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STUDIES ON SERUM SOMATOMEDIN ACTIVITY AND CARTILAGE RESPONSIVENESS IN THE REGULATION OF GROWTH

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ABSTRACT

STUDIES ON SERUM SOMATOMEDIN ACTIVITY AND CARTILAGE RESPONSIVENESS IN THE REGULATION OF GROWTH

This thesis describes the results of experimental investigation of aspects of the endocrine regulation of growth in the rabbit, rat and man. In particular the role of serum somatomedin and cartilage responsiveness to somatomedin have been examined. Throughout a relationship between the physiological control mechanisms, namely the stimulus and the degree of end-organ responsiveness, and the velocity of growth has been sought.

A longitudinal growth study of 69 New Zealand White rabbits between birth and adulthood provided data on the body weight, body length and 16 radiologically determined skeletal dimensions. In all respects female rabbits are larger than males from birth, or shortly thereafter, and remain so throughout life. A new mathematical approach to the analysis of longitudinal growth data has been developed and validated.

Serum somatomedin was measured by the porcine cartilage bioassay method in a cross-sectional study of rabbits at intervals from the intrauterine age of 23 days postcopulation (PC) to adulthood. The serum level rises from the youngest age studied to a plateau that begins between 100 and 150 days PC. No difference between male and females was found.

Cartilage responsiveness was measured *in vitro* in rabbit costal cartilage in a cross-sectional fashion at intervals from the intrauterine age of 15 days PC to adulthood. The uptakes of ^{35}S-sulphate and ^{3}H-thymidine were used as indices of responsiveness of the cartilage. It has been shown that the

response of the cartilage to serum somatomedin stimulation is primarily dependent on the age of the donor animal and not on the nature of the somatomedin stimulus. The degree of cartilage responsiveness is significantly correlated with velocity of the growth in length of the cartilage. Further evidence for an "inherent growth capacity" of cartilage was derived from the transplantation of rat costal cartilages into recipients of varying ages. It has been shown that the in vivo responsiveness of the transplanted cartilage is determined by the age of the donor animal and not that of the recipient.

Chondrocytes isolated from rabbit costal cartilage also exhibited age-dependent characteristics in their rate of incorporation of ^{3}H-thymidine in culture. Experiments comparing the effect on thymidine incorporation by isolated chondrocytes of native and heated-acidified normal adult human serum suggest that such serum may contain somatomedin inhibitors.

Serum somatomedin has been measured in normal children under the age of 4 years and falls within the range of normal adults. Serum somatomedin is low in children with severe protein-energy malnutrition (PEM) and rises, but not always to normal levels, with recovery. Intravenous administration of growth hormone in children with PEM does not change the somatomedin level, neither at the time of admission to hospital, nor after recovery.

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CHAPTER 1

SCOPE AND OBJECTIVES OF THE THESIS

"Only begin and then the mind grows heated;
only begin and the task will be completed."

Goethe

Growth is the hallmark of young animals. The features of growth and the factors that regulate it are the subjects of study of many disciplines. Ever since Von Bezold combined anatomical with chemical techniques in his 1857 publication on the composition of the tissues of the infant, the value of an interdisciplinary approach to the study of growth has been recognized. Yet as Tanner (1962) points out, few authors have ventured fully into the use of interdisciplinary methods.

The work of this thesis was planned to combine a somatometric, longitudinal growth study with an investigation of the role of somatomedin and cartilage responsiveness in the regulation of growth rate. This plan evolved when a general interest in growth, aroused by a study of Khoi children (Beaton 1969), focussed specifically on the endocrine regulation of growth. It was recognized that regulating factors operate on the velocity of growth, and that growth velocity is most effectively determined by longitudinal analysis.

In 1970, indeed as now, the precise nature of the endocrine control of growth in mammals was not understood. In particular, the role played by growth hormone (GH), although recognized to be of central importance, was an enigma. Prior to Salmon and Daughaday's discovery of a "sulphation factor" in 1956/7 it was

believed that GH exerted its growth-promoting effects by a direct action. Today two factors are known to be of major importance in determining GH stimulation of somatic growth; namely GH-dependent serum somatomedin (SM) activity and end-organ responsiveness. Somatomedin and its end-organs are the subject of an ever-increasing research interest. Of some 150 papers published in the field since 1957 more than half have appeared since 1970. Major reviews have appeared in the past 4 years (Daughaday 1971a;Grant 1972; Van den Brande 1973; Hall & Luft 1974; Van Wyk et al. 1974).

The central question which this work attempts to answer is: 'What are the relationships in normal, growing mammals between the velocity of growth, serum somatomedin activity and end-organ responsiveness?'

Chapter 2 discusses the nature of growth, its patterns and mechanisms. The important conceptual differences between growth and differentiation are described and a definition of growth is offered.

Chapter 3 examines the methods of describing and analysing growth, differences between cross-sectional and longitudinal analysis and the concept of physiological, as opposed to chronological, age. Growth characteristics of several species of mammals are described briefly.

In Chapter 4 selected aspects of the many factors that regulate growth are discussed. The interplay between endogenous and environmental factors is stressed. Special emphasis is placed on the role of GH in growth regulation, and this leads up to the key observation of Salmon and Daughaday on the mechanism of GH action on cartilage in vitro.

Chapter 5 reviews the present state of knowledge of SM. The sulphation factor hypothesis is placed in its historical

perspective. The bioassay systems currently used are critically examined and their respective merits contrasted. The recently developed radioreceptor and radioimmunoassays are described and compared to the bioassay. Physical properties, site of production and biological actions of SM are considered. The role of SM in the regulation of normal growth and in the causation of abnormal growth is explored. Finally the emerging concept of a family of growth-promoting peptides is discussed highlighting the main features of each of the potential family members.

The importance of the end-organ response in determining the outcome of hormone action is emphasized by placing it as a separate subject in Chapter 6. Current knowledge concerning changes in end-organ responsiveness, with particular reference to cartilage, is reviewed.

To examine relationships between growth velocity, serum SM and end-organ responsiveness, an animal model was necessary. The rabbit was chosen for a number of reasons. The rabbit is well suited to growth studies as it has a reasonably short growth period of about 6 months;litters are usually large, mothers tolerate interference with the young and the young are robust (Sawin 1950, Adams et al. 1966). Added to these favourable characteristics there is the relatively large size of the rabbit which provides workable quantities of serum and cartilage, even from foetal and neonatal animals. Furthermore, no complete longitudinal growth study appeared to have been made on the rabbit at the time this work was started (Tanner 1971, personal communication). It was envisaged that the growth data in itself would make a valuable contribution. No work has been published on the serum SM of the rabbit nor on the characteristics of its costal cartilage response to SM. In summary, the rabbit on theoretical grounds appeared to be a suitable choice of experimental

model. Chapter 7 describes the stock characteristics, housing, breeding and rearing procedures for the rabbits. Rats were used for one section of the work and the important features of the rats used are described.

After establishing a satisfactory breeding colony of rabbits a longitudinal growth study of body weight, body length and radiographically determined skeletal dimensions was executed during 1971/2/3. Norms of body weight growth were readily established by manual analysis immediately on completion of the study. All other measurements, viz. the skeletal dimensions, required development of a computerized method of analysis. Chapter 8 presents the method, results and discussion of the longitudinal study of rabbit growth.

Rabbits growing at the established normal body weight velocities provided serum and costal cartilage for the determination of serum SM activity and cartilage responsiveness. Perforce these studies were of a cross-sectional nature, extending from foetal to adult rabbits.

Chapter 9 describes the SM bioassay method used. The validation and quality control of the method are given in full detail. Results and discussion of the assays of the rabbit sera complete the chapter.

The in vitro cartilage response studies are described and discussed in Chapter 10. Costal cartilage from groups of rabbits between the foetal stage and adulthood was assayed for its ability to take up ^{35}S-sulphate ($^{35}SO_4$) and methyl-^{3}H-thymidine (^{3}HTdr). The cartilage was stimulated by both a serum pool derived from the donor animals of the cartilage and by a standard reference pool of human serum. Dose-response curves were obtained and time-sequence studies performed.

Cartilage was chosen as the end-organ in which to study end-organ responsiveness for a number of reasons. Linear somatic growth velocity is chiefly determined by the growth velocity of cartilage (Daughaday 1971b). The cartilage response to SM is the most fully documented tissue response, and therefore comparisons between the rabbit and other species would be of value. Cartilage is readily maintained in vitro, is resistant to hypoxia and consists of only one cell type; all factors favourable to in vitro determination of responsiveness (Daughaday & Mariz 1962). Costal cartilage is far more readily accessible than epiphyseal cartilage and therefore less prone to trauma and incomplete sampling during dissection. There is evidence to suggest that cartilage from different sites in the same animal behaves similarly during incubation. Thus costal, xiphoid, nasal and epiphyseal cartilage display quantitatively similar responses (Murphy et al. 1956; Salmon & Daughaday 1957; Herbai 1971a). Finally, the fates of the $^{35}SO_4$ and the ^{3}HTdr taken up by costal and epiphyseal cartilage have been well established and are the same for the cartilage from different sites. $^{35}SO_4$ is incorporated into chondroitin sulphate (Dziewiatowski et al. 1949; Dziewiatowski 1962; Katsura & Davidson 1966). ^{3}HTdr is incorporated exclusively into DNA (Cleaver 1967) and this rate of incorporation correlates very closely with mitotic rate (Mitchinson 1971). The rate of uptake of these two isotopes therefore indicates the rate of synthesis of cartilage matrix ($^{35}SO_4$) and the mitotic rate of the growing cartilage (^{3}HTdr).

In order to obtain further evidence for changes in end-organ responsiveness during growth costal cartilages from growing rats were transplanted into age-matched and adult rats. The uptake by transplanted and recipient costal cartilage of intraperitoneally administered $^{35}SO_4$ and ^{3}HTdr is reported and discussed in

Chapter 11. By this method the SM stimulus was held constant *in vivo* and growth-related changes in cartilage responsiveness assayed.

Chapter 12 describes the isolation and short-term culture of rabbit costal cartilage chondrocytes. The cells were stimulated by normal serum and heat-treated serum. The dose-response characteristics of their uptake of ^{3}HTdr and changes in uptake related to the growth-velocity of the donor animal are reported and discussed.

While this was primarily a study of normal growth, it was recognized that pathological disturbances of normal function can shed light on fundamental processes. Aspects of serum SM activity have therefore been studied in children with protein-energy malnutrition which is characterised by growth retardation. Serial changes in serum SM and GH in children recovering from malnutrition and the effects of exogenous GH infusion on serum SM are reported and discussed in Chapter 13.

Chapter 14 summarizes the main conclusions of the thesis.

CHAPTER 2

THE NATURE OF GROWTH

There is no better starting point for this chapter than Sir D'Arcy Thompson's (1917)comment on the nature of growth: "a somewhat vague word for a very complex matter", for in the words of this great student of growth lies the first issue, the definition of terms.

To early writers growth was essentially a quantitative phenomenon, an increase in the size and mass of the body, to be distinguished from qualitative changes such as alterations in shape and function. Thus Davenport (1899) wrote that "..... organic growth is an increase in volume, ... it is not differentiation" and was followed by De Beer (1924) stating that "growing implies getting bigger and this increase in size is what should strictly be termed growth .... Growth alone cannot be responsible for the process of development." The concept was thus introduced that growth and differentiation are two distinct processes which together contribute to the overall development of an organism (Needham 1942). As investigation progressed into understanding the subcellular mechanisms the distinction between the quantitative aspect of development, growth, and the qualitative aspect, differentiation, became blurred, leading J.Z. Young (1950) to suggest that "growth is the addition of material to that which is already organized into a living pattern." To this rather non-specific definition must be added the concept of differential growth in which several levels of organization may

be embraced, and which implies that the two processes are not mutually exclusive, but may occur concomitantly in the same tissue. Thus at cell-tissue level a population of identical cells may divide more rapidly (grow) in one direction than in another, leading to a change in organ shape (differentiation); whereas at the organelle-cell level an increase in the number of mitochondria would both increase cell size and modify cell function.

2.1. A Definition of Growth

To date no universally accepted terminology has emerged. The distinctions between growth and differentiation drawn earlier this century pervade common usage and understanding.

In this thesis growth is regarded as progress towards final adult size, in the course of which the differentiation of function and shape of the growing organism plays an integral, but not total, contributory and sometimes regulatory role. This concept has both phylogenetic and ontogenetic validity as Needham (1964) has extensively argued.

2.2. Mechanisms and Spatiotemporal Patterns of Growth

There are three fundamental mechanisms by which the growth of a multicellular animal occurs. In the first, the animal's growth may be the result of an increase in the number of cells as a consequence of mitosis; that is multiplicative (Huxley 1932) or hyperplastic growth. In the second, growth occurs as a result of increase in size of individual cells, without increase in number; that is auxetic (Needham 1942) or hypertrophic growth. Whereas in hypertrophic growth cell products accumulate intracellularly, in the third mechanism cell products are secreted into the extracellular compartment causing an increase in size. This occurs in cartilage matrix for example, by accretion (Huxley 1932).

In the rat (Winnick & Noble 1965, Enesco & Leblond 1962) and in man (Cheek 1968) the chronology of the respective contributions of these three processes has been demonstrated. For the rat the sequence is as follows: in utero and up to the time of weaning growth occurs predominantly by hyperplasia (phase I); after weaning hyperplasia and hypertrophy contribute about equally (phase II); and as adult size is approached hyperplasia gradually ceases and hypertrophy and accretion continue (phase III) until growth is complete. In man events appear to be similar to those in the rat, but studies are limited to skeletal muscle. In the rat various organs pass through the three phases at different times. The brain, for example, passes through phases I and II very rapidly, whereas the heart reaches phase III much later, and even well after the lungs.

While these generalizations concerning the mechanism and timing of cellular growth are most useful, sight must not be lost of the fact that growth also occurs at other levels of organization; by increases in size and/or number. This may range from molecular and organelle replication on the one hand to the hyperplastic growth of histological units such as acini on the other (Goss 1966).

2.3. Limitation of Further Considerations to Mammalian Growth

Much of our understanding of the nature of growth is based on the observation and analysis of the whole spectrum of animal life, exemplified in treatises such as On Growth and Form (D'Arcy Thompson 1917) and The Growth Process in Animals (Needham 1964). The literature is vast and continues to burgeon. Therefore in order to contain these introductory chapters and to relate them to the experimental work, further considerations are limited, with a few exceptions, to selected aspects of mammalian growth.

CHAPTER 3

THE DESCRIPTION AND ANALYSIS OF SOMATIC GROWTH

"Hoc iterum incrementum miro ordine distribuitur, ut in principio incubationis maximum est; inde perpetuo minuatur"

Haller

Our knowledge of the mathematical description and analysis of somatic growth is predominantly based on the large number of studies made on children this century, rather than on observations in other mammals (Tanner 1962). The pioneering work of Boas (1897), Crampton (1908) and numerous others since was crystallised by Tanner (1951) in a classic synthesis. This and later works (Israelsohn 1960 and Tanner 1962, for example) laid the foundations for a thorough understanding of the description and analysis of growth data.

The oldest published study of the growth of a child was made between 1759 and 1777 by Count Philibert de Montbeillard on his son. The animal stature measurements on the boy from birth to 18 years of age were expressed both as stature achieved each year, or distance, and stature gained each year, or velocity (Scammon 1927). This study illustrates two fundamental aspects of growth analysis: the difference between cross-sectional and longitudinal studies and the relative merits of velocity and distance data. These aspects are discussed further.

3.1. Cross-sectional and Longitudinal Studies

The Count's son was measured repeatedly over his entire post-natal growth period. This longitudinal method contrasts with the alternative cross-sectional method, whereby groups of children of different ages are each measured only once.

The two methods have their individual merits, but the important difference is that they do not give the same information and cannot be analysed in the same way. Cross-sectional studies are more easily and more rapidly completed, and give the necessary information to construct clinical standards, stature attained by normal children at given ages, for example (Tanner & Whitehouse 1959). Of necessity, longitudinal studies take many years to complete but yield the vital information on the velocity of growth with far fewer subjects than a cross-sectional study would yield with an equal degree of accuracy (Shuttleworth 1937). Longitudinal studies are essential, however, for the derivation of the variability of velocity, which is often of prime importance in the clinical assessment of growth (Tanner 1952). The not inconsiderable difficulties of obtaining an adequately large pure longitudinal sample result in many surveys becoming mixed longitudinal; that is, where not all subjects are followed for the specified period and are replaced by others entering during the course of the survey. Although formulae are available to permit the analysis of mixed longitudinal data, Tanner (1962) points out that it is frequently simpler to extract and report the pure longitudinal element. The growth data in this thesis are of a mixed longitudinal nature (Chapter 8).

3.2. Distance and Velocity Descriptors

While the distance curve of the Count's son's growth yielded a picture to be found on the doorposts of many homes, namely a

steady progression towards adult size, it was the velocity curve that showed the biologically significant features of his growth. Like all normal children his growth velocity steadily decelerated from birth to about the age of three years, was fairly stable until 12 years and underwent the characteristic adolescent growth spurt from 12 to 16 years of age. These changes in velocity, both positive and negative, are far more closely related to the underlying regulatory mechanisms and associated physiological and psychological changes than the distance achieved. Growth is a form of motion, and motion is clearly best characterized during progress, that is by velocity rather than by the final result; that is distance achieved.

Two further descriptors should be mentioned although their use has been minimal and the additional information they yield appears to be small (Tanner 1951). They are acceleration, which bears the same relationship to velocity, as velocity does to distance, and specific growth rate or multiplicative velocity. The specific growth rate relates velocity to the size of the growing organism and, for man at least, shows parallelism with the ordinary velocity curves and therefore yields no further information.

3.3. Growth Curves

Since growth is a form of motion it may be subjected to precise (often over-precise?) mathematical analysis. Curves may be derived deductively and compared to observed points to test hypotheses, or they may be empirically fitted. The constants in the resulting polynomials must have biological meaning. There is no value in an nth-termed, perfectly fitting equation in which no interpretation can be placed on the constants. The fundamental assumptions that must be made to permit curve fitting are not yet fully settled on biological grounds. To obtain a curve that describes a given population of individuals for comparison with

other populations, or as a standard against which individuals may be judged, a mean-constant curve must be derived (Merrell 1931). That is to say, the average curve must be derived from the individual curves, separately fitted, and not from the averages of individual measurements.

Whatever the nature of the functions derived all must recognize the essentially continuous nature of growth and changes in velocity that occur during the growth period. Consideration of the extensive literature (Medawar 1945; Israelsohn 1960; Kowalski & Guire 1974, for example) on this subject is beyond the scope of this thesis.

3.4. Chronological and Physiological Criteria

Individual animals, including man, vary considerably in the degree of physiological development achieved at the same chronological age. Thus one rabbit of 15 days of age may weigh 200g whereas another of the same strain, sex and nutritional status may weigh 250g. Alternatively one girl of 13 years may have passed her menarche, while another of that age has yet to reach this milestone. Crampton (1908) was one of the first to realise this phenomenon and introduced the term physiological age. The need for clearcut criteria of physiological age (also known as developmental maturity) has been increasingly recognized as the endocrine, biochemical and physiological aspects of growth have been explored.

Several systems of assigning physiological age have been developed. These have been developed mainly for clinical use and include:

Skeletal Age. To date this is the most widely used method for estimating physiological age. It is based on the radiological assessment of the numbers and shape of ossification centres in the hand and wrist (Greulich & Pyle 1959) or less commonly in the

hemi-skeleton. The use of skeletal age in paediatric endocrinology is well established (Mellman et al. 1959), but exactly what its endocrine control is and what its relationship is to other measures of physiological age is not yet established. Other than man full skeletal age standards are available only for the rat (Hughes & Tanner 1970b).

Dental Age. Dental age is based on the same principles as skeletal age, namely, the stages of formation and eruption of each tooth. In areas other than orthodontic practice its greatest value lies in the fact that it appears to be predominantly genetically determined, with environmental factors playing a small role (Tanner 1962). Animal studies are very limited in this field.

Morphological Age. The size of the whole or parts of the growing animal is the most extensively studied aspect of physiological age in many species. Thus body length or weight, head length or facial breadth, femur length or leg circumference, and so on are well documented. One of the problems in evaluating these gross characteristics is that each in turn consists of several components. The leg circumference, for example, encompasses bone, muscle and fat compartments and each of these may have different growth rates. For certain situations the study of the growth of one of these component parts may be the most appropriate, or it may be their allometric relationship that is revealing (Huxley 1932).

When sizes of one component are measured simultaneously in two or three dimensions (Hiernaux 1968) or in the same dimension of several different components, shape and shape changes are assessed. The full potential of the concept of shape age has yet to be developed.

Sexual Age. In view of the evolutionary significance of the age of achievement of reproductive ability the development of the sex organs and the outward pubertal manifestations in the secondary sex characteristics are of great importance, even though limited to a relatively brief period of the growth period. Testicular descent and vaginal opening in rats and breast development, pubic hair and genital development in man are biologically useful criteria which can be clearly related to sex steroid secretion and changes in end-organ responsiveness.

Functional/Metabolic/Biochemical Age. The preceding four categories of physiological age are all based on classical somatometric and somatoscopic techniques. To an increasing extent in the future physiological age will be assessed on functional, metabolic and biochemical criteria. The value of these approaches, which admittedly do not always span anything like the whole growth period, is exemplified in such studies as those on lung age in rabbits (Taeusch et al. 1972; Avery 1974), cartilage composition (Meyer 1969) and urinary creatinine excretion (Novak 1963).

Almost all functional age studies suffer from not being longitudinal in nature; from the labile nature of physiological measures compared to the stability of morphological measures and the error of reporting the few longitudinal studies in a cross-sectional fashion (Tanner 1962). Nevertheless, it must be recognized that many physiological studies are still contingent on invasive techniques that are incompatible with subsequent normal growth or on the sacrifice of the experimental animals, and are therefore cross-sectional. This is a problem encountered in this thesis, and as in other work there is no alternative at the present time.

3.5. Comparative Growth Studies

Growth studies in animals other than man are attended by a

number of difficulties that make execution, interpretation and inter-specific comparisons difficult.

The technique of measuring animals is largely undeveloped and only recently fully described and evaluated for the rat (Hughes & Tanner 1970a). Animals have fur and also they cannot cooperate like children. The realization of standardized, optimum nutritional, housing and breeding conditions is difficult, expensive and plagued by lack of data on species and strain-specific optimum conditions. Littersize is an important determinant of pre- and post-natal growth rates because of insufficiency of uterine vascular supply (Eckstein et al. 1955) and shortage of milk for large litters (Schultze 1954). Furthermore weaning in laboratory animals often occurs around a time of peak growth velocity and may therefore have a greater effect than the equivalent event in man (Tanner 1962). Many laboratory animals have high morbidity and mortality rates, rendering pure longitudinal series difficult to achieve, and then leaving open the question as to whether these animals represent the norm. Lastly, the shorter lifespan of laboratory and domestic animals compared to man renders many species more subject to seasonal fluctuations in growth.

These difficulties have made good quality (as defined earlier) growth studies the exception, with that on the rat by Hughes and Tanner (1970a; 1970b) standing out as a model for future work.

Data is available, chiefly for body weight, in several species other than man (summarized in Tanner 1962). These species may be divided into two groups: the primates, which appear very similar to man, and the non-primates, a group including mice, rats, rabbits, guinea-pigs, cows and sheep.

In man, chimpanzees and Rhesus monkeys birth occurs after the peak in weight growth velocity. In the non-primates birth occurs

shortly before the weight peak in guinea-pigs and sheep, and some considerable time before in rabbits, mice, rats and cows. Comparisons in terms of weight velocities may not be the most appropriate. In the rat, for example, peak weight velocity occurs some two weeks later than peak length velocity (Hughes & Tanner 1970a). This phenomenon also occurs in the rabbit, as will be shown in Chapter 8. For truly meaningful comparisons some type of overall developmental scoring system is required, such as that of Otis & Brent (1954), based on development of all organ systems in the mouse and man.

The differences between primate and non-primate are far more evident when the position of puberty, rather than birth, is examined in relation to growth rate. The primates attain puberty relatively much later than non-primates and undergo the adolescent growth spurt at this time. During this spurt the peak weight velocity may approach that of the intra-uterine peak. Although it is not nearly as dramatic as in man, there is some suggestion that the rat shows a pubertal growth change in that the decline in velocity of most linear measurements shows a diminution at or slightly after puberty (Hughes & Tanner 1970a).

A review of the details of published growth studies in the rabbit is given in Chapter 8.

CHAPTER 4

THE REGULATION OF GROWTH

"The very molecules appear inspired
with a desire for union and growth."

John Tyndall

A consideration of the factors and mechanisms regulating the rate of growth is constrained by the levels of organization that are examined. Ultimate control is centred in the genes through protein synthesis by replication, transcription and translation. Mitosis, tissue and organ growth and finally overall organism growth are the several levels at which further controls may be effected. This is a vast field and only a few topics germane to the overall theme of this thesis are elaborated.

4.1. Genes

Following gastrulation genes become progressively derepressed and their actions manifest, although our understanding is still largely based on accidents of nature rather than on normal development. Genes contribute to the regulation of early and late organogenesis (Danforth 1930) and also to the rate of early embryonic growth (Castle & Gregory 1929). The observations of Castle and Gregory are of particular interest to this thesis in that they were made on rabbits. They compared the ova, blastocysts and embryos of the large Flemish giant with those of the small Polish strain. In both strains ova were of the same size, but in the Flemish giant embryos cleave more quickly than in the Polish so that by 6 days the Flemish giant blastocyst is 47,8 μm in diameter

compared to the Polish 40,5 µm. This differential rate of growth persists through foetal and postnatal growth with the smaller strain retaining a lower growth rate and stopping growth earlier than the larger strain (Castle 1931).

