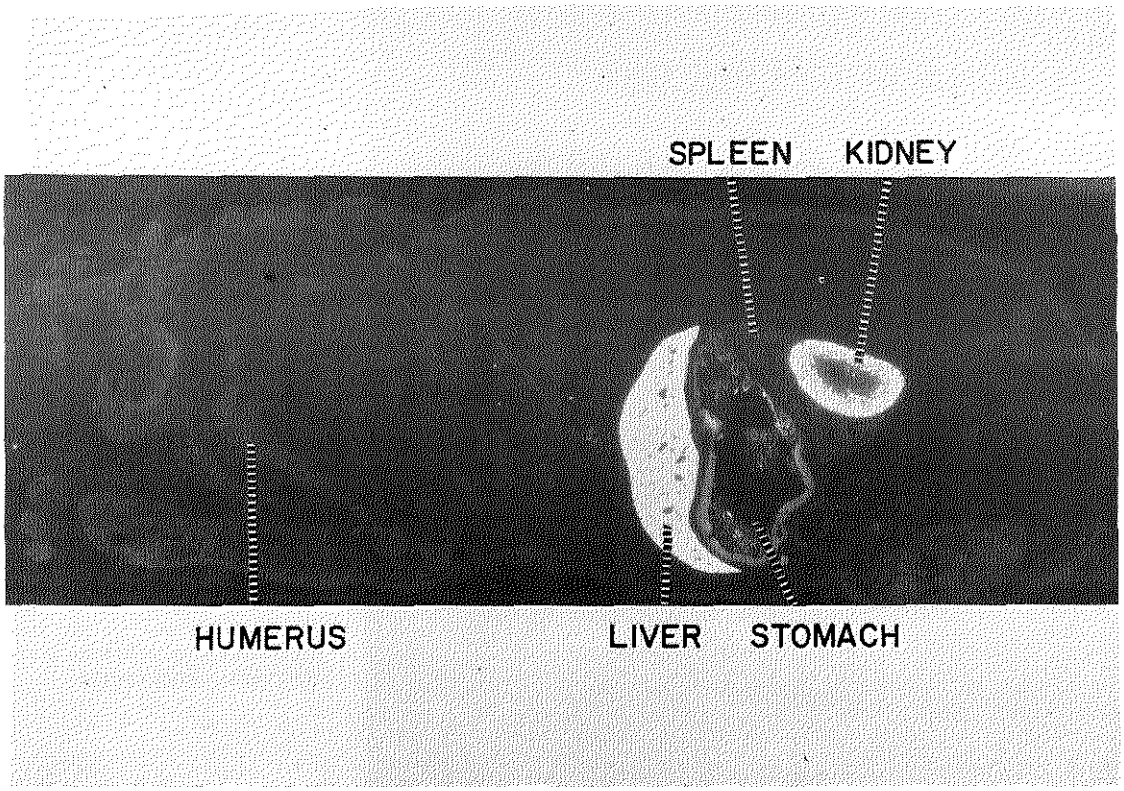

FIGURE 5

The quantitative determinations of radioactivity verify the patterns of distribution seen in the autoradiograms. Since the increased concentration of radioactivity in the adrenals and kidneys was limited to the cortices, the increase in the total counts per mg of organ was obscured because of the inclusion of the medullary tissue which did not have an affinity. This emphasizes the usefulness of the autoradiographic technique. It is clear from these studies that the accumulation of ^{125}I-HGH in the adrenals and liver is not due to contamination of the iodinated solution with ^{125}I-ACTH since it is blocked by the prior administration of unlabeled HGH. The reason for the surprising enhancement of the accumulation of ^{125}I-HGH by the adrenals after pretreatment with ACTH is not entirely clear, but one of us (JJVW) has observed large increases in the adrenal blood flow in dogs after the administration of ACTH intravenously. The enhanced accumulation of ^{125}I-HGH by the kidney after pretreatment with HGH has no clear explanation but conceivably might result from the saturation of tubular reabsorption sites. The finding that pretreatment with unlabeled HGH failed to suppress uptake of ^{125}I-HGH in the submandibular glands is confusing in view of the autoradiographic and electrophoretic findings.

Although it is not possible to infer a specific physiologic significance from the fact that growth hormone is rapidly concentrated in a given organ, such a finding may provide clues regarding the organs in which secondary growth factors are formed. The possibility that growth hormone is concentrated in the submandibular gland is particularly intriguing since a potent nerve growth factor and an epithelial growth factor have been extracted from submandibular glands of mice (16, 17). Furthermore, several mammals, including the rat, have been reported to respond to sub-

FIG. 6. Distribution of radioactive material in the kidney and adrenal cortex (same rat as in Fig. 4 and 5). The gradient of concentration of activity in the adrenal cortex appears to be greatest in the zona glomerulosa and least in the zona reticularis. No activity is present in the medulla. Activity in kidney is restricted to renal cortex.

mandibular adenectomy with a drastically reduced rate of linear growth (18). Neither loss of exocrine function nor anorexia due to dysphagia was apparently responsible for this growth arrest. The relevance, if any, of these observations to the early concentration of growth hormone in the submandibular gland remains to be explored.