In man the genes on the Y chromosome are responsible for the sex differences in skeletal development. These genes retard male skeletal age, thus permitting a longer growth period and therefore greater male size (Tanner et al. 1959).

4.2. Organ and Tissue Growth

Theories of regulation of organ and tissue growth include auto-inhibition, exogenous stimulation, local self-control and systemic overall control.

Weiss (1955) and Bullough (1965) have explored the role of inhibitory substances being produced by the organs whose growth they control, postulating an intrinsic growth capacity that is auto-inhibited. Opposing this concept of local control is that of a central regulator, possibly in the brain, that monitors and controls the growth of other tissues (Tanner 1963). Systemic control undoubtedly exists through endocrine, vascular and neurotrophic mechanisms, but how these factors relate to local physical and chemical factors is not clear.

In addition to these theories there are two factors that contribute to regulation. The one is a degree of genetic predetermination of size and growth rate. This genetic component has phylogenetic significance and is independent of transient environmental influences. The other basic determinant of organ and tissue growth is the functional demands made on the structure. This force can clearly operate only once the structure has reached a critical size so as to be able to function and respond to feed-back,

and so is superimposed on genetic and other control systems (Goss 1964).

4.3. Special Features of Local Regulation of Skeletal Growth

The great majority of our understanding of the local regulation of skeletal growth is derived from studies on long bone growth, whereas much knowledge of the endocrine and metabolic regulation derives from studies on cartilage from other sites, especially the costal region. A brief summary of the local factors influencing local bone growth is therefore included at this point and endocrine regulation is the subject of Chapter 5.

There is good evidence that the epiphyseal plates at each end of the long bones grow at rates determined by the local compression forces; growth being inversely related to longitudinal compression (Arkin & Katz 1956). Tension in the epiphyseal plate is also regulated by the overlying periosteum, which exerts a negative feed-back reaction force in response to being stretched by the proliferating underlying cartilage (Moss 1972).

4.4. Environmental Factors

Wolanski (1970) has stressed that writers frequently err by drawing too clear a distinction between genetic and environmental factors in the regulation of growth. The interface is usually blurred and little purpose is served by regarding factors as purely "nature" or purely "nurture", as it is the interplay of these factors and the adaptive responses of the organisms that are important.

In this context nutrition, day-length, ambient temperature, exercise, psycho-social environment and disease are relevant considerations in this thesis.

Nutrition. The consequences of undernutrition and maldistribution of dietary constituents have long been recognized in man (Howe & Schiller 1952) and in animals (Acheson & MacIntyre 1958). The effects of malnutrition are dependent on the phase of development during which it occurs and this phenomenon has been well documented in the rat (Widdowson & McCance 1960).

For laboratory and domestic animals the question of optimal nutritional conditions has already been alluded to above, and has been well-known for many years (Dunn et al. 1947). The only solution at present lies in carefully monitoring and reporting dietary constituents, feeding procedures and the resulting clinical and/or growth status of the animal colony.

Day-length. The stunting of rat growth following optic enucleation was shown by Browman in 1940. This and other evidence has been adduced to suggest that the seasonal fluctuations in growth of children are at least in part due to day-length (Marshall & Swan 1971). In chickens long hours of exposure and/or high intensities of illumination retard weight gain (Winchester 1964). In laboratory growth studies regular dark-light periods must therefore be maintained throughout the year.

Ambient Temperature. All other factors being equal extremes of ambient temperature retard growth in chicks (Brody 1945), cattle (Ragsdale et al. 1957) and rabbits (Johnson et al. 1957). The observations on rabbits provide yet another example of sexual differences in response to stress during growth in many species; the female always being more resistant (Tobias 1970; Williams et al. 1974). The body weight, body length and heart circumference of male rabbits reared at 28°C were 30% smaller than those reared at 9°C, whereas in the females there were no significant differences. Temperature plays an important role in determining the breeding patterns of rabbits as many strains have a low fertility during

hot summers and cold winters (Sawin 1950). An ambient temperature of 10°C to 19°C seems generally accepted as the optimum for rabbit production (Portsmouth 1962), although no definitive studies appear to have been done.

Exercise. Few firm conclusions can be drawn about exercise per se and its effects on growth (Tanner 1962). Whether it is reduced exercise that is a contributing factor to the reduction in growth that accompanies crowded living space is not clear in mammals. In continuously exercising animals, such as fish, reduction in opportunities for locomotion stunts growth (Brown 1946). The recommended floor surface area for rabbits is 0,23m^2/kg body weight (Adams et al. 1966).

Psycho-social Environment. Although the experimental evidence does not seem to be available for animals, the human growth response to an adverse psycho-social environment probably has analogies in other species. The classic observations of Widdowson (1951) showed that a congenial, loving atmosphere is more conducive to a child's growth than a rigid, hostile one, with other factors, including nutrition, being equal. Thus the handling and other environmental influences, such as noise and frequency of attendant's visits, to which animal colonies used for growth studies are exposed should be kept uniform, at least on theoretical grounds.

Disease. As in the previous paragraph, most experience concerning the effects of disease is based on human data. This is probably because animal studies are usually directed towards describing the normal situation, with diseased animals being excluded. In man acute illnesses, such as the exanthemata, have no detectable effect on growth (Tanner 1962). Chronic illness, such as congenital heart disease, retards somatic but not necessarily intellectual growth (Drash & Money 1968). Correction of the disease process results in varying degrees of "catch-up" growth depending

on the duration, severity and timing of the period of retardation (Tanner 1970).

4.5. Hormones and Their Target Tissues, with Special Reference to Growth Hormone.

The evolution of the thyroid hormones provides a clear illustration of the importance of examining hormones and their target tissues as an integrated complex so as to obtain an understanding of the control processes involved. Iodothyronines, for example, are present in biologically active forms as early in evolution as the protochordates and have remained unaltered in molecular structure into the most recent vertebrates (Salvatore 1969). These millenia of molecular stability plus the wide range of metabolic activities that the molecules initiate and control, exemplified by the host of processes under thyroid control in anuran metamorphosis (Eaton & Frieden 1969), imply that it is often the target tissues that are the major determinants of the outcome of hormone action. This role of the target tissues has just as much ontogenetic significance as it has phylogenetic significance (Barrington 1971), and therefore the function of the end-organ in the regulation of growth is examined further in Chapter 6.

Most of our current knowledge of the endocrine regulation of normal growth is derived from naturally occurring or experimentally induced hormone deficiencies or excesses and the resulting growth abnormalities. This somewhat paradoxical situation is reflected in the space allocated to a consideration of the role of normal hormone secretion during normal growth in two major paediatric endocrinology texts. Hubble (1969) devotes approximately three in 492 pages in his Paediatric Endocrinology and Wilkins (1965) two in 619 pages in The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence to this subject. Accordingly much of the

remainder of this chapter is based on pathological, rather than physiological, observations on growth hormone.

4.5.1. Growth hormone.

The importance of the pituitary gland in growth regulation was recognized at the beginning of this century when adenohypophyseal abnormality was linked to dwarfism and gigantism. In his book on the pituitary and its disorders Harvey Cushing (1912) suggested that the 'hormone of growth' was either over-secreted or under-secreted by the pituitary in states of abnormal growth. Evans and Long (1921) and Smith (1930) conclusively demonstrated a pituitary growth factor by producing gigantism in rats injected with anterior lobe extracts. These observations were developed into the hypophysectomised rat tibial epiphyseal plate bioassay for GH (Evans et al. 1943; Greenspan et al. 1949); this was an improvement on the 10 day body weight gain test (Marx et al. 1942). The molecule responsible for this cartilaginous proliferation was then purified and isolated (Li & Evans 1944) and has subsequently been fully characterized in several species (Grindeland & Ellis 1966; Li 1972). A peptide with GH-like activity has been synthesized (Li & Yamashiro 1970) but a fully active "core" of the natural molecule has not been identified (Kostyo 1974).

Growth hormone has a wide array of metabolic and anabolic actions. The metabolic actions involve carbohydrate and fat metabolism. They are related to the anabolic, or growth-promoting, actions on nucleic acid and protein synthesis insofar as the metabolic effects are protein-sparing. Much controversy still remains concerning the exact nature and mechanism of GH-induced metabolic events. The problem lies in the differences between acute and chronic administration, in vivo and in vitro observations and results obtained in hypophysectomised and normal animals (Turner & Munday 1975). A detailed consideration of the actions and regulation of GH is beyond the scope of this thesis, but some points are noted for comparison with somatomedin.

In liver and muscle from hypophysectomised animals GH stimulates both amino acid uptake and protein synthesis *in vitro* (Hjalmarson 1968; Clemens & Korner 1970; Kostyo 1968), whereas in muscle from normal animals only synthesis is stimulated (Reeds *et al*. 1971). Insulin stimulates both amino acids uptake and protein synthesis in normal muscle, but in muscle from hypophysectomised animals its effects on protein anabolism are minimal (Korner 1965). Furthermore, insulin and GH act synergistically on protein synthesis in normal muscle in vitro (Reeds *et al*. 1971). *In vivo* GH causes nitrogen retention in normal and hypopituitary subjects, with the earliest evidence of its effects being an increase in intracellular amino acids (Riggs & Walker 1960) and increased activity of liver Mg^{++}- activated DNA-dependent RNA polymerase (Jänne & Raina 1969). These effects begin within one hour of administration of GH. The protein anabolic effects of GH act in the same direction as those of insulin and the two hormones are mutually interdependent in the feed-fast cycle for protein homeostasis.

In contradistinction, the actions of GH on carbohydrate and fat metabolism are opposite to those of insulin. *In vivo* GH results in glucose intolerance and mobilises free fatty acids, although there is a very transient fall in blood glucose and free fatty acids with intravenous administration (Bornstein *et al*. 1968). In general, during fasting GH preferentially induces fat rather than glycogen utilization, and this is probably a protein-sparing manoeuvre as it decreases the need for gluconeogenesis (Glick 1969). Paradoxically, *in vitro* GH enhances glucose uptake in fat cells from hypophysectomised animals, but this effect is blocked by pretreatment with GH (Goodman 1965), and is therefore probably not physiologically relevant.

Growth hormone secretion is regulated by neural, hormonal and metabolic factors (Hunter 1972; Martin 1973). Stress,

exercise and slow-wave sleep result in bursts of secretion. In addition other apparently spontaneous day and night-time secretory peaks may occur and may be related to an underlying neural rhythm (Goldsmith & Glick 1970). A multiplicity of metabolic factors also influence secretion. Physiologically it is apparent that carbohydrate shortage stimulates and excess inhibits GH secretion. A variety of acute stimuli provoke secretion and these include insulin hypoglycaemia and arginine, 2-deoxy-D-glucose and L-dopa infusion.

Control is mediated by two hypothalamic hormones, namely GH-releasing hormone (Schally et al. 1967) and somatostatin (Brazeau et al. 1973, 1974; Hall et al. 1973). In addition to the established short feed-back loop of endogenous GH inhibitory auto-regulation (Hagen et al. 1972), a long feedback loop of SM inhibition has been postulated (Tanner 1972), but as yet there is no experimental evidence to support this. In fact, in the admittedly unphysiological situation of protein-calorie malnutrition where GH is elevated and SM depressed, this inverse relationship does not hold strictly during recovery with adequate feeding (Grant et al. 1973; Chapter 13 of this thesis).

One of the major difficulties in evaluating GH secretion is the wide and rapid fluctuations that plasma levels undergo during a 24 hour period. These fluctuations mean that single determinations of plasma levels are of comparatively little value and that secretory rates should ideally be measured. But because GH, like other peptide hormones, is degraded very rapidly to unidentified products in the body the precise secretory rates cannot be determined with certainty. The metabolic clearance technique gives a half-life of human GH of 27 minutes in adult and 17 minutes in rabbits (Parker et al. 1962), suggesting a turnover of 5mg/day. Continuous venous sampling methods have yielded somewhat lower secretory rates of 0,5 - 1,0mg/day (Frantz et al. 1969; Kowarski et al. 1971)

Although the association between growth and growth hormone has been recognized for over 60 years its exact role in regulating normal growth is still far from clear. The growth regulating role of GH is most easily considered during two phases: the intrauterine or antenatal phase and the postnatal phase of growth.

In 1947 Talbot and Sobel suggested on the basis of clinical observations in man that GH may not play an important function in growth during the first year after birth, and that growth occurred because of a carry-over of an intrauterine intrinsic growth capacity. While this view that human intrauterine growth proceeds independently of GH control has persisted largely unaltered until recently, there is good evidence concerning a definite role for GH in some mammalian species and little or no role in others. Evidence for a number of species will be reviewed.

Rat. Jost (1954) showed that decapitated foetal rats weighed 5 - 15% less than normal littermates, whereas brain aspiration, leaving the pituitary intact but removing the hypothalamus, results in 25 - 30% weight reduction (Swaab & Honnebier 1973). These ablation experiments must be interpreted with caution as radio-immunoassay studies reveal the first appearance of GH on the 19th day of gestation (Birge et al. 1967). Jost decapitated his foetuses at 15 - 18 days and Swaab and Honnebier theirs at 18 - 19 days. Equal circumspection must be applied to studies of pituitary content and concentration of GH, for these parameters simply reflect the nett balance of synthesis and secretion at one moment in time. Pituitary concentration rises from birth to approximately 80 days of age (Dickerman et al. 1972), although Birge et al. (1967) report a continuing rise of male levels into old age. Plasma GH levels steadily increase from 21 to 64 days of age with no sex differences (Dickerman et al. 1972). In vitro culture of pituitary glands has demonstrated that adult male pituitaries synthesize and

and release more GH than young male pituitaries, which in turn release more than weanling pituitaries(Burek & Frohman 1970). Pituitary GH synthesizing ability is inversely related to rat growth velocity. This illustrates the point that if GH secretion is causally related to growth velocity, then both secretory rate and utilization rate must be considered when evaluating this relationship.

Rabbit. Decapitation of rabbit foetuses on the 19th or the 23rd day of gestation does not significantly reduce body weight at 28 days gestation (Jost 1961, 1966), but does affect some pituitary target organs such as the thyroid which is reduced in weight (Bearn 1966).

Cattle. Ablative techniques have not been applied to the foetal calf, but spontaneous apituitarism in Guernsey cattle is associated with marked retardation of growth in utero (Kennedy et al. 1957). In Holstein bulls, neither plasma nor pituitary GH concentration were closely correlated with specific growth rate of body weight between birth and 12 months of age (Purchas et al. 1970).

Sheep. Destruction of pituitary function by either electrocoagulation (Liggins & Kennedy 1968) or stalk section (Liggins et al. 1973) results in foetal growth retardation. Measurements of plasma GH in the foetal lamb show no correlation with growth rate and fall rapidly at birth (Bassett et al. 1970). GH appears for the first time in the foetal pituitary at 50 days in the 147 day gestation period (Stokes & Boda 1968).

Rhesus monkey. The only study of experimental hypopituitarism appears to be that of Chez et al. (1970). These workers implanted a Yttrium-90 source in the pituitary and measured birth weights. Although mortality was high and numbers small these authors concluded that foetal hypophysectomy had no effect on birth weight.

Pig. Pituitary GH content has only been measured by bioassay (Baird et al. 1952; Nalbandov 1963). These workers have shown pituitary GH concentration to be greater in a strain of rapidly growing pigs than that in a slower growing strain. Serum GH levels fall with increasing age, weight and length (Siers & Hazel 1970).

Man. Some light has been shed on the role of the human pituitary and hypothalamus by studying the various anomalies of development and function that occur. In all, except isolated GH deficiency, the cause of the maldevelopment may have direct, nonendocrine effects on the growth of the foetus, unrelated to the coincidental pituitary defect; thus caution must be exercised in concluding a causal relationship.

Spontaneous foetal decapitation by amniotic bands (4 documented cases) and apituitarism (4 documented cases) is usually associated with normal birth weight (Reid 1960; summarized in Liggins 1974). In these cases adrenal and thyroid gland hypoplasia confirmed that the adenohypophyseal function was deficient. Many authors have long held that the human anencephalic achieves normal length and weight at term (Rimoin et al.1968). A recent careful assessment of 122 anencephalic foetuses showed at 30% reduction in weight velocity in anencephalics compared to normals (Honnebier & Swaab 1973) and another study showed a 25% weight reduction in anencephalics at 40 weeks gestation (Liggins 1974). Further evidence for abnormal growth in the anencephalic neonate is given by a quantitative histological study of the organs of 59 anencephalic newborns (Naeye & Blanc 1971). In the 37 prematurely born infants all organs were subnormal in size and a diminished number of cells for gestational age. In the 22 term or post-term infants some organs were of normal size, but most had a diminished number of cells. The hypothalamus was absent in all these cases, but the pituitary was found in all those full-term infants in whom

a careful search was made, but in only one of the premature infants. When found the anterior lobe ranged from one-tenth to one-half normal size and the concentration of acidophils was much lower than in the control infants. The finding of acidophils, at least in some cases, is in keeping with the detection of plasma growth hormone in anencephalics (Grunt & Reynolds 1970).

The anencephalic foetus, with multiple endocrine deficiencies, is a less suitable model for the study of GH dependent growth than the foetus with pure GH deficiency. Laron and Pertzelan (1969) have shown that in both familial isolated GH deficiency and in the familial dwarfism with high GH levels syndrome (Laron et al. 1966a) the birth weights of 12 of 14 cases were normal. It is of interest that 8 of the 14 were significantly short at birth, suggesting that GH, if it plays any role, has a greater influence on skeletal than on soft tissue growth.

The human pituitary acidophils appear at about the 9th to 10th week of gestation (Gitlin & Biasucci 1969) at which time the GH content becomes measurable by radioimmunoassay (Kaplan & Grumbach 1962; Matsuzaki et al. 1971). At this time GH can also be detected in the plasma (Kaplan et al. 1972). Pituitary levels rise to a term level of approximately 1,5mg/gland.

Human GH does not cross the placenta (Gitlin et al. 1965; Laron et al. 1966b). Rimoin et al. (1966) report a GH-deficient mother bearing a GH-deficient infant of normal length and weight at term. Maternal GH therefore plays no direct role in foetal growth. Jost (1966) has reviewed the evidence for the action of maternally administered GH on the foetus and concludes that it has little or no effect.

Lastly, human placental lactogen must be considered. This

peptide is synthesized by the placenta and has an amino-acid sequence very similar to growth hormone. At term umbilical venous serum has low levels compared to maternal serum and considerably higher concentrations are required for any growth-stimulating effects to be manifest (Kaplan & Grumbach 1965).

To summarize, the role of GH in the regulation of human foetal growth is probably significant when parameters such as cell number and size are considered and equivocal for parameters such as body length and weight. In other mammals such as the rabbit and monkey it appears to play no role, in rats a small role and in cows and sheep a significant role. However, all these studies have been based on body size, and observations on cell number and size are clearly needed before definite conclusions can be drawn.

In contrast to the confusion surrounding GH, the role of thyroxine in intrauterine skeletal development is quite unequivocal. Deficiency retards bone maturation, but has a less clearcut effect on body weight. The human hypothyroidic foetus is larger than normal at term while the lamb is smaller than normal. This is probably because of a smaller placental transfer of thyroxine in the sheep (Fisher et al. 1964; Hopkins & Thorburn 1971). GH plays a permissive role for the growth-stimulating actions of thyroxine (Leblond & Carriere 1955).

In the postnatal phase of growth there is no doubt about the importance of normal GH secretion for normal growth. This statement is based, as in the antenatal situation, mainly on pathological and experimental situations; the exact relationship of postnatal growth hormone secretion to growth velocity is still far from settled.

By hypophysectomising rats at successive intervals after birth it was shown that growth rate declined, but did not cease after

operation (Asling et al. 1950). Growth was restored by the injection of GH (Simpson et al. 1950). It is important to note that early hypophysectomy (6 days after birth) had less of an effect than late hypophysectomy (28 days after birth). In other words, factors regulating growth, other than GH, are relatively more important soon after birth than later in life. Identical conclusions can be drawn from Vezinhet's study on the rabbit (1968).

These experimental results are in agreement with observations on postnatal growth in GH-deficient children. Soon after birth growth retardation, although present, is slight and only becomes obvious at 2 or 3 years of age (Aarskog 1963). This progressive slowing of growth confused Seckel (1960) and caused him to conclude that GH was not necessary for normal growth in the first years of life after birth.

The postnatal ontogenesis of plasma GH levels is documented only in man. These measurements are beset by the difficulties of obtaining secretion rates and the lack of correlation between single plasma levels and secretory rate. All studies reported are cross-sectional. In the first year of life fasting levels are elevated above the subsequently similar prepubertal, pubertal and adult levels (Kaplan et al. 1968). The mean maximum rise in GH following insulin hypoglycaemia is three-fold higher in the adult (32,5ng/ml) than in the child (11,5ng/ml) and appropriate sex steroid administration can convert the child's response to adult levels (Youlton et al. 1969; Abrams et al. 1971). At puberty GH response to hypoglycaemia is greater than during prepubertal growth (Kaplan et al. 1968). The work of Finkelstein and his colleagues(1972a) has provided the best evidence to date that GH secretion rate is related to growth rate. Both the mean 24 hour secretion rate and the mean duration of secretory pulses were correlated with growth rate, but numbers were very small, 17 subjects ranging from 8 to 62 years of age, and the study was

cross-sectional. The half-life of GH in adults is about 25 minutes and in children 12 minutes (Cornblath et al. 1965; Glick et al. 1964).

The primate adolescent growth spurt (discussed in Chapter 3) is believed to be caused by increased androgen secretion, rather than by GH (Thompson et al. 1972) and although the spurt occurs in hypopituitarism (Rimoin et al. 1966), small amounts of GH appear to be necessary for this to occur (Scow & Hagan 1965). In hypopituitarism GH and androgens act synergistically (MacGillivray et al. 1974) and in hypophysectomised rats GH and thyroxine act synergistically (Becks et al. 1946).

After all skeletal growth has ceased GH secretion and response to provocative stimuli persists, even into old age (Abrams et al. 1971), but its metabolic role is not clear. In the adult dog GH increases bone matrix turnover (Harris & Heaney 1969).

4.6. Concluding Remarks

This chapter opened with an heuristic quotation that suggested an underlying, inherent capacity for growth in the tissues of the living organism. The sections of the chapter have highlighted some of the numerous observations that have been made in efforts to understand the processes that regulate growth. It is abundantly clear that there is both a balance and an interplay between "nature" and "nurture". Within the organism controls exist at many levels - nuclear, cytoplasmic, intercellular, tissue, organ, hormonal, vascular and nervous and between each and all of these levels of control an intricate array of positive and negative feedback systems operates. A host of environmental influences act on, modulate and modify the internal controls. Ranging from physical influences, such as day-length and even gravity, through chemical factors, such as diet and air-pollutants, to psychological determinants, such as maternal

attitudes, these environmental influences must be excluded as far as possible when internal controls are being investigated.

The investigation of the enigmatic, often paradoxical, relationship between the so-called growth hormone and growth acquired a new dimension in 1957. In that year a new hormone that mediated the growth-promoting actions of GH was described (Salmon & Daughaday 1957). Subsequent studies sparked off by this discovery are now opening up an additional level of organization at which growth is regulated. The following chapter is devoted to a discussion of this new hormone and a family of associated hormones.

CHAPTER 5

SOMATOMEDIN

"I must apologise for sending you so an imperfect account of this substance .... it may induce others to examine the subject with more attention."

Dr. Bostock 1827

5.1. The Sulphation Factor Hypothesis

In 1957 Salmon and Daughaday showed that serum from GH-treated hypophysectomised rats stimulated the synthesis of chondroitin sulphate in cartilage in an *in vitro* incubation system, while serum from untreated hypophysectomised rats, GH itself and a combination of both failed to stimulate synthesis. They postulated that a GH-dependent agent in serum was responsible for mediating the actions of GH on cartilage *in vivo* and named this agent "sulphation factor".

The work on which this hypothesis was based was preceded by a number of important observations on cartilage sulphate metabolism. Dziewiatowski (1949) showed that ^{35}S-sulphate was concentrated in the growing cartilage of young rats, and later showed that this concentration of sulphate was in the mucopolysaccharide, chondroitin sulphate (Dziewiatowski 1951; Dziewiatowski 1954). This sulphate incorporation into cartilage was then shown to be dependent on intact pituitary function, being drastically reduced by hypophysectomy, and restored by GH administration (Ellis *et al*. 1953; Denko & Bergenstal 1955). Daughaday's laboratory examined the effects of hypophysectomy in

detail. *In vivo* uptake of radioactive sulphate fell progressively after hypophysectomy, reaching a nadir 20 days after operation. Not only epiphyseal cartilage but also nasal septum cartilage was shown to actively take up sulphate in a GH dose-dependent manner. Most significantly, sulphating ability was shown not to be entirely specific to GH as thyroxine also stimulated sulphate uptake (Murphy *et al*. 1956).

These *in vivo* experiments were paralleled by *in vitro* work on cartilage sulphate uptake. Layton demonstrated uptake of ^{35}S-sulphate by chondrosarcomatous tissue (1949) and chick femoral cartilage (1950) in culture. In 1952 Boström and Mansson described a method for measuring ^{35}S-sulphate incorporation into calf costal cartilage. It was a modification of this technique that Salmon and Daughaday used and developed into a formal bioassay (Daughaday *et al*. 1959).

The original sulphation factor hypothesis has been extensively examined and confirmed by others including Salmon (1960a; 1960b), Almquist (1960), Kogut *et al*. (1963), Hall (1970), Van den Brande *et al*. (1971) and Van Wyk *et al*. (1972).

The range of biological effects of sulphation factor has been shown to extend well beyond cartilage sulphation and to include stimulation of thymidine incorporation into DNA (Daughaday & Rheeder 1966), uridine incorporation into RNA (Salmon & DuVall 1970a), proline incorporation into hydroxyproline of collagen (Daughaday & Mariz 1962), leucine incorporation into muscle and cartilage protein (Salmon & DuVall 1970b), glucose oxidation in fat (Hall & Uthne 1972) and to inhibit lipolysis (Underwood 1972). Purification procedures could not separate these activities into different molecular species. Therefore in 1972 the major workers in the field suggested a general term to replace the large number of different operational

designations "sulphation factor", "thymidine factor" and so on. The term suggested was "somatomedin"; "somato-" to connote the relationship with somatotrophin and also to the soma; and "-medin" to indicate that it mediates at least some of the actions of growth hormone (Daughaday et al. 1972). "Somatomedin" has now completely replaced "sulphation factor" in the literature.

5.2. Bioassay of Somatomedin

All current bioassay methods are modifications of the Salmon and Daughaday method (1957) which was based on the active incorporation of radioactive inorganic sulphate into chondroitin sulphate of cartilage in vitro.

As in all bioassays samples of serum (or plasma) of unknown, or relatively unknown, potency are compared with a standard preparation. For the purposes of the assay it is assumed that the unknowns differ only quantitatively from the standard. That is to say, it is assumed that the unknowns behave as dilutions, or concentrations, of the standard. Tests of this assumption, parallelism of standard and unknown dose-response curves, are part of the statistical evaluation of the assay.

A standard preparation is needed in each assay because of the natural variability of biological responses of the experimental substrate and because of inter-assay variability in technique. Optimum bioassay results are obtained by using tissue from one animal and measuring its response to several symmetrically spaced dilutions of standard and unknowns. Finney (1964)has fully defined the conditions and statistical evaluation of acceptable bioassays.

Somatomedin bioassays reported in the literature have not always been stringently ideal. The method of Daughaday et al. (1959)

used ten costal cartilage segments from each of eight rats and distributed these at random among the test samples. Chesley (1962) used but a single dilution by which to make comparisons between standard and unknowns. The comparison of statistically optimal assays of the same design from different laboratories is not possible because of the lack of an international reference standard (IRS). No IRS has been available up to the present time (Bangham 1975, personal communication) and so each laboratory sets its own local standard. This is usually a serum pool from one or more healthy, "normal" adults or from a blood bank, aliquotted and deep frozen. At least one author has used his own serum collected freshly for each assay run! (Chesley 1962).

These strict requirements for adequate design and analysis and the laborious and time-consuming nature of a SM bioassay have resulted in the development of a number of modifications of the assay. The important features of these are discussed, and summarized in Table 1.

The prototype assay used costal cartilage segments from rats hypophysectomised at 25 days of age. Uptake of labelled sulphate was shown to be linear in response to the logarithm of serum dose at concentrations between 1,7% and 10%. The index of precision for a reference pool used in the assay was 0,25 and 0,28 on two occasions (Daughaday et al. 1959). Almquist (1961) improved the precision of this assay by using one piece of cartilage from each of six hypophysectomised rats for each dose of serum to be assayed, so that each sample was assayed in sextuplicate. This reduced the index of precision to 0,14. A similar design was later used by Van Wyk et al. (1969) and Van den Brande et al. (1971).

The expense and problem of hypophysectomy was obviated by Yde (1968) who used 21-27 day old rats fasted for 48 hours before sacrifice and use in the assay. Sensitivity of fasted cartilage

TABLE 1 Summary of Current Approaches to Bioassay of Somatomedin

Authors	Tissue	Isotope	Total Incubation Time (hours)	Range of Sensitivity (% Serum)	Precision (index of precision)
Daughaday et al. 1959	Hypox. rat costal cartilage	$^{35}SO_4$	24	1,7 - 10	0,25 - 0,28
Almquist 1961	Hypox. rat costal cartilage	$^{35}SO_4$	24	1 - 20	0,14*
Van den Brande et al. 1971	Hypox. rat costal cartilage	$^{35}SO_4$	48	0,1 - 8,3	0,38
		$^{3}HTdr$	48	0,1 - 8,3	0,47
Van Wyk et al. 1969	Hypox. rat costal cartilage	$^{3}HTdr$	48	0,25 - 18	0,3
Yde 1968	Normal fasted rat costal cartilage	$^{35}SO_4$	24	5 - 15	0,209*
Alford et al. 1972	Normal fasted rat costal cartilage	$^{35}SO_4$	88	5 - 20	0,18
Fujisawa 1964	Hypox. puppy costal cartilage	$^{35}SO_4$	24	5 - 40	n.q.
Van den Brande & Du Caju 1971	Normal pig costal cartilage	$^{35}SO_4$	48 or 73	5,6 - 45,2	0,15
		$^{3}HTdr$	48 or 73	5,6 - 45,2	0,19
Hall 1970	Chick embryo pelvic leaflets	$^{35}SO_4$	6	2,5 - 40	0,20
Garland et al. 1972	Chick embryo pelvis isolated chondrocytes	$^{3}HTdr$	24	0,6 - 6,0	n.q.
Uthne 1973	Human glia-like cells	$^{3}HTdr$	48		
Wasteson et al. 1973	Human foetal lung fibroblasts	$^{35}SO_4$	6 to 50	3 - 50	n.q.

Note: Hypox. indicates hypophysectomised
* obtained after rejecting non-valid assays
n.q. indicates "not quoted" in reference paper.

was shown to be nearly equal to that of hypophysectomised cartilage. After rejection of those assays which were not statistically valid (22% of all assays) the mean index of precision was 0,209. Alford et al. (1972) simplified the Yde technique by digesting the cartilage after incubation and adsorbing the chondroitin sulphate onto paperstrips before scintillation counting. The problem of inter-animal variation was overcome using a single hypophysectomised puppy per assay (Fujisawa 1964). However, expense and the shallow dose-response curve have precluded further use of this technique. By preincubating hypophysectomised rat costal cartilage for 24 hours before addition of isotope Van den Brande et al. (1971) achieved maximum sensitivity, with the linear portion of the dose-response curve beginning as low as 0,1% volume/volume serum dilution. This assay also introduced simultaneous measurement of $^{35}SO_4$ and ^{3}HTdr uptake.

The advantages of a single, non-hypophysectomised animal cartilage source, enhanced sensitivity from preincubation and dual isotope uptake were combined in the porcine costal cartilage assay (Van den Brande & Du Caju 1972, 1974). By using a schedule of post-incubation in isotope solution variations in endogenous sulphate and thymidine pools are rendered unimportant and the labour involved in the assay is reduced. This assay has been extensively evaluated recently by Phillips et al. (1974) and was the assay used for the majority of studies in this thesis.

The expense and length of the rat assays prompted Hall (1970) to develop a simpler, less time-consuming assay. Hall used the pelvic leaflets of 11 to 12 day-old chick embryos to measure the incorporation of $^{35}SO_4$. This assay was based on the observation by Adamson and Anast (1966) that the variation in uptake between embryos of the same age was small and therefore, the precision of the assay compared favourably with others, the index of precision

being around 0,20. Hall's method was further simplified by the observation that omission of weighing of individual pelvic rudiments did not lower precision, and in fact actually seemed to improve precision for the overall mean of all assays in the laboratory of Schimpff and Donnadieu (1973).

The need for large quantities of homogeneous tissue to use in the assay has led at least three groups of workers to examine isolated cells in culture as possible substrates. Garland et al. (1972) isolated chick embryo pelvis chondrocytes by enzymatic digestion and measured ^{3}HTdr incorporation. Uthne (1973) used a line of human glia-like cells, obtained at brain biopsy, to measure ^{3}HTdr incorporation. Wasteson et al. (1973) used the same glia-like line and pulmonary fibroblasts in culture to assay sulphated polysaccharide synthesis. Of these three systems only Uthne's has been applied as a general assay system and has proved vital in the identification of the different SM species isolated to date (see below).

Of the several commonly used assay systems the hypophysectomised rat assay with preincubation has the greatest sensitivity, the porcine assay the greatest precision, and the chick pelvic assay is the least time consuming.

The SM bioassay technique has been applied successfully to a number of body fluids. Serum or plasma of several species has been investigated. In addition, serum extracts have been assayed for activity during purification procedures by several authors (see below). To date there have only been two reports on SM activity in other fluids; that of the author on adult cerebrospinal fluid (Beaton et al. 1975) and that of Anderson and Kastrup (1973) in human amniotic fluid. Attempts to show activity in urine extracts have been unsuccessful (Van den Brande 1972, personal communication).

All biossay systems are non-specific in that they measure the end-result of the addition of native plasma or plasma extracts to

metabolizing tissue. Not surprisingly therefore, a wide variety of factors influence the assay. Some of these have been extensively investigated, amino acid content and composition of the medium, for example. This can be controlled by addition of optimum amounts of amino acids to the incubation medium. Other factors, such as possible cofactors or inhibitors have only recently been suggested and remain quite unknown in importance and influence on the assay systems.

Amino acids stimulate sulphate uptake. In the rat cartilage serine, glycine, glutamine, valine, and threonine are particularly important (Koumans & Daughaday 1963, Almquist 1961; Salmon & Daughaday 1958; Boström et al. 1955b). In the chick glutamine and serine seem to be the most important (Hall 1970). All these amino acids and others, cystine,methionine, phenylalanine, tyrosine, arginine, lysine, histidine, tryptophan, isoleucine and leucine are added in approximately the concentrations found in normal human serum (Daughaday & Reeder 1966; Hall 1970; Van den Brande 1973). There appears to have been no investigation of the effects of adding amino acids in concentrations appropriate to the species of serum being assayed. Van den Brande et al. (1974), for example, used the same amino acid composition in assaying sera of animals as different as turtles, pigeons and dogs.

Homocystine stimulates cartilage sulphation and this has been suggested as a possible mechanism of the skeletal abnormalities found in homocystinuria (Dehnel & Francis 1972).

Glucose is added to the incubation media of all assays. The work of Herington et al. (1972) suggests that glucose is not acting only as an energy source, but also as a substrate for polysaccharide chain formation in embryonic cartilage.

Most hormones at physiological concentrations, and some even at supra-physiological concentrations, have little effect on the

SM assay. Salmon and Daughaday's (1957) original observations showed that GH in concentrations as high as 50 µg/ml had no effect on sulphation *in vitro*. At higher concentrations there is some effect of GH apparent on rat cartilage and more so on muscle (Salmon & DuVall 1970b). In chick pelvic leaflets human GH has no effect in concentrations up to 1,0 µg/ml (Hall 1970), but ovine GH stimulates significant sulphate incorporation in cultured chick chondrocytes in a dose-related linear response between concentrations of 0,01 and 1,0 µg/ml. (Meier & Solursh 1972). In spite of these apparently contradictory findings it must be stressed that at physiological concentrations GH does not stimulate SM (Daughaday 1971a). Human placental lactogen and prolactin similarly have no significant effect (Murakawa & Raben 1968).

In rat cartilage insulin does cause significant stimulation of sulphate uptake, leucine incorporation into chondromucoprotein, uridine incorporation into RNA and thymidine incorporation into DNA (Salmon 1960a; Salmon *et al*. 1967; Salmon *et al*. 1968). Supra-physiological concentrations of insulin and failure of highly specific insulin antibodies to reduce sulphating potency of serum (Salmon & DuVall 1970b) add further support to the view that insulin has an insignificant effect in the bioassay. Chick cartilage is completely refractory to insulin stimulation (Hall & Uthne 1971). Glucagon has no effect on cartilage sulphation *in vitro* (Salmon *et al*. 1968).

Glucocorticoids inhibit sulphate incorporation (Layton 1951; Eisenbarth & Lebovitz 1974; Tessler & Salmon 1975) and hydroxyproline synthesis (Daughaday & Mariz 1962) by cartilage *in vitro* and *in vivo* (Ebert & Prockop 1967). Whilst testosterone stimulates sulphation *in vivo* it has very slight effects *in vitro* (Salmon *et al*. 1963). Oestrogens inhibit sulphate uptake by normal cartilage (Herbai 1971b) but have no effect in cartilage from

hypophysectomised animals (Herbai 1971b; Wiedemann & Schwarz 1972).

A variety of non-hormonal factors also act on sulphation *in vitro*. These include the ionic composition of the medium (Adamson *et al*. 1964), pH (Klebanoff *et al*. 1958), straight chain fatty acids which inhibit sulphation (Delcher *et al*. 1973; Eisenbarth *et al*. 1973), heterocyclic compounds such as indoles (Liberti & Rogers 1970) and salicylates (Boström & Mansson 1955a). Manipulations of cyclic AMP activity, other than those acting directly on adenyl cyclase (discussed below), have resulted in contradictory results. Hall (1972) added dibutyrl-cAMP to chick pelvis and found no increase in sulphate uptake with increasing concentrations, while Adamson (1970) showed a 20-25% increase in amino acid transport, incorporation into protein and in sulphate incorporation in chick pelves. Uthne *et al*. (1974) blocked amino transport in muscle with the phosphodiesterase inhibitor theophylline, but showed that in the presence of a purified SM preparation this block did not occur.

The observation that free fatty acids inhibit SM *in vitro* is of considerable theoretical importance. If this effect can be shown *in vivo* it would suggest a mechanism whereby the lipolytic and glucose mobilizing effects of GH might be dissociated from its growth-stimulating effects. Thus stress, such as starvation, elevates GH levels resulting in lipolysis. This would, in turn, block SM activity so that substrate demands for growth are reduced. Protein-calorie malnutrition offers the opportunity to explore this relationship *in vivo*, and work in this field is reported in Chapter 13.

In recent years several authors have suggested that the reduced SM levels observed in fasting and malnutrition may be the consequence of a reduction of net serum activity due to inhibitors, rather than a reduction in absolute SM activity. Salmon (1972)

showed that there was a non-dialyzable heat-labile inhibitor in some but not all fasted rat sera. While Van den Brande has shown the presence of inhibitors in marasmic patients' serum (Van den Brande & Du Caju 1973; Van den Brande et al. 1974a)Grant et al. (1973) could not demonstrate inhibition by mixing experiments in two cases of kwashiorkor. A thermolabile inhibitor was demonstrated in normal adult human serum in the isolated chondrocyte system (Garland et al. 1972). Inhibitors of a molecular weight between 25 000 and 50 000 have been isolated by ultrafiltration of the perfusate of GH-stimulated isolated liver preparations (Dehnel & McConaghey 1974). The nature, origin and possible physiological role of these inhibitors are not clear at the present time.

Finally, it has been suggested that the loss of SM activity during purification procedures may possibly be due to loss of cofactors necessary for full expression of activity, rather than loss or degradation of the SM molecule(s) (Van Wyk et al. 1974, in the discussion section). This hypothesis is supported by the observation that mixing purified fractions with native serum of known SM potency results in a synergistic rather than additive increase in potency of the mixture (Van den Brande 1974, personal communication).

In summary, the SM bioassay has been refined and facilitated in the past 18 years by a number of methodological and statistical modifications. The important characteristics of these bioassays have been analysed and the multitude of non-specific factors that may influence the assay discussed. All assays suffer from non-specificity, relatively low sensitivity, variable precision and in being time-consuming. The lack of an international reference standard limits the value of comparison of results from different laboratories. Against all of these disadvantages must be weighed the fact that the bioassay yields unique information. The biological outcome of the net effects of serum can be accurately

measured under precisely controlled *in vitro* conditions. As Daughaday (1971b) has pointed out "progress in characterizing the action of a hormone on a tissue demands the removal of the hormonal target from the complex environment of the whole animal to the controlled conditions of *in vitro* incubation."

The application of radioreceptor assays and radioimmunoassays to SM means much more sensitive, precise and rapid results. However, these assays require the use of serum extracts or isolated membranes or measure only immunological activity of the molecule. As in the case of GH, these assays will yield vast and invaluable quantities of information concerning secretion, control and actions of SM. However, they cannot provide the information regarding the action of native serum on intact tissue in culture that the bioassay can offer. While good agreement between membrane binding ability and biological action has been demonstrated for several polypeptide hormones (Cuatrecasas 1972, Lefkovitz 1973) this is not yet the case for SM. With the availability of the three different types of assay, measuring three different aspects of SM action, it is important to select the appropriate assay(s) for the investigation of a particular situation.

5.3. Physical Properties and Purification of Somatomedin

Purification of SM has been slow as a consequence of the low biological activity in serum and the time-consuming bioassays that are needed at every step to identify the purification products. Early studies suggested that skeletal muscle and liver may be rich sources of SM (Boström *et al.* 1955c; Hall & Bozović 1969; Bozović *et al.* 1970; Hall *et al.* 1969, 1970; Salmon 1972), but these findings were not confirmed by Daughaday (1971a) and have not been pursued. Plasma or serum are the richest sources of SM and all purification has been performed starting with these materials.

Early work showed that SM is remarkably stable, retaining

activity after heating to 60°C for 15 minutes (Salmon & Daughaday 1957) and after prolonged storage at room temperature (Almquist 1961). Activity is lost in alkaline solution at 80°C for 30 minutes (Van den Brande 1973) but is retained at 100°C for 15 minutes in acid solution (Salmon & DuVall 1970b). Freezing, thawing and lyophilizing have no effect on activity (Daughaday & Kipnis 1966). Destruction of activity by pronase (Van den Brande 1973) or trypsin (Salmon 1972) digestion confirmed the peptide nature of the active molecule.

The first purification studies used small quantities of normal and acromegalic human and normal rat serum. Somatomedin was retained by both dialysis tubing (Salmon & Daughaday 1958) and ultrafiltration membranes with a pore size of 50 000 daltons (Van Wyk et al. 1971). Application of serum to Sephadex gels showed a higher molecular weight for SM than that of GH in serum (Bala et al. 1970; Van Wyk et al. 1969). In acid-ethanol SM activity appears at much lower molecular weights suggesting that it is normally bound to a carrier molecule (Hall & Uthne 1972; Van Wyk et al. 1971). These heating and acidifying steps alone or combined with filtration or Sephadex chromatography, resulted in 100-500 purification, but were quite unsuitable for large-scale purification. Acid-ethanol extraction was applied therefore as a first step and yielded satisfactory recovery of samples which paralleled the dose-response curve of the native plasma. Further purification of this acromegalic plasma by gel chromatography, ion-exchange chromatography, electrofocusing and high voltage paper electrophoresis continued to yield SM activity with progressive purity (Van Wyk et al. 1971).

Large scale purification of thousands of kilograms of outdated human plasma was therefore begun in 1971. In successive steps through Cohn fraction IV, acid-ethanol extracts and Sephadex G75 chromatography a single peak of activity at Kd 0,64-0,77 with

20 000 - 50 000-fold purification was achieved (Hall 1972). From this point two separate paths have been followed. Hall (1972) used ion-exchange chromatography on Dowex 50 followed by successive high-voltage electrophoresis on paper at pH 6,5, pH 3,6 and pH 2,0, achieving a 1- to 2-million-fold purification. This fraction stimulates chick pelvic sulphation, is a neutral peptide and is now termed somatomedin A (Uthne 1973). Isoelectric focusing of this fraction yielded a more acidic second peptide of slightly different molecular weight, but markedly different biological activity. Uthne et al. (1973) named this somatomedin B and showed that it stimulated thymidine incorporation into human glia-like cells, whereas somatomedin A did not. In his laboratory Van Wyk followed another purification procedure. The Sephadex G75 fraction of the acid-ethanol extracts was purified by continuous free-flow liquid electrophoresis followed by cation-exchange chromatography on carboxymethyl-cellulose. Electrofocusing between pH 7 and pH 10 of the most active fraction was followed by preparative electrophoresis at pH 2,2 on polyacrylamide gel and finally passage through Sephadex G75. Van Wyk's fractions yielded a basic peptide active on sulphation in rat cartilage but with no activity on chick cartilage. This is somatomedin C (Van Wyk et al. 1974).

Comparisons of the amino acid content of the most purified fractions of somatomedins A and C show differences. Whether these differences indicate two distinct peptides or simply chemical alterations as a result of the purification procedures is not known (Hall & Van Wyk 1974). Table 2 summarizes current concepts of the three somatomedins.

TABLE 2 Summary of the Characteristics of Somatomedins A,B,and C.

Somato-medin	Molecular Weight	Peptide Character-istics	Bioassay	Radio-receptor Assay	Radio-immuno-Assay
A	7500	neutral	chick pelvis sulphation	+	-
B	4500	acidic	human glia-like cell thymidine uptake	+	+
C	7500	basic	rat costal cartilage sulphation	+	-

Note : + denotes reported in the literature and
- not reported.

5.4. Radioreceptor and Radioimmunoassays for Somatomedin

The primary step in the action of polypeptide hormones is an interaction between part of the hormone molecule and the target cell membrane (Freychet et al. 1971). On the basis of this interaction several polypeptides, including insulin, growth hormone and adrenocorticotrophin, can be assayed by radioreceptor techniques (Lefkovitz et al. 1970; Tsushima & Friesen 1973; Freychet et al. 1971). A radioreceptor assay has been described for both somatomedin A (Hall et al. 1974) and somatomedin C (Van Wyk et al. 1974), based on the demonstration of competition of SM and ^{125}I-insulin for receptors in fat, cartilage and liver cell membranes (Hintz et al. 1972a).

Posner (1974) described a simple method for the preparation of particulate membrane fractions of human placenta, and the two radioreceptor assays use modifications of this preparation as the binding substrate. The somatomedin A assay requires purified ^{125}I somatomedin A and the somatomedin C assay ^{125}I - SM - C for competitive binding to the membranes.

The availability of larger quantities of somatomedin B (Uthne 1973) has made the development of a radioimmunoassay possible (Yalow et al. 1975). Prepared from Cohn fractions of human plasma this assay detects somatomedin B in 1 : 20 000 dilutions of human plasma, equivalent to concentrations of 25 pg/ml. Although there is only a single publication on the radioimmunoassay to date a wealth of new information has been acquired that was previously not accessible with the bioassay. In unextracted plasma somatomedin B is associated with plasma proteins at least as large as the gamma-globulins and has an electrophoretic mobility similar to the alpha-globulins. It appears only in primate plasma being detectable in human plasma at 1 : 20 000 and in monkey plasma at 1 : 5000 dilutions. There is no cross-immunoreaction at 1 : 20 in guinea pig, mouse, cow, rat, rabbit, dog, sheep or pig plasma. In other words there appears to be a species specificity similar to that for GH. This contrasts sharply with bioassay findings of no species specificity (Van den Brande et al. 1974b). The adult male fasting plasma concentration ranges from 4-20 µg/ml (mean 9,8 $\pm$ 1,5 µg/ml). Although there is overlap with the normal range levels are high in acromegaly, and fall with therapy, and low in hypopituitarism, suggesting GH dependence.

Although antibodies have been raised against somatomedin C in the guinea pig, the titres of the antisera are too low to be useful for radioimmunoassay at present (Van Wyk et al. 1974).

5.5. Site of Production of Somatomedin

Based on the thesis that SM production is GH-dependent, the localization of ^{125}I-labelled GH after injection has been used to suggest sites of SM production. A high concentration was found in liver, kidney, adrenal cortex and submandibular glands of hypophysectomised rats. Pretreatment with GH largely blocked

uptake in liver and adrenal cortex, suggesting specific binding in these organs. Reassuringly GH was not taken up by epiphyseal plates of long bones (Mayberry et al. 1971).

As previously discussed tissue extracts have yielded equivocal results and the best evidence is derived from organ perfusion or culture.

Liver. Perfusion of isolated livers with GH-enriched medium results in the generation of SM activity (McConaghey & Sledge 1970; McConaghey 1972; Williams & Hughes 1974). Rat liver slices (Hintz et al. 1972b) and liver microsomes (Hall & Uthne 1971) also release SM activity. Three different in vivo approaches also suggest that the liver is a source of SM or is at least necessary for full expression of SM activity. Partial hepatectomy in the rat results in a fall in serum SM activity which is followed by a gradual rise that parallels regeneration (Uthne et al. 1971; Uthne & Uthne 1972). In man catheterisation of the hepatic vein shows higher SM levels in the hepatic serum than in arterial serum (Girard et al. 1974). Hepatic insufficiency is associated with reduced serum SM in man (Wiedemann et al. 1974; Wu et al. 1974).

Kidney. Perfusion of the rat kidney with GH-enriched medium results in SM production (McConaghey & Dehnel 1972; Beaton & Rabkin 1975, unpublished observations). As the kidney plays an important role in the degradation and excretion of GH (Wallace et al. 1972) the growth retardation of the nephrectomised lamb foetus has been attributed to interference with SM production (Thorburn 1974); although this has not been proven by serum assays. The recovery of growth rate and the closely correlated rise in serum SM that occurs in children after renal transplantation suggests an important renal contribution to serum SM activity (Saenger et al. 1974).

Reduction of normal growth rate in monkeys following

extirpation of the submandibular glands (Narasimhan & Ganla 1968) and somatomedin-like activity in muscle extracts (Hall et al. 1970) suggests a possible role for these organs in somatomedin mediated growth.

For all of these organs identity between the SM of the organ perfusate or extract and serum SM has not been established and has been queried by at least one group for the liver (Hintz et al. 1972b). Only precise molecular characterization, by radioimmunoassay will permit proof of the exact origins of SM.