Growth hormone has been reported to increase adrenal weight and increase the width of the zona reticularis (19). Recent evidence suggests that GH plays a significant role in the release of aldosterone from the adrenals of chronically salt restricted, hypox rats (20).

Aldosterone release can be demonstrated with adrenals from intact rats during *in vitro* incubation but not with adrenals from hypox rats (21). Adrenals from hypox rats regained their ability to secrete aldosterone after treatment with monkey GH. Furthermore, serum from such GH-treated animals, but not GH itself, increased aldosterone release *in vitro* from adrenals of intact rats (21).

These observations on GH and aldosterone secretion complement the discovery that GH does not act directly to stimulate cartilage growth *in vitro* (7, 8). Since radioactivity does not accumulate in the epi-

TABLE 1. ^{125}I-HGH distribution/^{131}I-HSA distribution

	Organ	Pretreatment		
		None	ACTH	HGH
6 min after ^{125}I-HGH	Spleen	0.56	0.57	0.55
	Pancreas	1.03	0.79	0.98
	Kidney	3.37	2.94	5.50
	Liver	1.51	1.44	0.72
	Adrenals	1.31	2.96	0.73
	Bone	0.46	0.46	0.54
	Thymus	0.35	0.38	0.44
	Submandibular glands	1.77	1.39	1.59
20 min after ^{125}I-HGH	Spleen	0.58	0.63	0.59
	Pancreas	1.43	1.29	1.29
	Kidney	1.90	2.06	3.37
	Liver	2.30	2.37	0.72
	Adrenals	1.34	3.01	0.55
	Bone	0.47	0.41	0.45
	Thymus	0.89	0.78	1.28
	Submandibular glands	1.79	1.65	1.27

Values are the ratio of the distribution of ^{125}I-HGH to the distribution of ^{131}I-HSA at each of 2 time intervals after injection of labeled material. The distribution of radioactivity was calculated as the ratio of the radioactivity found per mg of tissue to the radioactivity administered per mg of whole animal. Nonlabeled ACTH or HGH was given 6 min prior to the ^{125}I-HGH.

physeal cartilage following the injection of tritiated or iodinated GH *in vivo* (2, 4–6), the concentrations of radioactivity in the zona glomerulosa of adrenals from rats studied here suggest that separate pathways or mechanisms could exist through which GH exerts its influence on these diverse functions.

These studies provide some meager clues regarding the site of origin of the growth hormone-dependent sulfation and thymidine factors which promote growth in skeletal tissue. The possibility that either the submandibular glands or the adrenals may be required for the formation of growth hormone-dependent skeletal growth factors is being investigated and will be reported subsequently.

Acknowledgment

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Somatomedin: Proposed Designation for Sulphation Factor

The action of growth hormone (GH) on skeletal tissue was proposed to be mediated through a secondary substance, described by the operational term 'sulphation factor".[1] The observations underlying this hypothesis have been amply confirmed and extended. A GH-dependent plasma factor stimulates in cartilage not only the incorporation of sulphate into chondroitin sulphate, but also the incorporation of thymidine into DNA[2], proline into the hydroxyproline of collagen[3] and uridine into RNA[4]. GH has substantially no *in vitro* effect on cartilage metabolism.

In plasma sulphation factor (SF) circulates associated with the large molecular components but extracts of considerably smaller size with sulphation factor activity have been prepared by denaturating plasma protein by boiling[4] or by extraction with acid ethanol.[5] Van Wyk et al.[5] purified their acid ethanol extracts by gel filtration, ion exchange chromatography and electrophoresis. After an approximate 25,000-fold purification, the biological activity could be attributed to a neutral peptide with a molecular weight of about 8,000 (ref. 6).

The spectrum of biological action of SF has been extended by experiments with partially purified SF. Hall and Uthne[7] demonstrated that injections of SF active extracts induced widening of the tibial epiphyseal cartilage. The biological actions of SF are not limited to cartilage. Salmon and Du Vall[8] showed that partially purified SF had insulin-like actions on isolated rat diaphragm and Hall and Uthne[7] have shown that similar SF preparations stimulate conversion of ^{14}C-glucose to $^{14}CO_2$ by rat adipose tissue. The insulin-like activity cannot be neutralized by anti-insulin serum and follows SF activity through multiple fractionation procedures[5], suggesting that SF is identical with or very similar to the smaller molecular weight component of the non-suppressible insulin-like activity (NSILA-S) extensively studied by Jakob, Hauri and Froesch.[9] NSILA-S stimulates thymidine incorporation in rat costal cartilage.[10] Recently Salmon and Hosse[11] reported that a serum fraction with SF activity stimulated HeLa cell growth.

Although the mechanism of production and ultimate physiological role of SF remain to be defined, its importance in the growth and anabolic responses of both skeletal and certain non-skeletal tissues can be in no doubt. After consideration of many alternatives to the operational terms 'sulphation factor' or 'thymidine factor', we propose the more general term, 'somatomedin'; the prefix, 'somato', connotes both a hormonal relationship to somatotropin and, also, to the soma which is the target tissue of this agent. 'Medin' is included in the name to indicate that it is an intermediary in somatotropin action.

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