The possibility that SM may be a breakdown product or active fragment of the GH molecule has been investigated. Available evidence does not support this suggestion, but none is yet conclusive. Only precise identification of the amino acid sequence will finally decide this point. Failure to cross-react with GH antisera (Daughaday & Kipnis 1966), induction in hypophysectomised rats by a non-GH factor from the tapeworm, Spirometra mansonoides (Garland et al. 1971) and complete temporal separation of related serum changes in SM and GH (Hall 1971) all make it unlikely that SM is derived from the GH molecule.

5.6. Factors Influencing Serum Somatomedin Levels

Serum SM levels may be affected by changes in the rate of production, rate of removal or degradation and the concentration of the serum binding-protein.

Hypophysectomy results in a fall in serum SM. In the rat the half-life is 3 - 4 hours (Daughaday et al. 1968) and in man it is 9 - 18 hours (Almquist & Falkheden 1961). Somatomedin activity is restored by injection of GH within 8 - 12 hours in the rat and mouse (Daughaday et al. 1968; Herbai 1971a) and within 3 - 6 hours in man (Hall 1971; Schimpff et al. 1974). These hypophysectomy findings are confirmed by the half-life of

3 - 4 hours of somatomedin A following partial hepatectomy in the rat (Uthne 1973). The response to GH in the hypophysectomised animal is an active process involving protein-synthesis as it is blocked by cycloheximide (Uthne 1973). The response is also blocked by oestrogen pre-treatment (Wiedemann & Schwarz 1972).

No consistent significant negative correlation has been shown between GH and SM, as may be expected from general endocrine principles of negative feedback control and as has been postulated (Tanner 1972). In normal children and acromegalic adults (Hall 1972) and in malnourished children (Grant et al. 1973) there is no correlation between basal GH levels and SM. These observations mean little in view of the different half-lives of the two hormones; minutes for GH and hours for SM. Adequate conclusions can be based only on secretion rates and dynamic studies with pure SM. There are two indirect items of evidence suggesting negative feedback. Hepatectomy in mice results in changes in the pituitary suggestive of increased GH synthesis and secretion (Zotter 1972) and in an increase in serum DNA synthesis-stimulating potency, identical to that caused by GH (Llanos et al. 1971).

There is much less information regarding removal of SM from serum. Successive incubations of cartilage in serum suggest inactivation or binding of SM and effective reduction in SM potency in the remaining fluid (Daughaday et al. 1968). This does not occur with the GH present in the medium, further indicating that GH and SM are separate molecules.

The role of binding-proteins in regulating the serum level of SM is purely speculative and there is no information on the nature, affinities or control of these proteins. It is possible that GH modulates the levels of and/or SM binding to these proteins.

That SM is not entirely GH-dependent has been recognized from

the outset. Children with no detectable immunoreactive GH almost always have low but measurable SM levels (Daughaday & Parker 1963). This incomplete dependence on GH has been highlighted by the finding that the spargana of the tapeworm Spirometra mansonoides contain a substance that induces SM production and subsequent growth (summarized in Daughaday & Garland 1972). This worm growth factor is not immunologically related to GH and does not act on cartilage directly. After 4 - 6 weeks antibodies against the factor develop, SM levels fall and growth slows (Steelman 1971).

A variety of physiological factors influence serum levels. In the absence of measures of secretion rates the effects of the factors on production, utilization or protein-binding remain undetermined. Furthermore, all of the factors discussed below have been described using bioassay techniques. Therefore, the possible effects of inhibitors remain largely unexplored and these may be long- or short-term, cyclical or continuous.

Circadian rhythm. Daughaday et al. (1959) observed no consistent diurnal variation in three adults, but Du Caju and Van den Brande (personal communication) have some evidence to suggest a pattern of diurnal variation that is significant, with levels being highest in the early morning. These workers have data also to show that SM levels are inversely correlated with plasma cortisol levels. Further relationships have not been elucidated and await more data. The in vivo uptake of $^{35}SO_4$ (Simmons 1968) and mitotic activity (Simmons 1964) in epiphyseal cartilage of mice shows a marked diurnal rhythm, being maximal between 15h00 and 18h00.

Fasting. Fasting for 48 hours or more results in a fall in serum SM in rats (Daughaday & Kipnis 1966; Salmon 1972). Salmon (1972) has shown this fall to be due to inhibitors appearing in the serum.

Stress. Repeated bleeding causes a rise in serum SM in rats (Boström et al. 1971; Bozovic & Boström 1969). The significance of this finding is not clear as GH responses were not measured.

Nutritional status. Chronic malnutrition of the protein-calorie deficiency type results in a fall in SM in the face of elevated GH levels, as already discussed (Grant et al. 1973). In childhood obesity SM is normal, while GH levels are often low (Du Caju & Van den Brande 1973; Van den Brande & Du Caju 1973).

Pregnancy. The early studies of Chesley (1962) and Daughaday et al. (1959) suggested that SM was unaltered in pregnancy, but the more precise assay used by Tato et al. (1975) recently has shown a reduction during pregnancy and an even further reduction during delivery. The values during delivery fall within the hypopituitary range. The authors suggest that oestradiol may be responsible for the low levels at delivery.

Age. Age-associated changes in SM are discussed later in this chapter.

5.7. Biological Actions of Somatomedin

Much of the work on the biological actions of SM has been conducted with whole serum or serum extracts of variable purity. The only criteria for assigning the observed actions to SM have been GH-dependence of the activity and the failure of GH itself to cause similar effects.

In vitro cartilaginous actions. Since the early work of Daughaday which demonstrated cartilage sulphation, many other actions in cartilage have been ascribed to somatomedin. ^{14}C-leucine is incorporated into chondromucoprotein (Salmon et al. 1968) and ^{14}C-U-proline is converted into ^{14}C-hydroxyproline-collagen (Daughaday & Mariz 1962, Henneman 1971) under the stimulus of SM. The synthesis of both RNA and DNA is accelerated

by SM (Daughaday & Reeder 1966; Salmon et al. 1968; Murakawa & Raben 1968). In chicken cartilage transport of alpha-aminoisobutyric acid, cycloleucine and metabolizable amino acids is stimulated by SM (Adamson & Anast 1966).

When protein synthesis is blocked by actinomycin or puromycin, sulphation is also inhibited (Haba & Holtzer 1965; Salmon et al. 1967), indicating that sulphation occurs as the terminal process in the synthesis of the sulphated proteoglycan molecule.

Several studies have shown that the various metabolic activities in cartilage are stimulated by one purified moiety (Salmon & DuVall 1970b; Van Wyk et al. 1969, 1971).

Whereas GH has no effect on all of these processes in vitro, the growth and metabolic activity of both mammalian and chick limb-buds is stimulated by GH in culture (Leong 1974, personal communication; Hay 1958; Renda & D'Este 1968).

In vitro non-cartilaginous actions. Somatomedin is insulin-like in its actions on adipose tissue, stimulating ^{14}C-glucose conversion to $^{14}CO_2$ (Hall & Uthne 1971) and inhibiting adrenalin-induced lipolysis (Underwood et al. 1972). Hall and Uthne's study was one of the first to suggest that SM and soluble non-suppresible insulin-like activity (NSILAs) may be the same, or very similar, molecules. This similarity is discussed later. Partially purified preparations stimulate amino acid transport and protein synthesis in rat diaphragm muscle (Hall & Uthne 1972; Salmon & DuVall 1970b). Uthne's later studies (1973) confirmed the insulin-like effects of SM on protein synthesis in muscle, and convincingly showed that no lag period occurs as it does with GH, the effects of which are also anabolic (Kostyo 1968).

In vitro mitogenic actions. Salmon and Hosse (1971) demonstrated that partially purified SM stimulated Hela cell growth, but stressed that not all the growth-promoting activity of serum

was resident in the SM fraction. Other authors have shown the stimulation of DNA synthesis and mitosis in a variety of cells including human glia-like cells (Uthne 1973), foetal rat liver cells (Leffert & Paul 1972), rat ovarian tumour cells (Clark et al. 1972), human skin and lung fibroblasts, meningocytes and cat lung fibroblasts (Westermark et al. 1973) and mammary gland epithelium (Hsueh & Stockdale 1974). Each of these groups has used only one or other of the somatomedins (usually impure), and whether the mitogenic effects in each tissue system can be ascribed to only one or to all the somatomedins is not known at present.

Cell membrane interactions. Because many of the actions of insulin and SM are similar, investigators examined the possibility that the two may compete for the same receptor site on the plasma membranes of responsive cells. Competitive binding, at physiological concentrations, occurs between insulin and SM in fat, liver and cartilage cell membranes (Hintz et al. 1972a; Marshall et al. 1974). Moreover, the relative binding affinities reflect the biological potencies of the hormones in the responding cells. Thus in fat cells, for equivalent binding, the ratio of SM to insulin was 50 microunits of insulin to one unit of SM, and for glucose oxidation 20 microunits to one unit. This contrasts with the situation in cartilage where small amounts of SM completely displace insulin from the binding sites, and for sulphation one unit of somatomedin is equivalent to 30 milliunits of insulin. Furthermore, in fat cells the effects of SM and insulin are strictly additive (Clemmons et al. 1974).

The conclusion that SM and insulin share common receptors and that this is the mechanism of the similarity of their biological actions is further strengthened by the demonstration that SM inhibits adenyl cyclase in several tissues including fat, liver, spleen lymphocytes and chondrocytes (Tell et al. 1973) and the

observations that high concentrations of both exogenous and endogenous cAMP inhibit sulphation in chick pelvis (Rendall *et al*. 1972).

These findings heighten the paradox of a GH-dependent molecule having insulin-like actions *in vitro* while GH itself antagonizes insulin *in vivo* (Froesch *et al*. 1967). Until working quantities of pure SM are available for *in vivo* use this paradox will not be resolved.

In vivo actions. Shortage of pure SM has permitted only one very limited *in vivo* study. Somatomedin A induced tibial epiphyseal widening and skin thickening in hypophysectomised rats, but did not increase body weight (Hall & Uthne 1972). The failure to stimulate weight gain may be due to the use of Somatomedin A which is primarily active in chick cartilage and only slightly active in rat cartilage *in vitro*.

In spite of impressive *in vitro* evidence it remains to be rigorously established that GH mediates its effects on growth *in vivo* via the somatomedin mechanism.

5.8. Role of Somatomedin in the Regulation of Normal Growth

By examining changes in serum SM with age from birth to old age in a cross-sectional manner several authors have attempted to infer a role for SM in the regulation of growth velocity. Beyond describing a broad and somewhat variable trend such studies offer little information as they are based on single determinations on relatively small numbers of patients.

In cord blood SM has been described as approximately equal to that of the corresponding maternal serum (Daughaday *et al*. 1959; Chesley 1962; Hintz *et al*. 1974) or significantly higher (Tato *et al*. 1975). In Tato and collaborators' study in the newborn, levels are low to undetectable, rising into the normal adult range by the 4th day of life and falling somewhat between 23 days and 15 months of age. There is general agreement that from about one year

of age to puberty SM rises progressively (Van den Brande & Du Caju 1973; Kogut et al. 1963; Almquist & Rune 1961), although Daughaday et al. (1959) and Daughaday and Parker (1963) found no clearcut trend. Only one study has suggested a pubertal rise in SM, but the finding of a similar rise at 8 years makes the significance of the pubertal rise dubious (Almquist & Rune 1961). No difference between males and females has been shown. Adult levels persist into old age.

These age-associated changes in SM mean that clinical studies should always be related to age-specific control levels, yet only Van den Brande (1973) has consistently done this.

Recently serum SM has been related to growth velocity rather than to chronological or height achieved age (Hall & Filipsson 1975). As discussed in Chapter 3 this is the most meaningful correlation to seek on theoretical grounds. In this study no correlation was found between age and somatomedin A ($r = -0,11$) but a highly significant correlation was shown between SM and gain in stature in the year prior to assay ($r = 0,67$).

The in vivo age-associated changes have been investigated in the rat and the mouse. Injected $^{35}SO_4$ is maximally taken up by the tissues at birth, after which there is a progressive decline in uptake (Layton & Denko 1952). Dziewiatowski (1953) showed an increasing ability to incorporate $^{35}SO_4$ in rat embryos as they grew heavier. He also showed that postnatally $^{35}SO_4$ was much more rapidly and intensely concentrated in the femur by 10 day-old rats compared to 30- or 300-day old animals (Dziewiatowski 1954). Herbai (1971a) demonstrated that the costal cartilage uptake of $^{35}SO_4$ was highly correlated with the body weight gain in the preceding week in mice ($r = 0,84$ for males, $r = 0,80$ for females).

A possible explanation for the increase with age in serum SM is suggested by the observation that liver membranes of 3 day-old

rats bind 5 to 6 times less GH than do those of 105 day-old rats. This increase in binding capacity is not due to changes in affinity but to an increase in the number of active binding sites (Du Caju & Desbuquois 1974).

All work to date that attempts to relate serum SM to somatic growth rate suffers from drawing on indirect evidence. Human serum SM, for example, is assayed by measuring sulphation of rat costal or chick pelvic cartilage. This activity is then correlated, in the best studies, with stature velocity over the past year; stature velocity being chiefly determined by hyperplasia in the epiphyseal plate, a mitotic rather than a matrix synthesis function. Ideally this question should be approached by a longitudinal study in which growth velocity of a particular tissue is correlated with the response of that tissue to SM.

5.9. Role of Somatomedin in the Causation of Abnormal Growth

Claude Bernard's "experiments of nature" and therapeutic intervention in a variety of diseases have yielded insight into the role of SM in the causation of abnormal growth.

Hypopituitarism. Somatomedin is low in hypopituitarism and after hypophysectomy or pituitary ablation (Daughaday et al. 1959; Daughaday & Parker 1963; Kogut et al. 1963; Hall 1970; Hall & Olin 1972). GH administration causes a prompt rise in SM (Daughaday 1971a;Hall 1971). During long-term treatment there is a good correlation between growth rate and level of serum SM (Hall & Olin 1972). The initial growth rate and SM responses during the first year of therapy are usually not matched in subsequent years. This phenomenon was attributed to the development of GH antibodies by Parker et al. (1964), but the more recent work of Hall and Olin (1972) does not support this explanation. Experimentally GH antibodies can be shown to inhibit the growth of newborn rats (Duquesuoy & Good 1970).

Emotional deprivation dwarfism is associated with low GH secretion and low SM levels (Du Caju & Van den Brande 1973). Exogenous GH has little growth-stimulating effect (Tanner et al. 1971; Frasier & Rallison 1972); the added complication of malnutrition in many cases makes interpretation of the role of SM in the genesis of growth retardation in this syndrome difficult (Powell et al. 1967).

The syndrome of high immunoreactive GH levels and dwarfism illustrates a failure of SM generation, inherited on an autosomal recessive basis (Laron et al. 1966a; Daughaday et al. 1969; Van den Brande et al. 1974a). Treatment of these patients with GH does not cause any increase in growth rate or in SM (Laron et al. 1971).

Acromegaly is usually accompanied by high SM levels Daughaday et al. 1959; Hall 1970), the degree of clinical activity correlating fairly well with the SM level (Hall 1972). Rats with GH-secreting tumours have elevated serum SM (Peake et al. 1968).

Somatomedin measurements in a variety of other conditions have yielded interesting, but as yet unexplained findings. In cerebral gigantism levels are low (Lecornu 1973; Du Caju & Van den Brande 1973), in craniopharyngioma patients post-operative levels are normal (Du Caju & Van den Brande 1973) in spite of low GH secretion (Finkelstein et al. 1972b), and in Turner's syndrome levels are normal or elevated (Almquist et al. 1963) with normal GH secretion (Donaldson et al. 1968).

Short stature, normal GH and normal SM in the Congo pigmy suggest a diminished end-organ response compared to other genetically distinct, populations of greater stature with similar GH and SM levels (Rimoin et al. 1969; Daughaday et al. 1969). The role of nutrition in the causation of the pigmy short stature has not been carefully examined. Although SM studies have

not been made in the Kalahari Bushman, nutrition may play a role in his short stature in view of the secular trend for increasing stature since 1900 that has been demonstrated (Tobias 1962).

5.10. Relationship of Somatomedin to Other Growth Peptides

In recent years a number of growth-stimulating factors have been described. This group has several features in common : all are peptides of fairly low molecular weight, they are tissue-specific to a greater or lesser degree, many are insulin-like in their metabolic actions, they evoke their responses by interaction with the cell membrane and they promote growth. Hershko et al. (1971) have termed this group of actions the "positive pleiotypic response."

Somatomedins A, B and C and a number of other peptides fit into this general grouping and a consideration of the features they share and differ in suggests a broader biological role for the somatomedins than was originally envisaged by the sulphation factor hypothesis.

Insulin. In 1945 Young showed insulin promoted protein anabolism and in hypophysectomised rats insulin and a carbohydrate-rich diet stimulates both body weight gain and skeletal growth (Salter & Best 1953). Further high doses of insulin can partially replace serum in stimulating DNA synthesis and mitosis in chick embryo fibroblasts in culture (Temin 1967). Evidence already discussed shows that insulin and SM are quite distinct molecules (Laron et al. 1972).

Nonsuppressible Insulin-like Activity (NSILAs). The immunoreactive insulin of serum contributes only 7% of the total insulin-like activity (ILA) in serum when assayed by glucose oxidation in the epididymal fat pad (Froesch et al. 1967; Jacob et al. 1968). That portion of the ILA which is soluble in acid-ethanol (NSILAs) also stimulates thymidine uptake by rat

cartilage (Raben et al. 1972) and some workers now believe that NSILAs and somatomedin C are the same or very similar molecules. The additional observations that NSILAs is also partially GH-dependent and that it induces cartilage sulphate suggest that NSILAs should be classed as a somatomedin, or vice versa (see Froesch in discussion section of Van Wyk et al. 1974). On the other hand there are sufficient differences in amino acid composition to show that NSILAs and the somatomedins are distinct from each other (Labhart et al. 1972).

Nerve Growth Factor (NGF). A peptide purified from mouse submandibular glands stimulates growth in sympathetic nerve cells in vitro and in vivo (Levi-Montalcini & Angeletti 1968). This NGF stimulates RNA and protein synthesis and glucose uptake in these cells, and does not do so by stimulating adenylate cyclase (Hier et al. 1972). The close amino acid similarities between proinsulin and NGF and the fact that high doses of insulin mimic the actions of NGF suggest a possible common ancestral precursor molecule (Frazier et al. 1972). NGF has no sulphation activity (Hambley et al. 1974).

Multiplication-Stimulating Activity (MSA). A factor that stimulates chicken embryo fibroblast multiplication has been purified 6000-fold from calf serum and found also to have NSILAs-like activity on rat adipose cells (Dulak and Temin 1973). MSA also stimulates DNA uptake in fibroblasts and competes with SM and insulin for binding sites in rat liver membranes (Smith & Temin 1975). MSA is produced by rat liver cells growing in a serum-free medium and therefore does not appear to be GH-dependent (Smith & Temin 1975). Other recent reports suggest that MSA or MSA-like activity is GH-dependent for maximum stimulation of growth of fibroblasts of man (MacGillivray et al. 1975) and chicks (Cohen et al. 1975). Recently platelets have been shown to be a rich source of fibroblast growth-promoting activity (Kohler & Lipton 1974).

Epidermal Growth Factor (EGF). EGF, like NGF, has been purified from mouse salivary gland tissue. This heat-stable protein of molecular weight 6400 stimulates growth of cells of ectodermal origin, amino acid transport and RNA synthesis (O'Keefe et al. 1974). It binds to cells at sites that are different from those GH, insulin and NGF. The increase in concentration in the submaxillary glands with age suggests a role in adult life as well as during the growth period (Bynny et al. 1972).

Erythropoietin. Erythropoietin is a partially GH-dependent peptide (Peschle 1972) that stimulates the growth of the erythroid cells both in vivo and in vitro (Gordon 1971).

Granulocytosis-inducing Factor. The mouse submaxillary gland contains a third distinct peptide that stimulates granulocyte proliferation in culture (Angeletti et al. 1965).

Other less well characterized factors have been described in serum (Paul & Walter 1975; Granström 1974; Pickart & Thaler 1973; Bradley & Metcalf 1966) and in the pituitary (Corvol et al. 1972; Malemud & Sokoloff 1974; Rudland et al. 1974) that are essential to or stimulate the growth of various cells in culture.

5.11. Concluding Remarks

The somatomedins fall into the broad grouping of growth factors. Whether the somatomedins should also be considered hormones, in the classical sense of the term, cannot yet be decided. When the exact origin of serum SM is identified and when the physiological in vivo role of the somatomedin group is elucidated the correct appellation can be decided upon. It is possible that each tissue produces its own self-specific somatomedin or somatomedin-like factor or factors and that these are GH-dependent. It is further possible that all the metabolic actions that have been identified and that are not related to growth

are physiologically insignificant and that the only significant actions are those related to the growth of the somatomedin-specific tissue. Generalizations are difficult to make at the present because of the piecemeal nature of the information available about each of the somatomedins and other growth-promoting peptides, and the lack of evidence concerning their *in vivo* role.

On the basis of the facts reviewed in this chapter a broad definition of somatomedin can be suggested : A somatomedin is a peptide that is at least partially under the control of growth hormone, that is insulin-like in its actions and that stimulates growth in at least one tissue type. The omission of the requirement that sulphation be stimulated means that peptides that do not act on cartilage can also be included in this category.

On the basis of this definition somatomedins A, B and C, NSILAs and MSA must all be regarded as part of the "somatomedin family" (Van Wyk *et al*. 1974). As information concerning the precise structure of the somatomedins becomes available a more useful classification may be possible, as is the case with the prostaglandins.

CHAPTER 6

THE END-ORGAN RESPONSE

"... our birth is nothing but our death begun"
Edward Young

In Chapter 4 brief mention was made of the necessity to regard hormone action as the net result of hormone-target or end-organ reaction. The role of the end-organ as an important determinant of endocrine function remains largely unexplored. In 1953 Alfred Jost wrote that he was "unaware of any systematic assays throughout development ..... other than those concerned with the gonadal response" that had investigated "the reactivity of the target organs." The early work of Moore and Morgan (1943) and of Joseph Gillman (1948) had demonstrated that the foetal genital organs pass through a period of a sensitivity during which their properties are irreversibly changed by hormonal influences. In recent years attention has remain focused on the maturation of sexual organs. Odell and others have shown that in the postnatal male rat the response of testosterone secretion to luteinizing hormone (LH) stimulation increases with age. This changing testicular responsiveness to LH is an important factor in determining sexual maturation (Odell et al. 1974).

In mammalian tissues other than the genital tract there are scattered reports of changes in end-organ sensitivity, indicating that the phenomenon has generalized applicability. For example, GH-stimulated alpha-aminoisobutyric acid transport in diaphragm occurs in young rats but not older rats (Kipnis & Reiss 1960). Similarly, GH induces 2-deoxyglucose uptake by rat diaphragm in

young but not in older rats (Riddick et al. 1962). The sensitivity of amphibian organs during metamorphosis shows that at any one time in the same animal the prevailing concentration of thyroxine has dramatically different effects, depending on the responding target. The tail and gills undergo necrosis, while the limbs differentiate and grow (Schwind 1933). In the tadpole not only is the absolute direction of the response to thyroxine different in different targets, but the threshold also changes. Thus the dual controls of changing end-organ response and changing thyroxine concentration, result in an ordered sequence of differentiation and growth (Balinsky 1970).

In phylogenetic, rather than ontogenetic perspective the importance of the end-organ in determining the extent and direction of response to hormonal action is also clearly seen. The hypothalamic peptide arginine vasotocin, for example, is found in all classes of vertebrates except mammals. When the evolutionary step from water to land was heralded by the appearance of the amphibia, water balance was achieved by adaptation and specialization of end-organ response to arginine vasotocin; this peptide had been present since the time of the lampreys. The skin, kidneys and bladder of the amphibia evolved responses to the hormone not present in those forms which remained in the water (Barrington 1971).

Although much remains to be unravelled it is probable that changes in the response of the end-organ during development are major determinants of the patterns of growth and differentiation that occur. In the growing mammal the weight of evidence suggests that changes in cartilage sensitivity occur during the growth period; this is reviewed in the next section.

6.1. Cartilage

Cartilage and bone cells arise from a common stem cell and

the differentiation of this ancestor appears to be intrinsically, that is genetically, determined. This differentiation is nevertheless triggered by microenvironmental factors (Hall 1970b) and the rate of division of chondroblasts appears also to be influenced, inter alia, by microenvironmental negative feedback controls (Jackson 1970). Skeletal organogenesis is also subject to local regulation (Lacroix 1951). Thus there is little evidence for pure, or primary, genetic regulation of skeletal growth, but there is reasonable evidence of local, or microenvironmental, controls, acting by modifying the genome.

In vitro studies of cartilage segments indicate that age is an important determinant of responsiveness. Two studies show that the uptake of $^{35}SO_4$ in serum-free medium declines with increasing age in the rat (Heins et al. 1970) and in the pig (Phillips et al. 1974). The work of Heins et al. also showed that cartilage response to SM (as human serum) varies with age. Foetal rat costal cartilage was not stimulated by the addition of serum. The high uptake without serum suggests that the cartilage may be synthesizing chondroitin sulphate at maximum rates and therefore further stimulation is ineffectual. However, the only concentration at which serum was added was 10%, and the possibility remains that higher concentrations may have caused stimulation. The sulphation of costal cartilage from newborn rats and rats weighing 10g, 45g and 88g was significantly stimulated by the addition of 10% serum, in contrast to the refractoriness of foetal cartilage. Commenting on these observations Daughaday (1971a) states that "responsiveness in rat cartilage .... is greatest during the period of active postnatal ... growth in the rat." While this study is unique in the SM and growth regulation literature it nevertheless does not take account of several important points. The first has already been mentioned, namely the nature of the stimulus to which the cartilage

was subjected. A range of serum concentrations would have offered more information on the response characteristics of the cartilage. The threshold and slope of the dose-response curve may be of greater significance in interpreting age-related changes than the stimulation achieved with serum at a single concentration. Secondly, while the serum used retained its potency throughout the experimental period, this potency was measured in the hypophysectomised rat assay. It is possible that this serum contained inhibitory material to which foetal cartilage, but not postnatal normal and hypophysectomised cartilage, was sensitive. Although the authors demonstrated that serum from hypophysectomised rats also did not stimulate foetal cartilage, this serum was only used in a single concentration and was not tried on cartilage from older rats. Thirdly, cartilage response was related only to body weight and not to any parameter of the velocity of growth. Therefore, while Daughaday's comment, that responsiveness is greatest during the period of active growth, may be a correct generalization, it indicates that no further conclusions can be drawn regarding the relationship between responsiveness and velocity of growth. Fourthly, as stressed earlier, it is the hormone-tissue interaction that is the most important determinant of hormone action and therefore it is possible that in Heins' study the serum of cartilage donor rats would have been a more appropriate stimulus than human serum. Finally, the use of chondroitin sulphate synthesis as the most appropriate index of the responsiveness of growing cartilage is open to question. It is possible that mitosis and DNA synthesis, and therefore ^{3}HTdr incorporation, is a more appropriate index when the whole span of the growth period is being considered.

The work on changes in cartilage sensitivity in pigs (Phillips et al. 1974) reveals only that the percentage response to 40% human serum of foetal costal cartilage is greater than

that of 6-9 month-old pigs. The cartilage of pigs older than two years cannot be stimulated under these conditions.

Injection of $^{35}SO_4$ into intact animals of different ages and measurement of uptake by growing cartilage is an index of cartilage responsiveness as modified by its metabolic and hormonal milieu. This technique demonstrates a declining uptake as age increases after birth in rat tibial epiphysis (Murphy et al. 1956), rat femoral epiphysis (Dziewiatowski 1954) and rat costal cartilage (Salmon et al. 1963). In the mouse costal cartilage uptake rises in males until five weeks of age and then declines and in females falls off slowly from three weeks of age (Herbai 1971a).

By comparing the uptake of $^{35}SO_4$ by normal and osteoarthritic human articular chondrocytes in culture in response to stimulation by normal and heat-inactivated sera Schwartz et al. (1974) have clearly shown that the biosynthesis of sulphated proteoglycans is controlled by both serum and cellular factors.

The direct experimental evidence for changes in end-organ responsiveness is therefore scanty, but the theoretical argument is strong and supported by many authors who write of "alteration in end-organ potential" (Daughaday et al. 1959), of "decreasing ability .... to respond to somatomedin" (Tanner 1972) and of growth depending on "both humoral growth factors and on the sensitivity of the individual to these factors" (Hall & Filipsson 1975).

CHAPTER 7

EXPERIMENTAL ANIMALS

7.1. Rabbits

New Zealand white rabbits were used in the study. The breeding stock of 12 does and 3 bucks were purchased from a South African champion breeder who had maintained the strain in a large, closed colony for 18 years by line-breeding (Portsmouth 1962). The unusually long time for which the strain had been maintained and the similarity of the physical size and appearance of the breeder's herd suggested a high degree of homogeneity.

Housing. The experimental colony was housed in a brick-walled room 5m by 9m and 4m high. Overhead artificial lighting was used in a 12 hour light - 12 hour dark cycle. Temperature was maintained at 18 $\pm$ 1°C by a reverse-cycle air-conditioner. Humidity varied between 55% and 70%.

Breeding stock were kept in galvanized mesh cages measuring 130cm x 70cm x 60cm, giving a floor surface of 0,91m^2. After weaning, growing rabbits were kept in galvanized mesh cages measuring 70cm x 70cm x 45cm, giving a floor surface of 0,49m^2. These cages sizes exceed the Universities' Federation for Animal Welfare (UFAW) recommended minima (Adams et al. 1966). Breeding and growing cages were built on mobile racks in tiers of four. There were 12 breeding and 25 growing cages.

Sawdust in the excreta trays was changed daily. Trays, cages and the house floor were washed down weekly with a Hycolin® - water mixture.

Feeding. All stock were fed pelleted rations containing 18% protein (Delmas Milling Rabbit Pellets) which meet the recommended nutrient requirements of growing rabbits (National Research Council 1954). Hoppers were filled twice daily. Adults were given just sufficient rations to ensure a steady body weight and all growing and lactating stock sufficient to ensure hoppers always contained some pellets. Fresh green carrot tops were supplied to all stock in the evening. Fresh water was provided by an automatic supply system.

Mating procedure. Does were introduced to the buck's cage and copulation observed. If the buck's ejaculation was successful the doe was examined 13 days later by abdominal palpation (Adams 1960). If she was pregnant a wooden nesting box measuring 60cm x 40cm x 40cm containing dry grass was provided on the 28th day of pregnancy. If the doe was not pregnant she was put to the buck again on the 13th day and the procedure repeated.

A random mating policy was adopted and records kept of all matings, pregnancies and litters' progress (Sawin 1950).

Does were considered sterile if 5 or more matings were unsuccessful and were destroyed. Three of 27 breeding does, including the original 12, were sterile (11%); this compares favourably with the figure of 18% reported by Sawin and Curran (1952).

During the four years that the colony was maintained a total of 121 litters were born. Of these 104 were born on the 33rd day of gestation, 14 on the 32nd day and 3 on the 31st day. Ninety-nine litters were raised successfully, the remainder either being still-born (11), neglected (6), cannibalized (3) or dying because of maternal failure to lactate (2).

The sex ratio at birth was 1,02, as also found by Hammond (1925) with 331 female and 337 male rabbits being born.

The average litter size was 5,5 at birth and in the successfully raised litters average size was 5,6 at weaning (Table 3).

TABLE 3 Summary of litters born and reared

Litter Size	Number of Litters	
	At Birth	At Weaning
1	1	-
2	4	2
3	4	1
4	19	16
5	31	28
6	33	30
7	17	16
8	8	5
9	3	1
10	1	-
total	121	99
average size	5,5	5,6

Rearing Procedure. All litters were born in the early hours of the morning and were inspected on the day of birth. The dead and runted babies were removed, all babies sexed and the normal ones marked and weighed. Marking was effected by staining either crown, neck or rump with gentian violet, mercurochrome or brilliant green solutions.

The young were weaned at eight weeks, which was at least two weeks after maternal milk supply started to diminish. At weaning colony serial numbers were tattoed into the left ears with Indian ink. Young were housed in pairs until 12 weeks and then separately.

Rabbits were handled for experimental purposes only by the author and the attendant.

For both the growth and endocrine studies only a doe's second and subsequent litters of four to seven young born on the 33rd day

of gestation were used. This reduced the well recognized variability that litter size and length of gestation have on birth size and neonatal growth (King 1915; Gates 1925; Spencer & Coulombe 1965; Kumaresan et al. 1967; Duncan 1969; Park et al. 1971) and also reduced variability resulting from different numbers of animals per cage prior to weaning (Hughes & Nowak 1973). All other offspring were either raised for breeding stock or slaughtered at six weeks.

The rabbits of the colony maintained excellent health. There were no deaths due to disease and only five rabbits became ill in the four year period. Four pregnant does developed the "wet dewlap" syndrome, a suppurative skin condition from which no pathogen could be isolated and which responded rapidly to shaving of the affected area (Sandford 1957). One young rabbit developed non-specific diarrhoea which responded rapidly to a kaolin and pectin mixture.

7.2. Rats

The rats were a Sprague-Dawley strain supplied by the University of the Witwatersrand Central Animal Unit.

The breeding colony is housed at ambient temperatures with natural lighting. A random mating policy is applied.

Litters were raised in 45cm x 20cm x 20cm aluminium cages until weaning. Thereafter males and females were housed separately in groups of ten in 60cm x 60cm x 20cm aluminium cages. The diet was standard rat pellets. All experiments were performed within a six week period. Only litters of five to eight animals were used. Rats were delivered on the day of experimentation.

CHAPTER 8

A LONGITUDINAL STUDY OF THE GROWTH OF THE RABBIT

Rabbits of all ages are extensively used for laboratory experiments and rabbit husbandry is a rapidly expanding agricultural industry. In spite of this interest in the rabbit little is known of the precise characteristics of rabbit growth and no thorough longitudinal growth study has been carried out.

Previous work includes that on body weight by Minot (1908), Castle (1929), Lowrance (1953a,b) and Sawin and Crary (1960). Waterman (1943) has measured body weight and crown-rump lengths of a series of foetuses from 17 to 31 days gestational age. Over very limited age ranges data is available for metacarpal and metatarsal length and breadth (Crary & Sawin 1949), tibial and calcaneal lengths (Crary & Sawin 1949) and skull length for a single rabbit (Selman & Sarnat 1953). Ear length growth has been reported by Bronson (1958). The only work on skeletal maturation describes the number of epiphyseal centres in the hind- and fore-limbs of foetal rabbits (Kotas et al. 1971). Several papers describe external and dry skeletal dimensions of fully grown rabbits (Baumgartner & Sawin, 1943; Latimer & Sawin, 1959, 1962, 1963). In none of these studies, all of which were on adults, was the reproducibility of the measurements reported. Furthermore, most of the work of Sawin and his collaborators was on highly inbred and mutant strains of rabbit and therefore not of general applicability.

This chapter presents reliable techniques for measuring the body length on a specially designed measuring board and numerous skeletal dimensions by radiography. Further, a novel method of

deriving distance and velocity statistics is described and validated.

8.1. Materials and Methods

Animals. The rabbits used in this study were the New Zealand Whites described in Chapter 7. Fourteen litters of 14 proven does sired by four different bucks and born on the 33rd day of gestation were studied. Litter sizes ranged between four and seven. A total of 78 (40 male and 38 female) newborn rabbits entered the study. Sixty nine (37 males and 32 females) completed the study. Those not completing the study either died under anaesthesia (5), all as neonates, or as a result of falling from their cages (2) or were excluded because they became permanently ataxic after anaesthesia (2). During the course of the study some animals were not measured on every occasion for technical reasons (omission from the transport list and poor x-ray exposure, for example) or one or more measurements could not be made on the x-ray plate for technical reasons (animal too lightly anaesthetized and raising its head, for example). The study was thus a mixed longitudinal one and the group is referred to as the 'mixed longitudinal group'.

Control series. All other animals in the colony were routinely weighed at birth and every sixth day thereafter until used for experimental purposes or breeding, or culled because of pressure for accommodation. The weights of 134 animals (63 males and 71 females) that reached 180 days post-copulation (PC) age were extracted from the colony records. These weights served as a control series both to assess possible effects of anaesthesia and to check the validity of the method of analysis of the radiologically studied group. This control series is referred to as the 'pure longitudinal group'.

Examination frequency. All litters were examined on the day of birth, usually between 23h00 and 01h00. Thereafter litters were examined on Tuesdays or Wednesdays between 19h00 and 22h00 at weekly intervals until approximately 90 days PC and at fortnightly intervals until approximately 200 days PC.

The examination schedule was determined by the availability of the radiological facilities. The apparatus was a Siemens unit with 0,6mm focal spot and Tridoros 5s generator at the Johannesburg Hospital. The facilities were only available after normal working hours and because large numbers of rabbits had to be transported several kilometers to and from the Hospital this could be organized only on prearranged days of the week. An exception was made for the newborn litters which were always examined within 24 hours of birth.

Examination Technique. Each rabbit was weighed and then anaesthetized with ether. Up to 80 days PC the animal was firmly held between the anaesthetist's forearm and body and an appropriate sized beaker with ether-damped gauze in the bottom held over nose and mouth. Older animals were placed in a converted balance box to which a flow-meter and ether vapourizer were attached. This modification of Sherrard's (1966) box induced anaesthesia which was of slow onset and produced no struggling. It thus permitted several rabbits to be under induction of anaesthesia simultaneously. Anaesthesia was induced before the evening feed and in suckling rabbits at least two hours after removal from the mother.

Apparatus. A lever-arm balance calibrated in 1g divisions up to 5kg was modified by adding a grocer's scale pan to hold the rabbits. The balance calibration was checked with standard weights at weekly intervals.

Body length was measured from nose tip to tail tip on a

specially constructed measuring board, illustrated in Figure 1.



Figure 1. The measuring board used for body length on anaesthetized rabbits. A black rabbit is shown for photographic clarity.

Initial experiments showed that the rabbit could be far more consistently positioned and that measurements were more reproducible in a supine rather than prone position. The 140cm x 40cm laminated wood base carries the perspex V-shaped body holder. The holder has an angle of 110° and is 58cm long. The head of the holder is closed with a perspex sheet and at the foot is a guide-hole for the sliding rod. The body holder end of this steel rod is fixed to a triangular plate which fits exactly into the bottom third of the V of the body holder. The other end slides in a groove alongside a brass metric rule calibrated in mm. When the plate of the rod is against the head of the body holder the other end of the rod is opposite zero on the rule.

Although rabbits that are frequently handled can be measured while conscious, more consistent results are obtained when they are anaesthetized. The anaesthetized animal is placed supine in the holder, the neck extended and the nose tip pushed against the head board by pressure of the left thumb and forefinger of the examiner being applied to the angles of the mandible. The right hand ensures that the body of the animal lies symmetrically, and then grips and gently pulls the tail out parallel to the

base of the holder. An assistant slides the rod up to the tip of the tail and the examiner indicates when the pressure is adequate to have made bony contact. The assistant then reads off the body length.

The still anaesthetized animal was then transferred to a 6mm thick clear perspex sheet on which were scored fine vertical and horizontal lines to guide positioning for x-ray. The animal was placed on its ventral surface and held in the position shown in Figure 2 with cellotape across limbs, head and tail. Foam rubber wedges were used to secure the symmetrical positioning of the body.



Figure 2. Position of the rabbit for radiographic examination.

The left manus was placed prone, with digits separated, on the perspex so that the humerus was approximately at right angles to the body axis and to the forearm bones. The right manus was pulled caudally at 45° to the main body axis and fixed in a supine position. The left foot was positioned with its lateral border uppermost in such a way that approximate right angles were formed between femur and body axis, femur and tibia and tibia and foot. The right hind limb was pulled caudally at 45° to the body axis and fixed with the sole of the foot uppermost. The snout and tail were fixed in the axis of the body.

The perspex plate was then placed on the 91 x 36cm film cassette, the beam centred on the mid-lumbar spine at 130cm from the film and exposure made. A Halsey cassette with a Titan grid was used throughout. Films were identified by leaded figures giving the rabbit number and the date. Exposures varied from 50 Kv, 3,2 mAs for 0,01 sec in the newborn to 55 Kv, 5 mAs for 0,015 sec in large animals. Films were developed by an automatic processing unit.

At the completion of the study, measurements were made on the radiographs with a caliper reading to 0,1mm (Tanner & Whitehouse 1955) obtained from British Indicators, Herts, England. The following skeletal dimensions were measured (Craigie 1948):

Skull length: from the tip of the nasal bones to the posterior border of the foramen magnum.

Humerus length, right: from the most proximal point of the articular surface of the head to the most distal point of the articular surface of the trochlear.

Humerus breadth, right: the diameter of the narrowest point of the shaft.

Radius length, right: from the most proximal point of the articular surface at the elbow to the most distal point of the articular surface at the wrist.

3rd metacarpal length, right: the distance between the articular surfaces.

5th lumbar vertebra: the length was considered the distance between the articular surfaces of the body in the sagittal plane and the breadth the diameter of the body at its mid-point.

11th rib length: the rib was divided into two or more segments, the inferior borders of which were reasonably straight, from the vertebral articulation to the costo-chondral junction. Each segment was measured along its inferior margin. The segment lengths were summed to give total bony length. The curvature of more proximal ribs was too great to permit measurement of their lengths.

Ilium length, right: from the tip of the iliac crest to the ilio-ischial junction.

Ischium length right: from the ilio-ischial junction to the distal tip of the ilium.

Bi-iliac width: the maximum distance between the most lateral points of the iliac crests.

Bi-acetabular width: the maximum distance between the articular surfaces of the acetabula.

Bi-ischial width: the maximum distance between the distal points of the ischia.

Femur length, right: from the most proximal point of the articular surface of the head to the most distal point of the articular surface of the condyles.

Tibia length, right: from the most proximal point of the

articular surface at the knee to the most distal point of the articular surface at the ankle.

Calcaneus length, left: from the tip of the tuberosity to the articular surface with the cuboid.

In young rabbits these landmarks could not always be precisely defined as radiographic appearances were blurred due to incomplete calcification. Where a reasonable estimate of the landmark could not be made by the observer the measurement was not recorded.

8.2. Reliability of the measurements

Body length. To estimate the reliability of the method of measuring body length ten 38 - 43 days PC and ten full-grown rabbits were measured five times by the author and by a second observer. The animals were completely removed from the measuring board and repositioned for each measurement. For adults the within-observer maximum difference was 0,6% and between observers 1%. For the young rabbits the maxima were 1% and 1,4% respectively.

Radiographic measurements. To estimate the reliability of the radiographic measurements the author remeasured a 10% random sample one year after the first measurements and a second observer measured the same 10% sample. The reproducibility of a measurement is expressed as the mean for all ages of :

$$100 \times \frac{\text{difference between measurements of a given age}}{\text{mean of measurements at that age}} .$$

The reliabilities are shown in Table 4.

TABLE 4. Reliabilities of within- and between observer measurements

Measurement	Within- observer	Between observers
Skull length	4,7	5,1
Humerus length	5,7	6,1
Humerus breadth	11,6	14,2
Radius length	6,3	7,0
3rd Metacarpal length	7,0	7,9
5th Lumbar vertebra length	2,8	3,6
5th Lumbar vertebra breadth	2,9	3,4
11th Rib length	4,7	5,3
Ilium length	5,6	7,3
Ischium breadth	5,2	6,9
Biliac width	2,1	2,4
Biacetabular width	3,6	4,0
Bi-ischial width	2,0	2,1
Femur length	2,3	2,8
Tibia length	2,5	3,0
Calcaneus length	6,3	9,8

8.3. Data analysis

Because of the constraints placed on the times when radiographs could be taken rabbits were examined on day 33 PC and on varying days at the predetermined intervals thereafter. For example, litter A may have been examined on days 33, 40, 47, 54, etc., whereas litter B may have been examined on days 33, 38, 44, 51, etc. This variability made direct calculation of the distance and velocity statistics impossible and interpolation was necessary.

Two approaches to interpolation in this situation have been reported (Goldstein 1970). The first, developed by Patterson (1950), depends on using the measurements of the whole sample in

the region of the target date to derive a suitably weighted adjustment for the unknown values of the individual. The second method considers the observed measurements available for each individual near the target date and the desired value can be obtained by the application of standard interpolation formulae. This second method of individual adjustment avoids introducing biased estimates for individuals whose growth curves are different from the one representing the group. The analysis of the data in this study is based on this principle of individual adjustment.

All data was encoded on computer punch-cards; each card holding the data for one dimension, e.g. body length, of one rabbit from birth to completion of the study.

A frequency table of the days on which examinations were made was derived and from this the days at which interpolations were to be effected were selected. The selection was such as to have as many actual examination days coincide with interpolation days as possible and to give approximate 7 day intervals until 90 days PC and 14 day intervals between 90 and 200 days PC. On this basis days 33, 39, 45, 53, 60, 67, 74, 80, 88, 101, 118, 132, 156, 171 and 199 were chosen. In applying interpolation formulae caution must be exercised when dealing with the end-points of the data. In this study the results for days 33 and 199 were not used due to lack of necessary information about these end-points, respectively for the periods before and after each. However, distance results for day 33 could be obtained directly from the raw data as all rabbits were examined on day 33 PC.

Interpolations at each of the chosen points were then made for each rabbit. The interpolation method used was a spline fit described by Pennington (1965). This method fits a section of a third-order polynomial through adjacent points which constitute the interval containing the point, or points, at which the

interpolation is to be made. Appendix A contains a listing of the general form of the computing programme and the 2 subroutines used to accomplish the interpolation. The means of each measurement at each age were calculated from the interpolated data to provide the coordinates from which the 'distance curves' were plotted. In addition standard deviations and t-test statistics for differences between males and females were calculated.

The ordinates from which the 'velocity curves' were plotted were obtained as follows. The interpolation formula used for the spline fit was differentiated and this first derivative formula was then used to obtain velocity estimates at the desired points. It should be noted that the nature of the spline fit used constrains the first and second derivative of the interpolating function to be continuous.

For body weight and body length velocities were also calculated as 'actual increments'; that is, the increment from one examination to the next was calculated for each individual rabbit and these increments used to compute the velocities.

In the pure longitudinal group control series body weight velocities were calculated as 'actual increments'.

8.4. Results

Body weight (Figures 3, 4, 5, 6; Table 5)

The velocities of body weight growth, determined for the pure longitudinal group as 'actual increment' velocity and for the mixed longitudinal group as both 'actual increment' and 'first derivative' velocity, for males and females are shown in Figures 3 and 4. Inspection shows that there is no difference in either shape or variability between the curves of the pure and mixed longitudinal groups. Further examination shows that the two different techniques of analysis of the velocity of the mixed

longitudinal group yield the same curve and degree of variability.

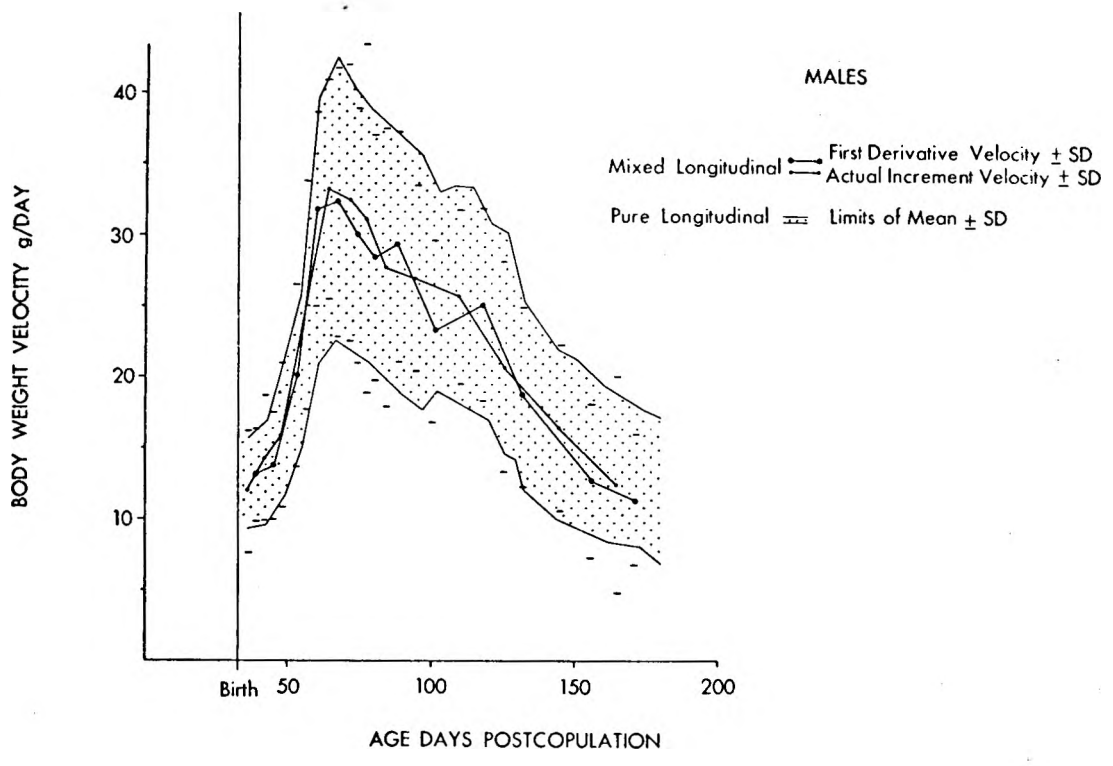


Figure 3. Body weight velocity of male rabbits of both the mixed longitudinal and pure longitudinal series.

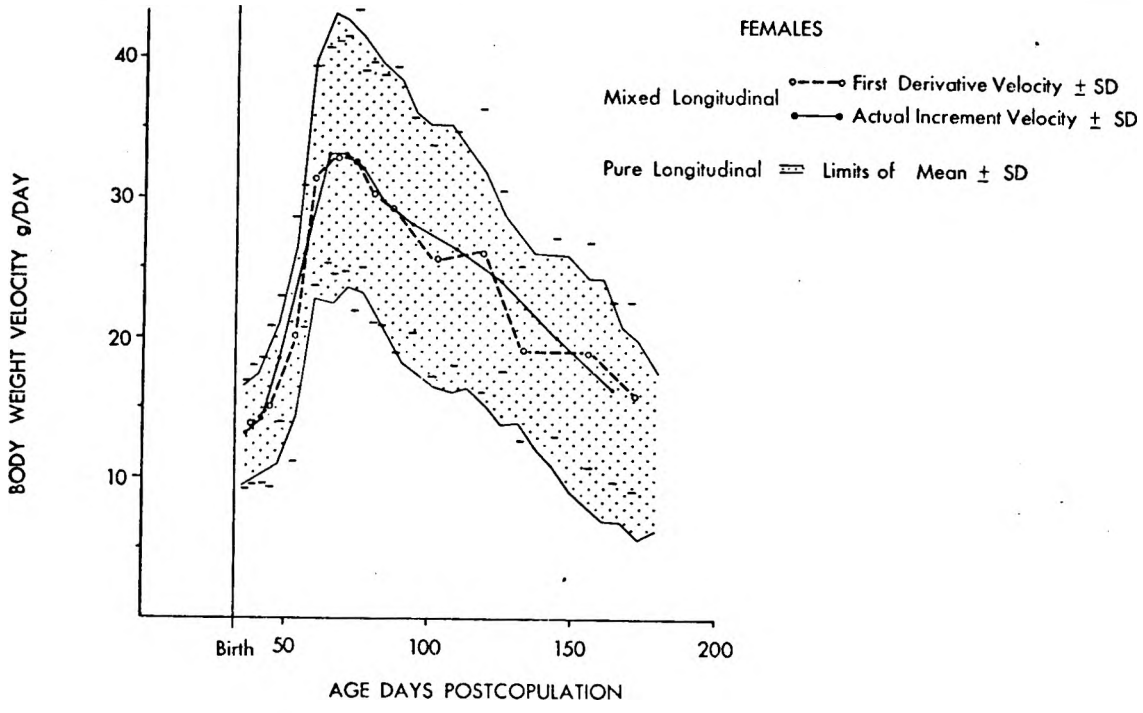


Figure 4. Body weight velocity of female rabbits of both the mixed longitudinal and pure longitudinal series.

Females are larger than males from birth onwards, with the difference becoming more marked from 100 days PC onwards (Figure 5).

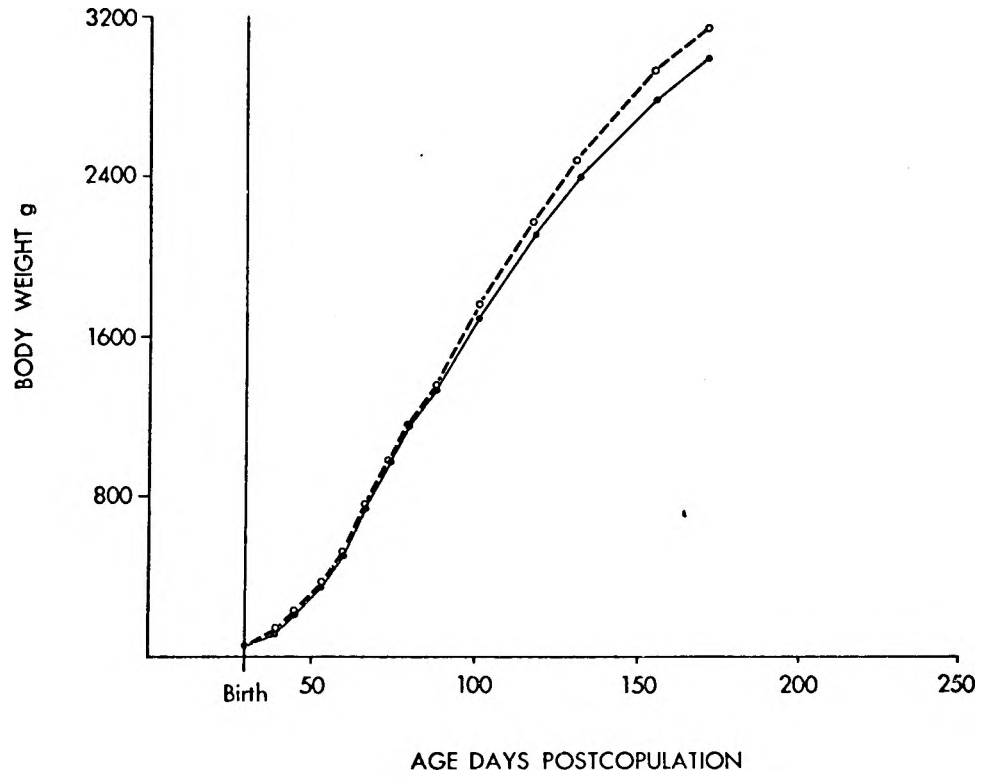


Figure 5. Body weight of male (●—●) and female (o--o) rabbits.

Increase in body weight continues well beyond the point at which this study was terminated and colony records showed a mean adult weight of 4,8kg for females and 4,2kg for males. At no age does the sex difference achieve statistical significance ($p > 0,05$).

The shape of the velocity curve is similar in both sexes, rising sinusoidally after birth to a peak at 67 days PC. It then declines, more slowly in females, with a definite arrest in the rate of decline between about 85 and 130 days PC. This 'shoulder' is most clearly seen in Figures 3 and 4. It coincides with testicular descent in the male which occurs between 90 and 110 days PC. There is no clearcut pubertal event in the female rabbit, but the timing is similar in the two sexes (Templeton 1968).

TABLE 5. Body weight distance and velocity data (means with standard deviation below and coefficients of variation).

AGE	DISTANCE (g)						VELOCITY (g/day)			
	MALE			FEMALE			MALE		FEMALE	
(days pc)	n	mean SD	C.V.	n	mean SD	C.V.	mean SD	C.V.	mean SD	C.V.
33	37	60,1 6,2	10,3	32	60,4 7,3	12,1	-		-	
39	37	136,0 32,0	23,5	32	143,0 39,9	27,9	13,1 3,2	24,4	13,8 4,2	30,1
45	37	210,0 33,7	16,1	32	225,1 44,3	19,7	13,7 3,8	27,4	15,1 5,7	38,0
53	37	336,2 53,7	16,0	32	357,3 74,9	21,0	20,1 6,4	31,6	19,9 8,7	43,7
60	37	519,9 74,0	14,3	32	533,8 107,3	20,1	31,8 6,8	21,4	31,5 7,8	24,7
67	37	751,8 118,7	15,8	32	766,1 122,9	16,0	32,3 9,4	29,2	32,9 8,4	25,4
74	37	962,1 187,3	16,0	32	988,2 165,6	16,8	30,0 8,9	29,6	32,6 10,7	32,7
80	37	1130,6 255,7	22,6	32	1177,2 216,7	18,4	28,4 8,6	30,4	30,3 9,3	30,6
88	37	1350,9 321,4	23,8	32	1403,1 272,8	19,4	29,3 8,2	28,0	29,2 103	35,1
101	37	1701,4 317,3	18,7	32	1762,8 316,3	18,0	23,2 6,4	27,5	25,5 8,2	32,2
118	37	2134,9 296,4	13,9	32	2193,5 361,0	16,5	25,1 6,8	27,0	26,2 10,1	38,3
132	37	2432,0 318,6	13,1	32	2522,6 310,0	12,2	18,6 6,3	34,0	18,9 6,2	32,7
156	37	2799,1 305,1	10,9	32	2963,0 357,6	12,1	12,7 5,4	42,4	18,8 8,0	42,2
171	37	3021,7 283,2	9,4	32	3168,0 345,5	10,9	11,4 4,6	39,8	15,8 6,7	42,2

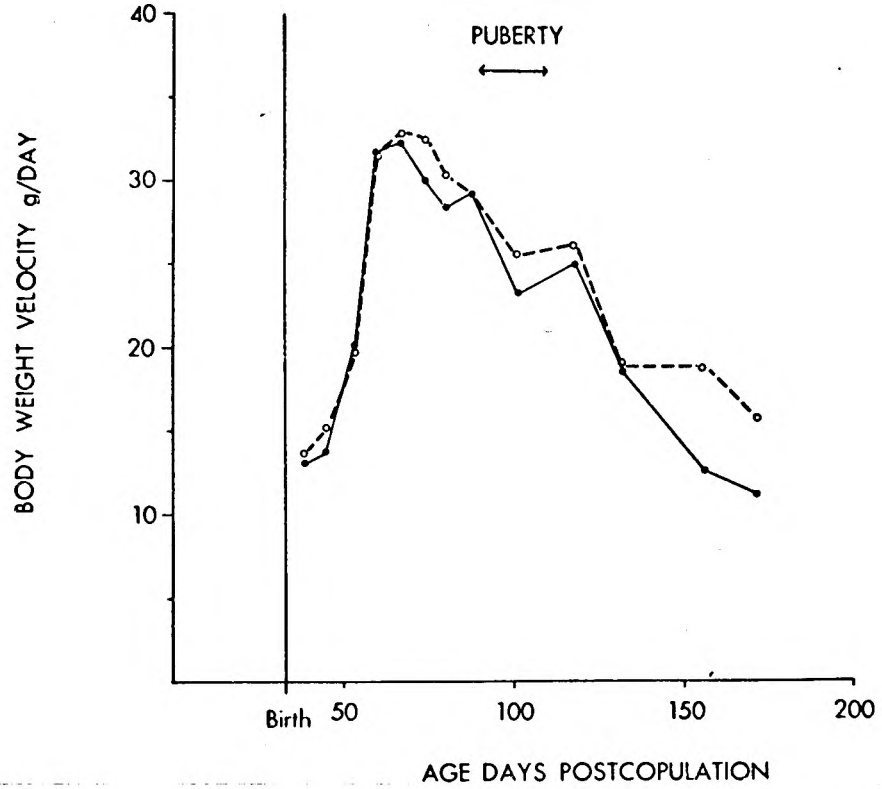


Figure 6. Body weight velocity of male (●—●) and female (o—o) rabbits.

Body length (Figures 7, 8, 9; Table 6)

Body length is greater in the female throughout the growth period, but not statistically so ($p > 0,05$). Growth ceases around 171 days PC with the female being 3% longer than the male. (Figure 7).

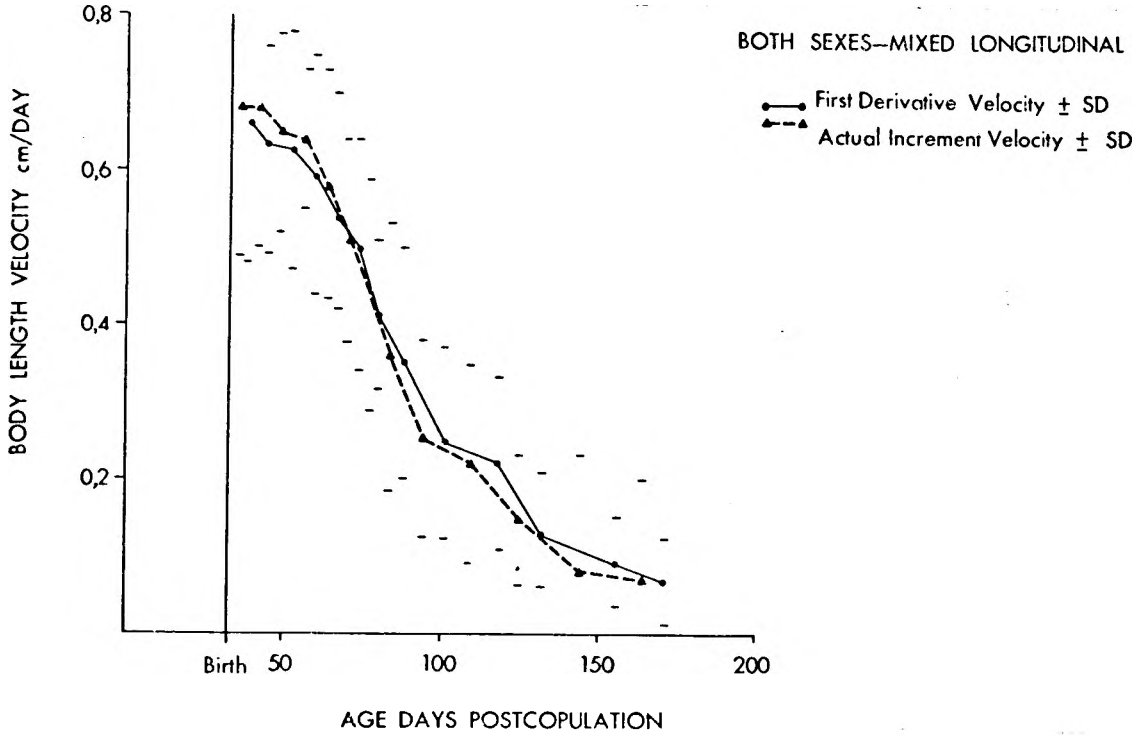


Figure 7. Body length velocity of both sexes combined of the mixed longitudinal series.

TABLE 6. Body length distance and velocity data (means with standard deviations below and coefficients of variation).

AGE	DISTANCE (cm)						VELOCITY (cm/day)			
	MALE			FEMALE			MALE		FEMALE	
(days pc)	n	mean SD	C.V.	n	mean SD	C.V.	mean SD	C.V.	mean SD	C.V.
33	37	15,2 3,4	22,4	32	15,4 3,3	21,4	-		-	
39	37	18,1 6,2	34,3	32	20,2 7,0	34,7	0,64 0,59	92,2	0,68 0,26	38,2
45	37	21,6 8,7	40,3	32	24,5 7,8	31,8	0,61 0,45	73,8	0,66 0,20	30,3
53	37	26,3 11,4	43,3	32	29,2 10,8	37,0	0,58 0,24	41,4	0,65 0,10	15,4
60	37	30,1 13,0	43,2	32	33,6 13,4	39,9	0,56 0,13	23,2	0,62 0,13	21,0
67	37	34,3 14,0	40,8	32	37,5 14,2	37,9	0,52 0,14	26,9	0,55 0,10	18,2
74	36	37,4 13,8	36,9	32	41,1 13,7	33,3	0,47 0,18	38,3	0,52 0,11	21,2
80	36	40,0 13,7	34,3	32	43,6 13,5	31,0	0,41 0,18	43,9	0,43 0,13	30,2
88	37	43,1 12,7	29,5	32	46,1 11,4	24,7	0,33 0,15	45,5	0,38 0,15	39,5
101	37	46,8 11,2	23,9	32	48,5 11,7	24,1	0,26 0,11	42,3	0,32 0,13	40,6
118	37	49,8 9,9	19,9	32	51,3 10,4	20,3	0,23 0,12	52,2	0,25 0,10	40,0
132	37	51,9 9,6	18,5	32	53,6 9,4	17,5	0,13 0,07	53,8	0,14 0,08	57,1
156	37	54,5 10,8	19,8	32	56,2 10,7	19,0	0,09 0,07	77,8	0,10 0,06	60,0
171	36	54,9 10,6	19,3	32	56,8 9,7	17,1	0,07 0,05	71,4	0,07 0,06	85,7

The velocity curve for both sexes combined was calculated by both 'first derivative' and 'actual increment' methods. Figure 8 shows that the two methods again yield curves of very similar shape, position and variability.

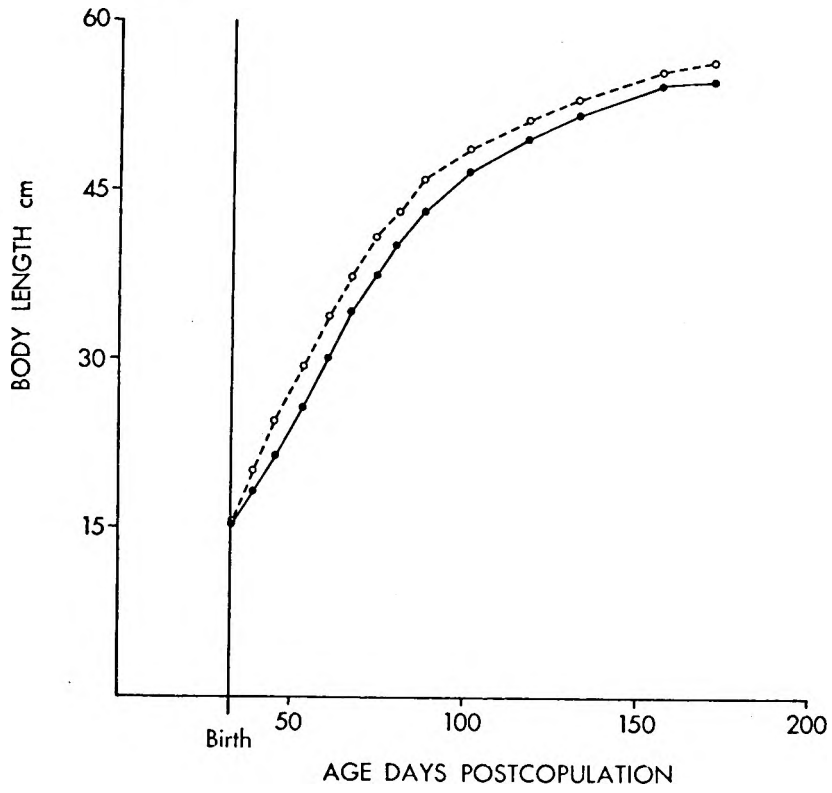


Figure 8. Body length of male (●—●) and female (o--o) rabbits.

Both males and females show a slowing in the rate of decline in velocity at the time of puberty, with female velocity being significantly greater than male at 101 days PC (Figure 9).

Skull length (Figures 10, 11; Table 7).

The female skull is longer than the male from birth until growth is complete, but the difference does not reach statistical significance ($p > 0,05$).

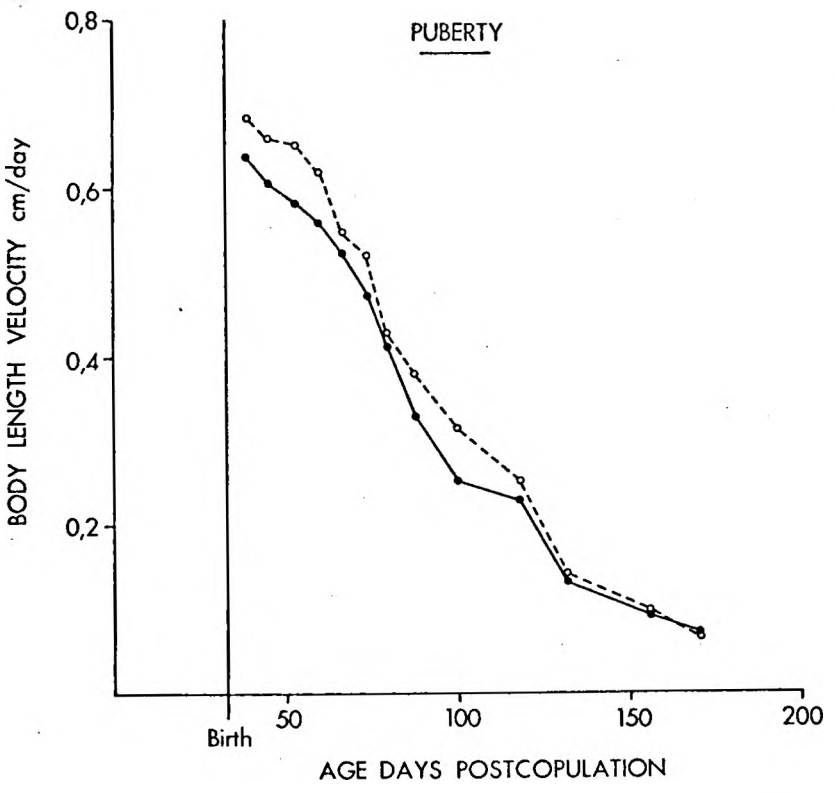


Figure 9. Body length velocity of male (●—●) and female (o--o) rabbits.

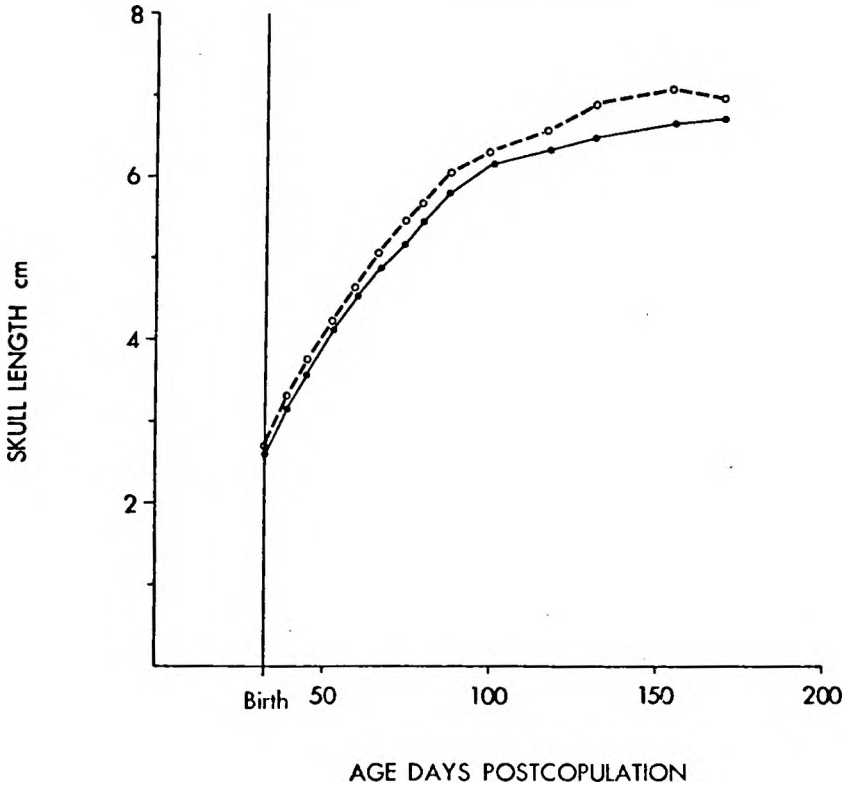


Figure 10. Skull length of male (●—●) and female (o--o) rabbits.

TABLE 7. Skull length distance and velocity data (means with standard deviation below at each age).

AGE (days pc)	n	DISTANCE (cm) MALE	n	FEMALE	VELOCITY (cm/day) MALE	FEMALE
33	32	2,59 1,19	27	2,65 1,08	-	-
39	32	3,17 1,37	27	3,32 1,26	0,094 0,033	0,101 0,031
45	36	3,59 1,43	32	3,75 1,28	0,079 0,035	0,079 0,038
53	37	4,11 1,56	32	4,24 1,41	0,074 0,049	0,066 0,029
60	37	4,56 1,70	32	4,68 1,52	0,067 0,034	0,071 0,039
67	37	4,89 1,88	31	5,10 1,64	0,052 0,035	0,058 0,029
74	37	5,18 2,04	32	5,43 1,74	0,057 0,031	0,061 0,039
80	36	5,44 2,13	32	5,69 1,85	0,058 0,026	0,062 0,035
88	36	5,80 2,26	32	6,08 1,95	0,047 0,047	0,053 0,039
101	36	6,19 2,43	32	6,39 2,28	0,040 0,022	0,036 0,029
118	36	6,37 2,47	31	6,60 2,31	0,022 0,017	0,027 0,021
132	36	6,54 2,55	31	6,94 2,40	0,014 0,015	0,023 0,015
156	35	6,71 2,76	31	7,11 2,59	0,016 0,028	0,018 0,018
171	35	6,73 2,94	30	7,09 2,73	0,015 0,016	0,017 0,043

The velocity curve shows a rapid fall after birth with a small, early puberty 'spurt' in both sexes between 75 and 85 days PC.

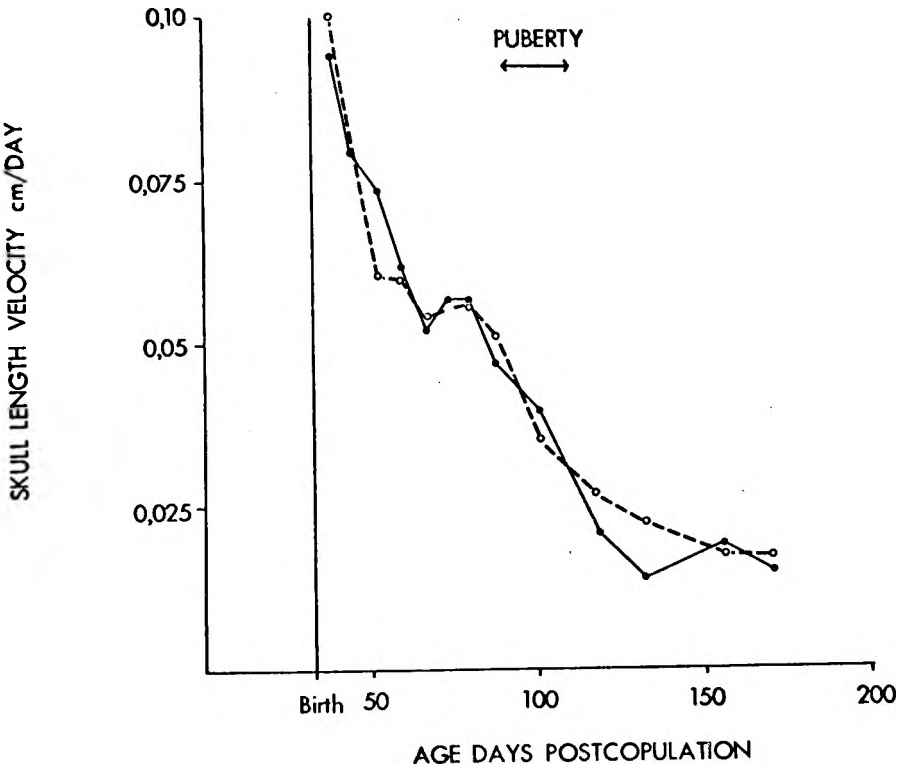


Figure 11. Skull length velocity of male (●—●) and female (o--o) rabbits.

5th lumbar vertebral length and breadth (Figures 12, 13; Table 8).

In both dimensions the female 5th lumbar vertebra is larger than the male at birth and remains so through to adulthood, but at all ages $p > 0,05$. There is no obvious pubertal change in velocity.

11th rib length (Figures 14, 15; Table 9).

The female rib is longer than the male from birth and this becomes more marked after 80 days PC, but at all ages $p > 0,05$. Pubertal velocity changes are more marked in the female, where a reversal of the progressive fall occurs, while male length velocity only plateaus for the duration of puberty. Female velocity is significantly greater than male at 88 and 101 days PC.

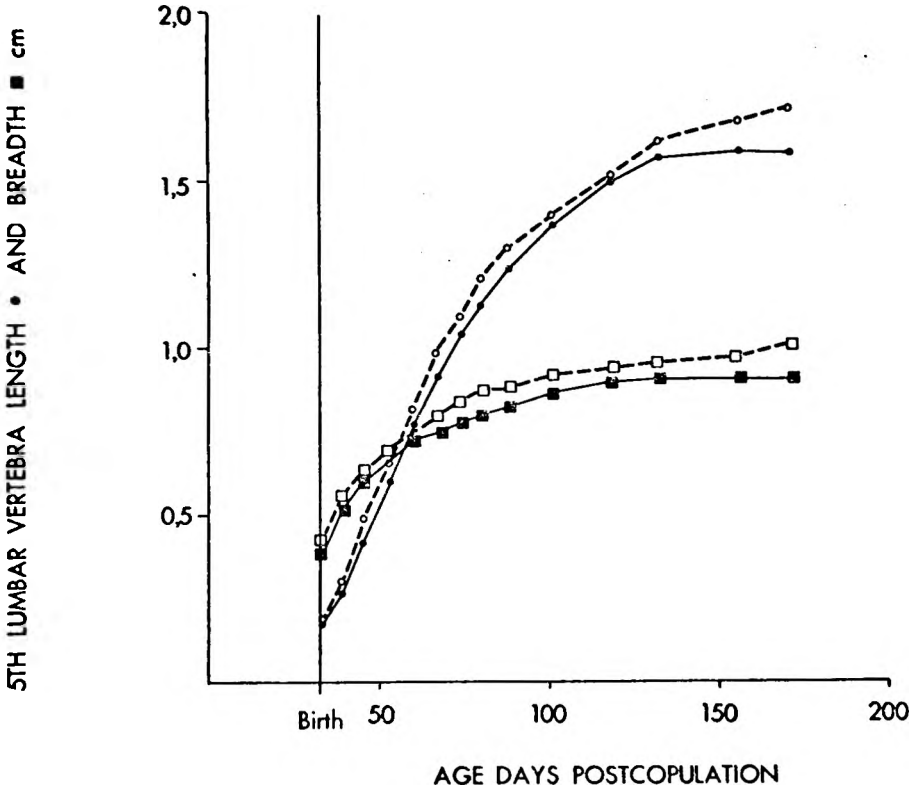


Figure 12. 5th lumbar vertebra length and breadth of male (●—●) and female (o--o) rabbits.

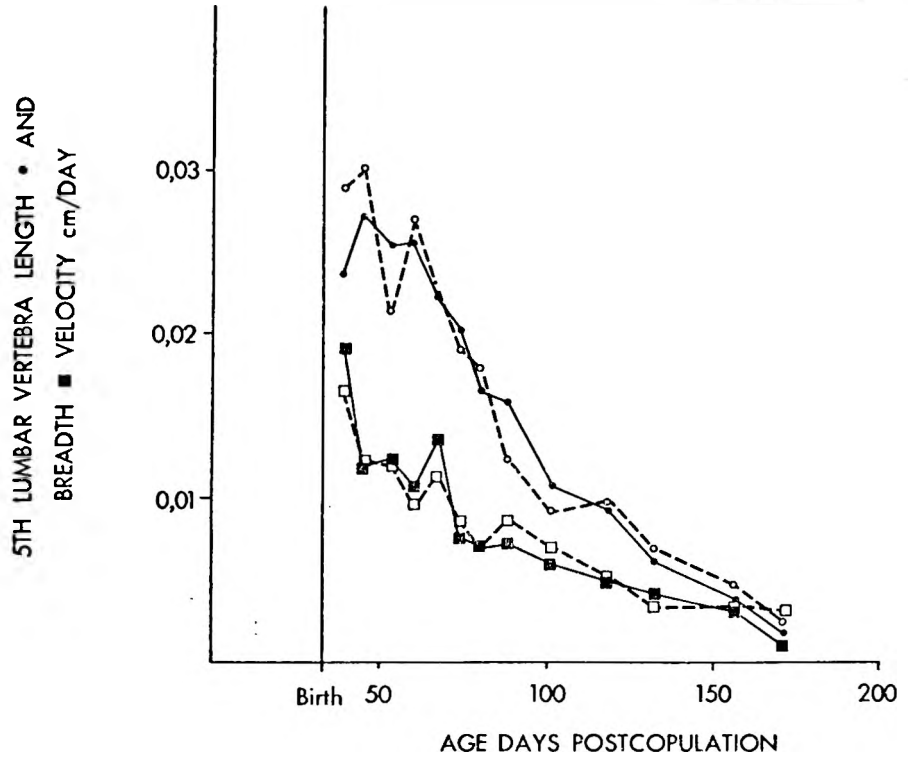


Figure 13. 5th lumbar vertebra length and breadth velocity of male (●—●) and female (o--o) rabbits.

TABLE 8. Lumbar vertebra length and breadth distance and velocity data (means with standard deviations below at each age group). Numbers of animals in each group are the same as those in Table 9.

AGE (days pc)	LENGTH				BREADTH			
	DISTANCE (cm)		VELOCITY (cm/day)		DISTANCE (cm)		VELOCITY (cm/day)	
	M	F	M	F	M	F	M	F
33	0,17	0,19	-	-	0,38	0,42	-	-
	0,38	0,35			0,45	0,42		
39	0,27	0,30	0,024	0,029*	0,52	0,56	0,019	0,016
	0,39	0,35	0,010	0,009	0,46	0,41	0,008	0,008
45	0,42	0,48	0,027	0,031	0,60	0,63	0,011	0,012
	0,43	0,39	0,012	0,009	0,48	0,43	0,006	0,007
53	0,60	0,66	0,026	0,021	0,66	0,70	0,012	0,012
	0,49	0,44	0,010	0,007	0,50	0,45	0,007	0,008
60	0,77	0,82	0,026	0,027	0,73	0,75	0,011	0,010
	0,54	0,48	0,008	0,010	0,52	0,46	0,006	0,006
67	0,91	0,98	0,022	0,023	0,75	0,80	0,014	0,012
	0,61	0,54	0,010	0,012	0,55	0,48	0,012	0,008
74	1,04	1,10	0,020	0,019	0,78	0,84	0,008	0,009
	0,66	0,58	0,011	0,008	0,58	0,50	0,005	0,006

continued overleaf

TABLE 8. Continued

AGE (days pc)	LENGTH DISTANCE (cm) M	F	VELOCITY (cm/day) M	F	BREADTH DISTANCE (cm) M	F	VELOCITY (cm/day) M	F
80	1,13	1,21	0,017	0,018	0,80	0,87	0,007	0,007
	0,70	0,61	0,007	0,007	0,59	0,51	0,006	0,008
88	1,24	1,30	0,016	0,012	0,83	0,88	0,007	0,009
	0,75	0,67	0,011	0,005	0,61	0,55	0,005	0,005
101	1,37	1,40	0,011	0,009	0,87	0,92	0,006	0,007
	0,80	0,73	0,011	0,007	0,63	0,59	0,005	0,007
118	1,50	1,52	0,009	0,010	0,90	0,94	0,005	0,005
	0,86	0,77	0,008	0,010	0,64	0,59	0,006	0,003
132	1,57	1,63	0,006	0,007	0,91	0,96	0,004	0,004
	0,89	0,81	0,005	0,005	0,65	0,60	0,004	0,003
156	1,59	1,69	0,004	0,005	0,91	0,98	0,004	0,004
	0,92	0,82	0,003	0,002	0,68	0,60	0,004	0,004
171	1,58	1,73	0,002	0,002	0,91	1,01	0,001	0,003
	0,96	0,84	0,005	0,004	0,69	0,61	0,004	0,007

* female mean significantly different from male mean at 5%

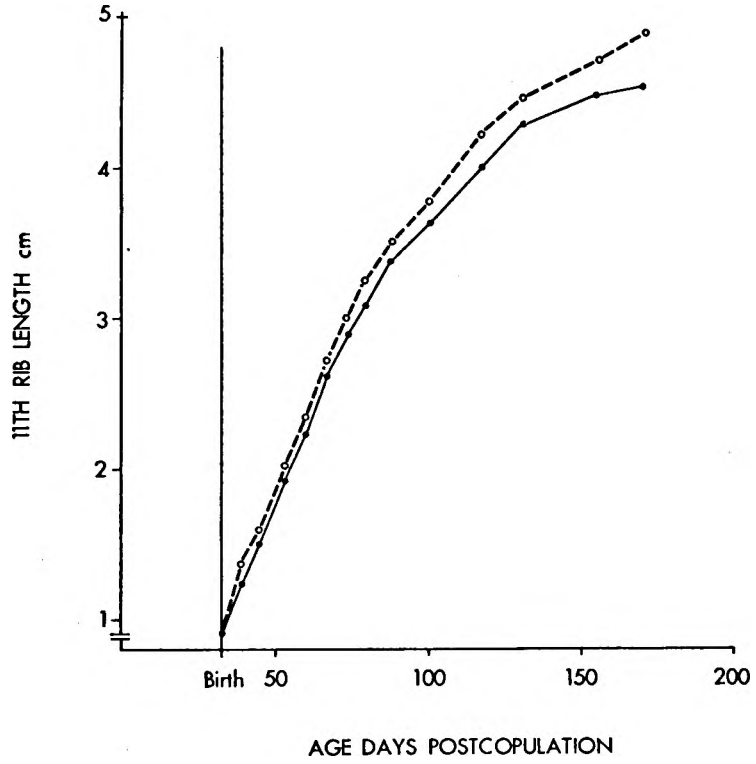


Figure 14. 11th rib length (right) of male (●—●) and female (o--o) rabbits.

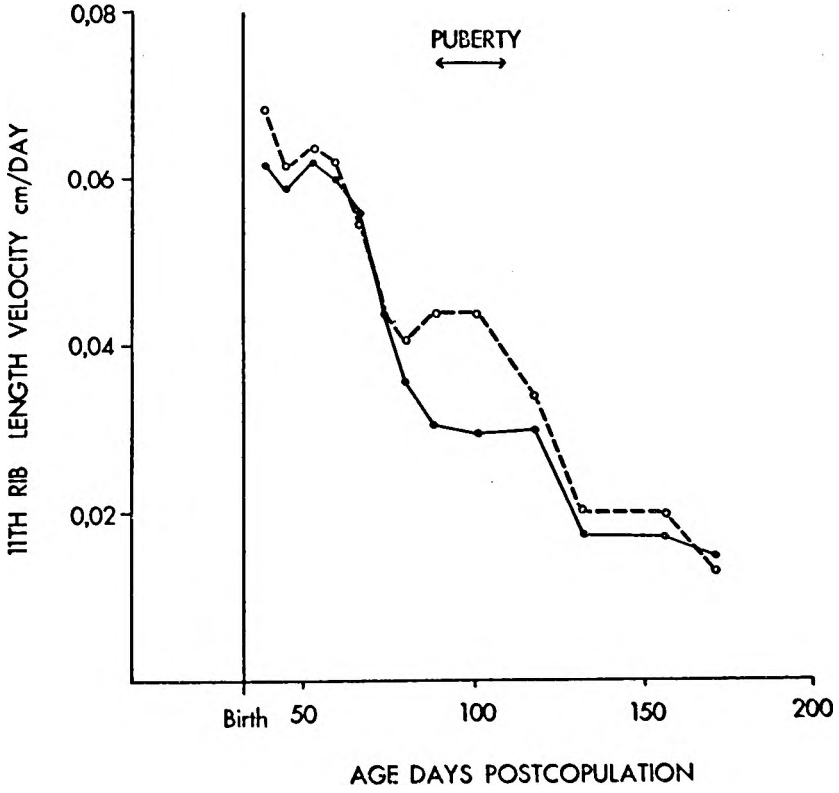


Figure 15. 11th rib length velocity of male (●—●) and female (o--o) rabbits.

TABLE 9. 11th rib length distance and velocity data (means with standard deviations below).

AGE (days pc)	DISTANCE (cm) n	MALE	n	FEMALE	VELOCITY (cm/day) MALE	FEMALE
33	37	0,90 0,64	32	0,92 0,60	-	-
39	37	1,22 0,75	32	1,38 0,72	0,062 0,036	0,068 0,038
45	37	1,49 0,78	32	1,60 0,74	0,059 0,024	0,061 0,022
53	37	1,92 0,91	32	2,01 0,83	0,062 0,028	0,064 0,041
60	37	2,23 0,99	32	2,34 0,91	0,060 0,026	0,062 0,028
67	37	2,61 1,17	32	2,72 1,03	0,056 0,020	0,054 0,031
74	36	2,89 1,28	31	3,03 1,12	0,044 0,024	0,044 0,036
80	37	3,08 1,36	31	3,26 1,18	0,036 0,020	0,041 0,033
88	37	3,37 1,47	31	3,50 1,27	0,031 0,015	0,044* 0,030
101	37	3,63 1,57	30	3,79 1,47	0,030 0,018	0,044* 0,028
118	36	4,01 1,71	31	4,24 1,60	0,031 0,014	0,034 0,031
132	36	4,28 1,80	31	4,48 1,67	0,017 0,014	0,020 0,012
156	37	4,49 1,98	30	4,72 1,75	0,017 0,011	0,020 0,015
171	36	4,54 2,07	31	4,93 1,81	0,016 0,011	0,013 0,013

* female mean significantly different from male mean at 5%

Pelvic dimensions (Figures 16 to 25; Tables 10,11).

In all dimensions females are either slightly greater than or similar to males between birth and 101 days PC, but differences do not achieve statistical significance ($p > 0,05$). Thereafter the female pelvic measurements become progressively larger than the males, ranging from a 19% difference for iliac length to 4% for bi-acetabular width at 171 days PC when growth has ceased.

All the female velocity curves show a distinct pubertal slowing in their rate of decline. In the male this pubertal phenomenon is clearcut only for bi-iliac and bi-ischial width.

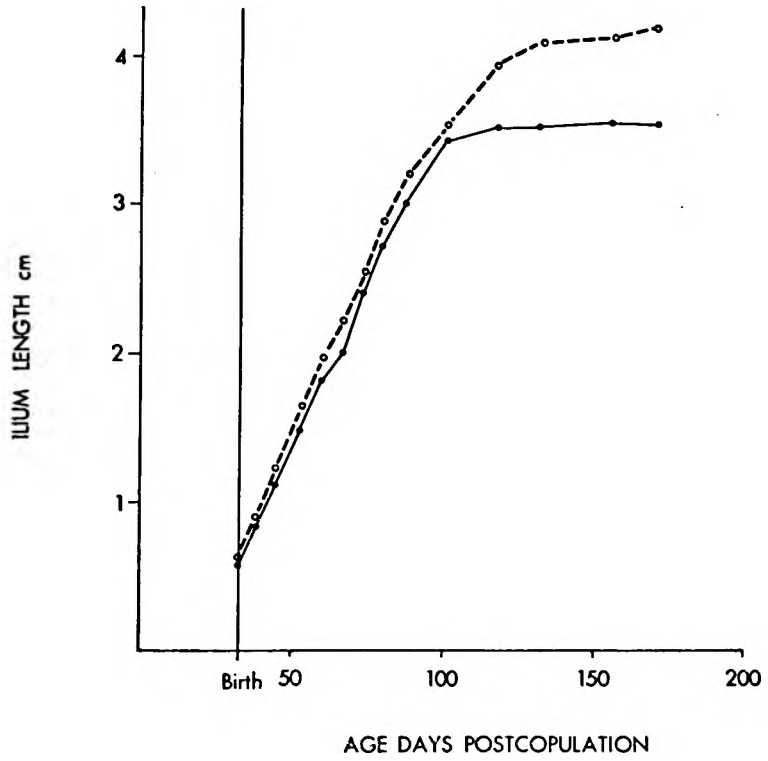


Figure 16. Ilium length (right) of male (●—●) and female (o--o) rabbits.

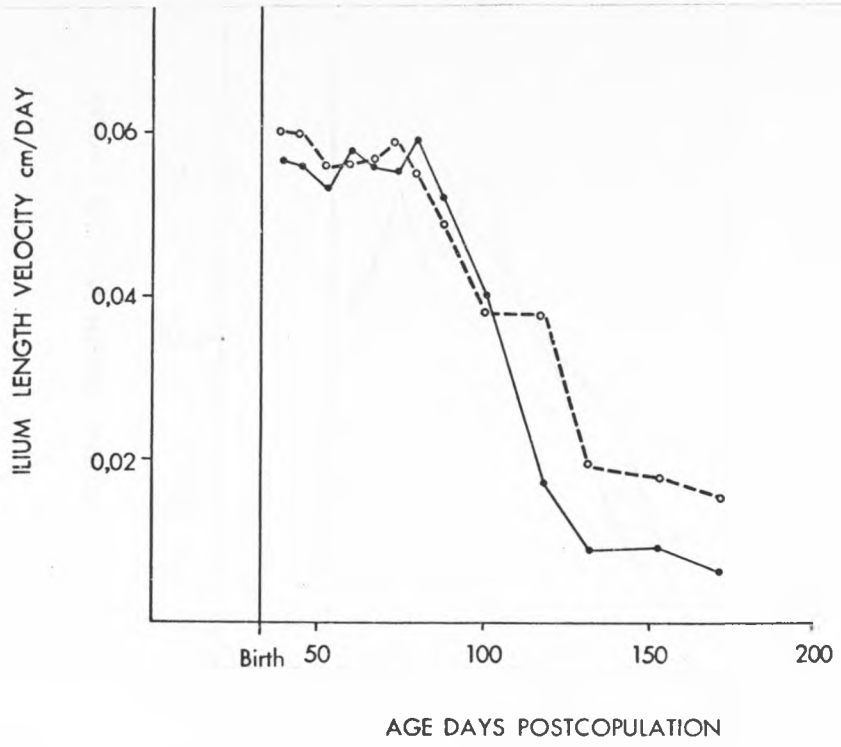


Figure 17. Ilium length velocity of male (●—●) and female (o--o) rabbits.

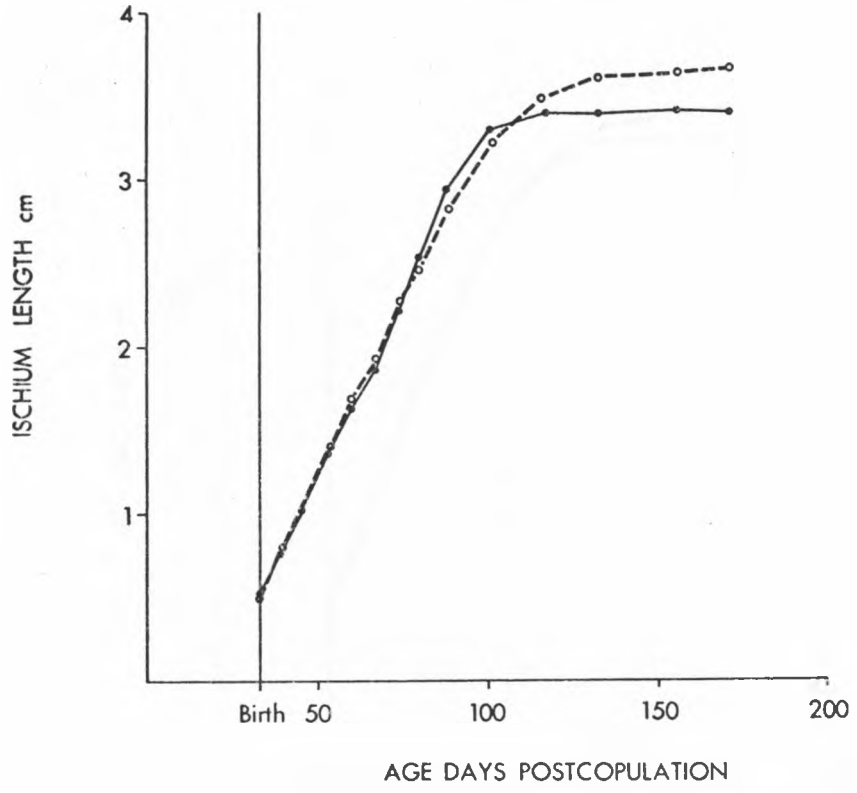


Figure 18. Ischium length (right) of male (●—●) and female (o--o) rabbits.

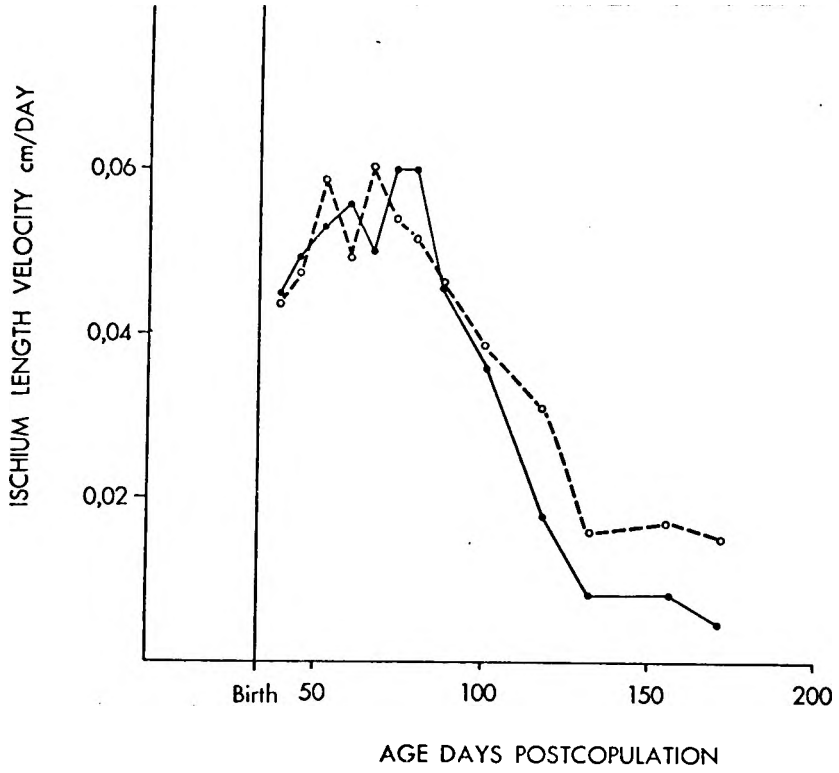


Figure 19. Ischium length velocity of male (●—●) and female (o--o) rabbits.

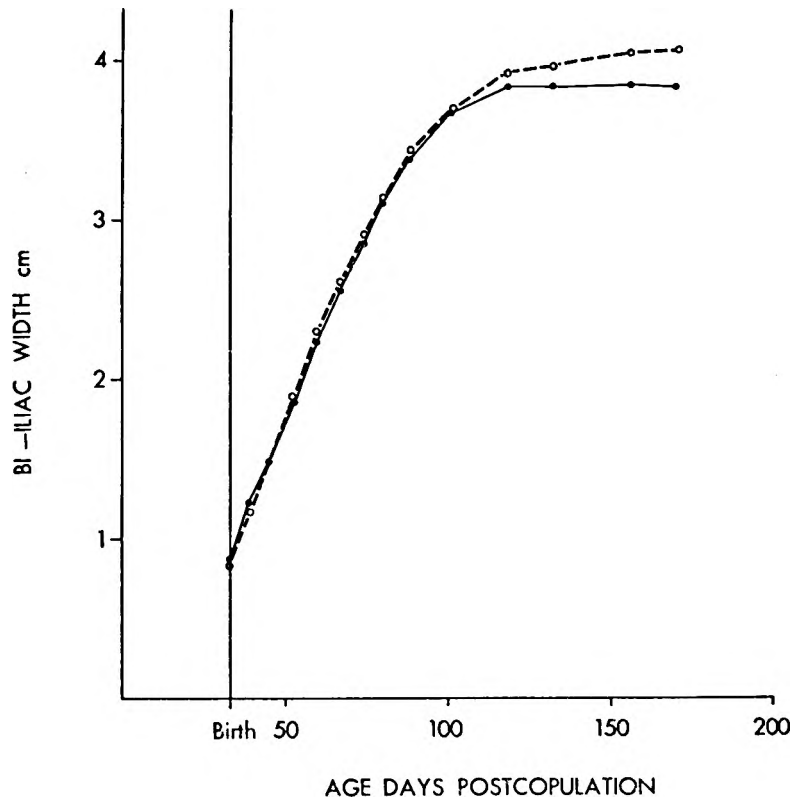


Figure 20. Bi-iliac width of male (●—●) and female (o--o) rabbits.

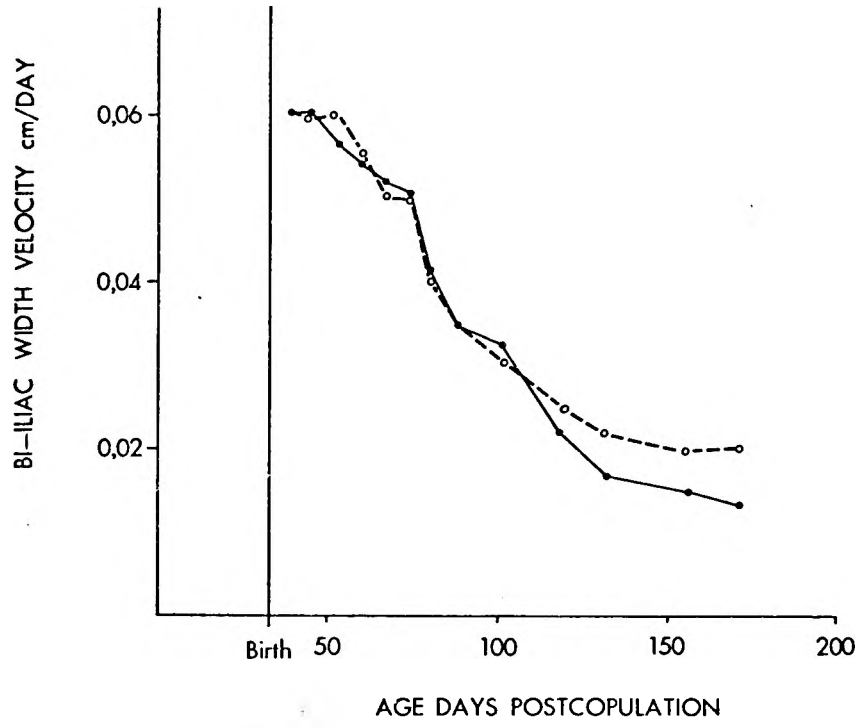


Figure 21. Bi-iliac width velocity of male (●—●) and female (o--o) rabbits.

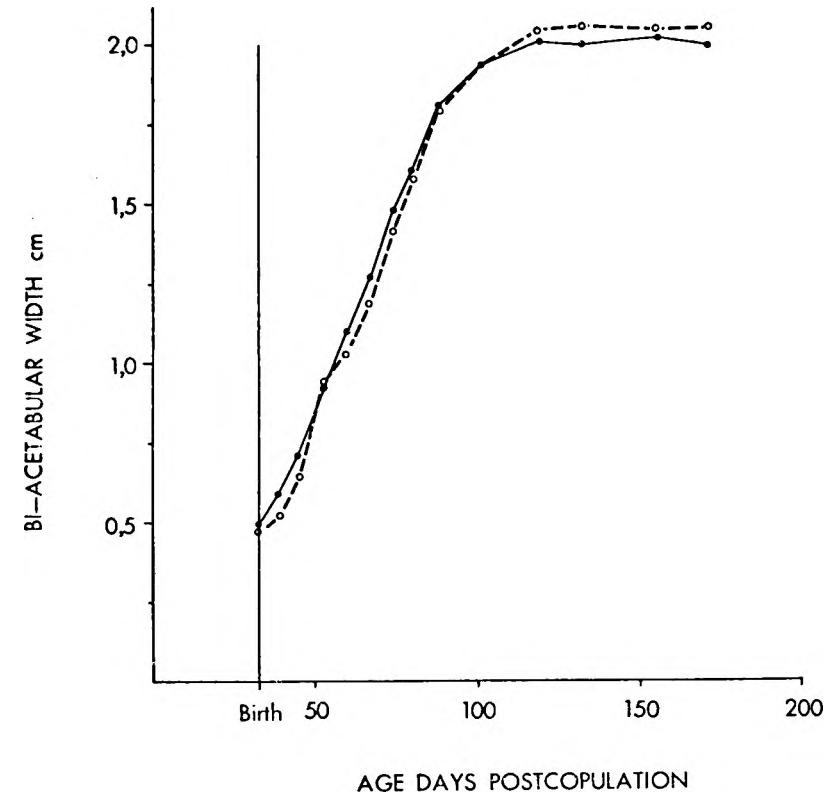


Figure 22. Bi-acetabular width of male (●—●) and female (o--o) rabbits.

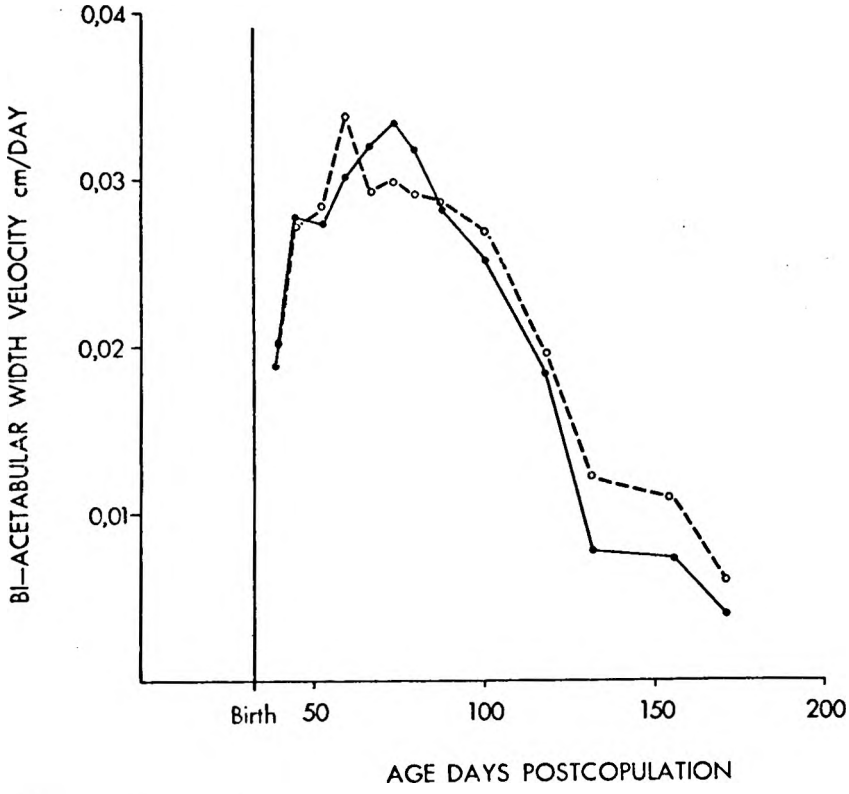


Figure 23. Bi-acetabular width velocity of male (●—●) and female (o--o) rabbits.

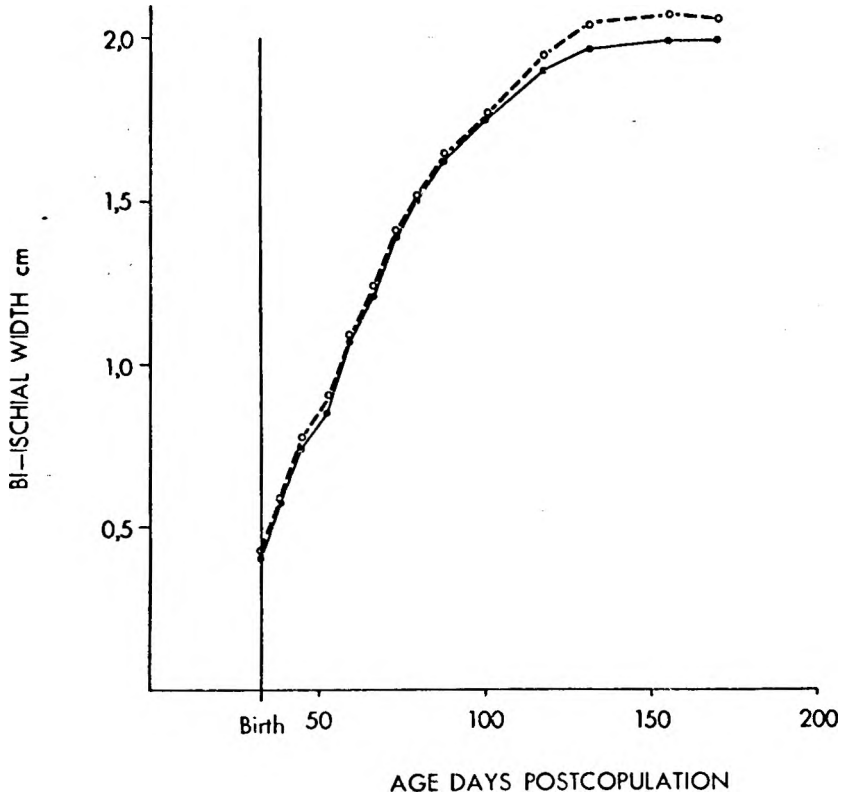


Figure 24. Bi-ischial width of male (●—●) and female

TABLE 10. Pelvic dimensions distance data in cm, (mean and standard deviations at each age). Numbers of animals in each group are the same as in Table 9.

AGE (days pc)	ILIUM LENGTH		ISCHIUM LENGTH		BI-ILIAC WIDTH		BI-ACETABULAR WIDTH		BI-ISCHIAL WIDTH	
	M	F	M	F	M	F	M	F	M	F
33	0,57	0,63	0,54	0,52	0,85	0,84	0,49	0,47	0,41	0,43
	0,64	0,58	0,54	0,54	0,65	0,65	0,52	0,61	0,38	0,40
39	0,84	0,91	0,80	0,77	1,23	1,16	0,59	0,52	0,57	0,59
	0,70	0,60	0,57	0,55	0,73	0,69	0,51	0,59	0,50	0,48
45	1,12	1,23	1,02	1,05	1,49	1,49	0,71	0,64	0,74	0,77
	0,81	0,70	0,66	0,64	0,81	0,77	0,55	0,63	0,51	0,56
53	1,49	1,63	1,36	1,39	1,86	1,90	0,92	0,84	0,85	0,91
	0,94	0,82	0,76	0,74	0,93	0,90	0,64	0,71	0,60	0,71
60	1,82	1,96	1,63	1,69	2,23	2,30	1,10	1,04	1,07	1,09
	1,07	0,92	0,86	0,84	1,05	1,03	0,69	0,79	0,68	0,73
67	2,08	2,22	1,87	1,91	2,57	2,61	1,27	1,19	1,21	1,24
	1,24	1,03	1,00	0,93	1,23	1,16	0,78	0,87	0,77	0,89
74	2,41	2,56	2,23	2,26	2,85	2,89	1,48	1,42	1,89	1,91
	1,40	1,18	1,13	1,01	1,35	1,27	0,87	0,95	0,89	1,00
80	2,72	2,89	2,54	2,47	3,13	3,14	1,60	1,57	1,50	1,52
	1,56	1,29	1,28	1,16	1,47	1,36	0,93	1,02	0,76	0,79

continued overleaf

TABLE 10 continued

AGE (days pc)	ILIUM LENGTH		ISCHIUM LENGTH		BI-ILIAC WIDTH		BI-ACETABULAR WIDTH		BI-ISCHIAL WIDTH	
	M	F	M	F	M	F	M	F	M	F
88	3,07	3,20	2,95	2,80	3,40	3,42	1,81	1,79	1,63	1,64
	1,78	1,54	1,47	1,39	1,62	1,61	1,04	1,18	0,78	0,81
101	3,45	3,55	3,30	3,21	3,70	3,71	1,94	1,94	1,75	1,77
	2,01	1,69	1,64	1,57	1,83	1,77	1,16	1,35	0,80	0,96
118	3,52	3,97	3,43	3,49	3,84	3,94	2,03	2,05	1,95	1,93
	2,20	1,84	1,76	1,69	1,96	1,84	1,24	1,41	0,90	0,91
132	3,54	4,10	3,40	3,62	3,83	3,98	2,00	2,07	2,08	2,01
	2,37	2,08	2,00	2,00	2,25	2,11	1,36	1,54	1,00	1,03
156	3,57	4,17	3,42	3,65	3,86	4,14	2,05	2,05	2,10	2,03
	2,52	2,20	2,11	2,19	2,42	2,30	1,46	1,66	0,97	0,99
171	3,53	4,21	3,40	3,68	3,82	4,15	2,01	2,10	2,01	2,11
	2,71	2,32	2,38	2,04	2,57	2,39	1,61	1,70	0,93	1,10

TABLE 11. Pelvic dimensions velocity data in cm/day, (mean and standard deviations at each age). Numbers of animals in each group are the same as in Table 9.

AGE (days pc)	ILIUM LENGTH		ISCHIUM LENGTH		BI-ILIAC WIDTH		BI-ACETABULAR WIDTH		BI-ISCHIAL WIDTH	
	M	F	M	F	M	F	M	F	M	F
39	0,057	0,061	0,045	0,045	0,061	0,060	0,019	0,020	0,021	0,020
	0,013	0,015	0,015	0,020	0,015	0,027	0,010	0,011	0,011	0,010
45	0,056	0,060	0,049	0,047	0,061	0,059	0,028	0,027	0,027	0,027
	0,014	0,011	0,015	0,018	0,017	0,023	0,017	0,012	0,019	0,018
53	0,052	0,056	0,053	0,059	0,057	0,061	0,027	0,028	0,029	0,031
	0,022	0,025	0,017	0,095	0,017	0,027	0,011	0,011	0,013	0,016
60	0,058	0,057	0,056	0,050	0,054	0,056	0,031	0,034	0,028	0,029
	0,025	0,024	0,019	0,025	0,021	0,021	0,017	0,021	0,020	0,017
67	0,056	0,057	0,050	0,063	0,051	0,051	0,032	0,029	0,031	0,032
	0,025	0,027	0,021	0,028	0,021	0,022	0,017	0,001	0,012	0,019
74	0,056	0,059	0,060	0,054	0,051	0,050	0,034	0,030	0,032	0,031
	0,042	0,044	0,037	0,043	0,035	0,049	0,028	0,019	0,026	0,030
80	0,060	0,055	0,061	0,052	0,042	0,041	0,032	0,029	0,024	0,024
	0,052	0,050	0,050	0,046	0,057	0,049	0,028	0,026	0,021	0,019

continued overleaf

TABLE 11 continued

AGE (days pc)	ILIUM LENGTH		ISCHIUM LENGTH		BI-ILIAC WIDTH		BI-ACETABULAR WIDTH		BI-ISCHIAL WIDTH	
	M	F	M	F	M	F	M	F	M	F
88	0,052	0,049	0,046	0,047	0,035	0,036	0,028	0,029	0,019	0,020
	0,040	0,041	0,036	0,050	0,030	0,023	0,025	0,023	0,020	0,021
101	0,041	0,039	0,036	0,038	0,033	0,031	0,025	0,027	0,019	0,020
	0,017	0,027	0,023	0,019	0,022	0,028	0,016	0,011	0,021	0,023
118	0,017	0,038	0,018	*0,036	0,022	0,025	0,019	0,020	0,017	0,014
	0,011	0,028	0,010	0,020	0,060	0,019	0,022	0,013	0,016	0,010
132	0,012	0,019	0,009	0,017	0,017	0,019	0,008	0,012	0,007	0,009
	0,010	0,013	0,015	0,005	0,028	0,017	0,005	0,005	0,006	0,005
156	0,013	0,018	0,009	0,018	0,015	0,022	0,007	0,011	0,006	0,008
	0,009	0,014	0,006	0,022	0,009	0,008	0,008	0,012	0,006	0,006
171	0,010	0,016	0,005	0,016	0,013	0,021	0,004	0,006	0,004	0,010
	0,006	0,023	0,004	0,009	0,007	0,009	0,009	0,008	0,006	0,009

* female mean significantly different from male mean at 5%

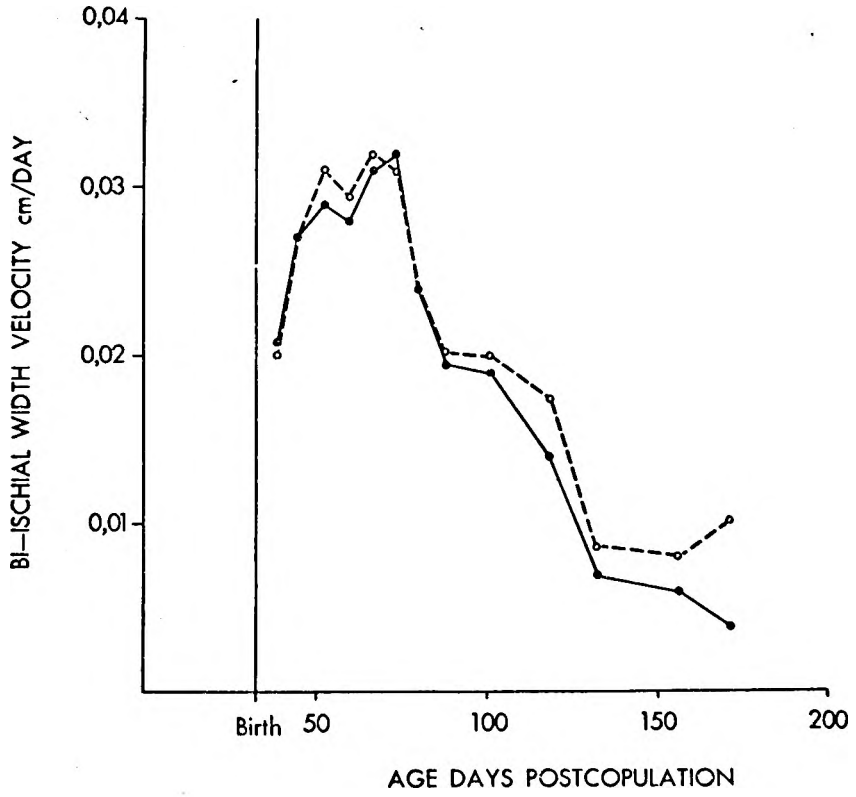


Figure 25. Bi-ischial width velocity of male (●—●) and female (o--o) rabbits.

Humerus length and breadth (Figures 26, 27; Table 12).

Females are larger than males throughout, although not statistically so ($p > 0,05$). A small rise in length velocity is seen in both sexes between 67 and 80 days PC.

Radius length (Figures 28, 29; Table 13).

Females are larger than males throughout, although not statistically so ($p > 0,05$). No clearcut change in velocity occurs in relation to puberty.

3rd metacarpal length (Figures 30, 31; Table 14).

Again females are larger than males throughout, although not statistically so ($p > 0,05$). Soon after birth velocity falls off steeply with two episodes of a decline in the rate of fall, at 60 - 74 and 95 - 110 days PC.

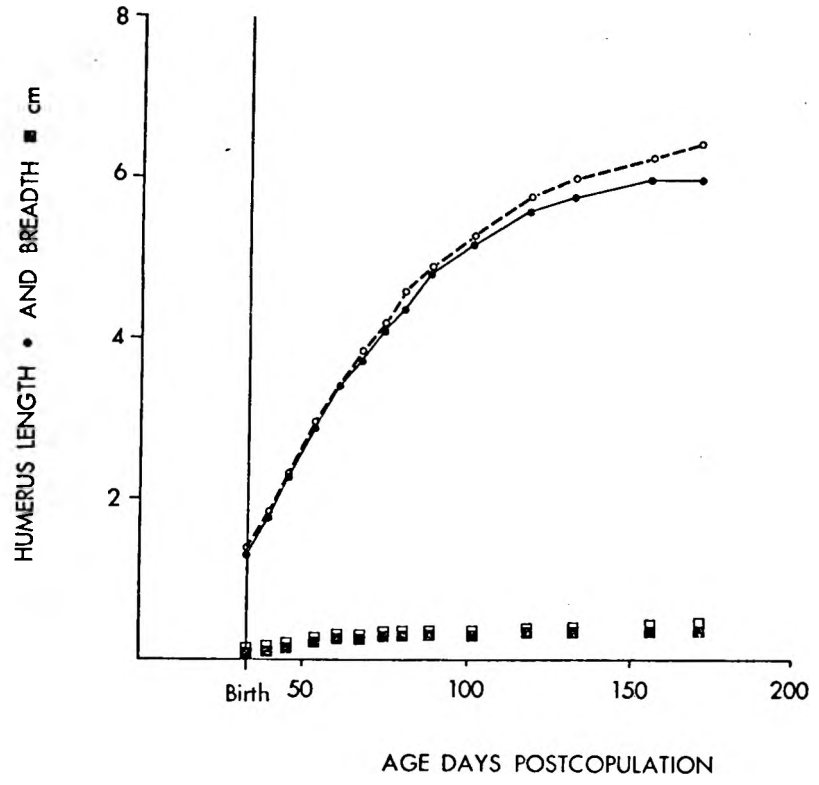


Figure 26. Humerus length (right) and breadth of male (●—●) and female (o--o) rabbits.

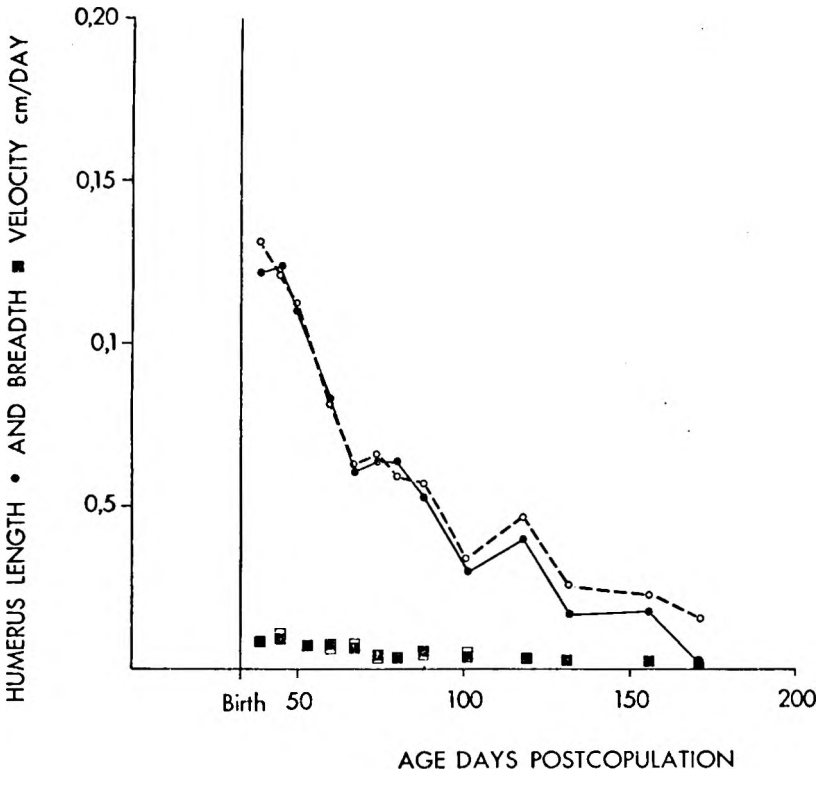


Figure 27. Humerus length and breadth velocity of male (●—●) and female (o--o) rabbits.

TABLE 12. Right humerus length and mid-shaft breadth distance and velocity data (means with standard deviations below at each age group).

AGE (days pc)	n	LENGTH DISTANCE (cm) M	n	F	LENGTH VELOCITY (cm/day) M	F	BREADTH DISTANCE (cm) M	F	BREADTH VELOCITY (cm/day) M	F
33	30	1,30	26	1,44	-	-	0,05	0,08	-	-
		0,78		0,77			0,34	0,31		
39	31	1,75	28	1,92	0,172	0,131	0,10	0,13	0,008	0,008
		0,93		0,89	0,039	0,030	0,33	0,30	0,004	0,005
45	36	2,27	30	2,30	0,174	0,121	0,15	0,18	0,010	0,010
		1,03		1,00	0,049	0,032	0,36	0,31	0,004	0,005
53	37	2,88	32	2,96	0,110	0,112	0,21	0,24	0,007	0,007
		1,19		1,10	0,034	0,032	0,36	0,33	0,003	0,004
60	37	3,40	32	3,42	0,080	0,081	0,24	0,28	0,007	0,006
		1,35		1,20	0,039	0,041	0,38	0,34	0,004	0,003
67	37	3,74	32	3,87	0,061	0,064	0,27	0,30	0,006	0,007
		1,51		1,33	0,030	0,044	0,40	0,35	0,003	0,004
74	37	4,07	32	4,20	0,064	0,066	0,28	0,32	0,004	0,003
		1,65		1,44	0,047	0,034	0,41	0,36	0,003	0,003
80	37	4,38	31	4,56	0,064	0,060	0,29	0,32	0,003	0,004
		1,78		1,53	0,026	0,035	0,42	0,36	0,002	0,003

continued overleaf

TABLE 12. continued

AGE (days pc)	LENGTH						BREADTH			
	DISTANCE (cm)				VELOCITY (cm/day)		DISTANCE (cm)		VELOCITY (cm/day)	
	n	M	n	F	M	F	M	F	M	F
88	37	4,82	32	4,92	0,053	0,058	0,31	0,34	0,005	0,004
		1,92		1,64	0,032	0,024	0,44	0,39	0,003	0,002
101	36	5,19	32	5,30	0,030	0,034	0,32	0,34	0,003	0,004
		2,09		1,92	0,022	0,020	0,44	0,41	0,003	0,003
118	36	5,59	31	5,80	0,042	0,047	0,35	0,37	0,003	0,002
		2,22		2,06	0,023	0,019	0,45	0,42	0,003	0,001
132	37	5,89	32	6,06	0,017	0,026	0,37	0,39	0,002	0,002
		2,33		2,15	0,011	0,008	0,46	0,42	0,002	0,002
156	36	6,07	32	6,38	0,019	0,023	0,36	0,41	0,002	0,002
		2,51		2,23	0,012	0,009	0,48	0,42	0,002	0,002
171	37	6,09	31	6,54	0,002	0,015	0,36	0,42	0,001	0,002
		2,63		2,28	0,008	0,013	0,50	0,43	0,002	0,002

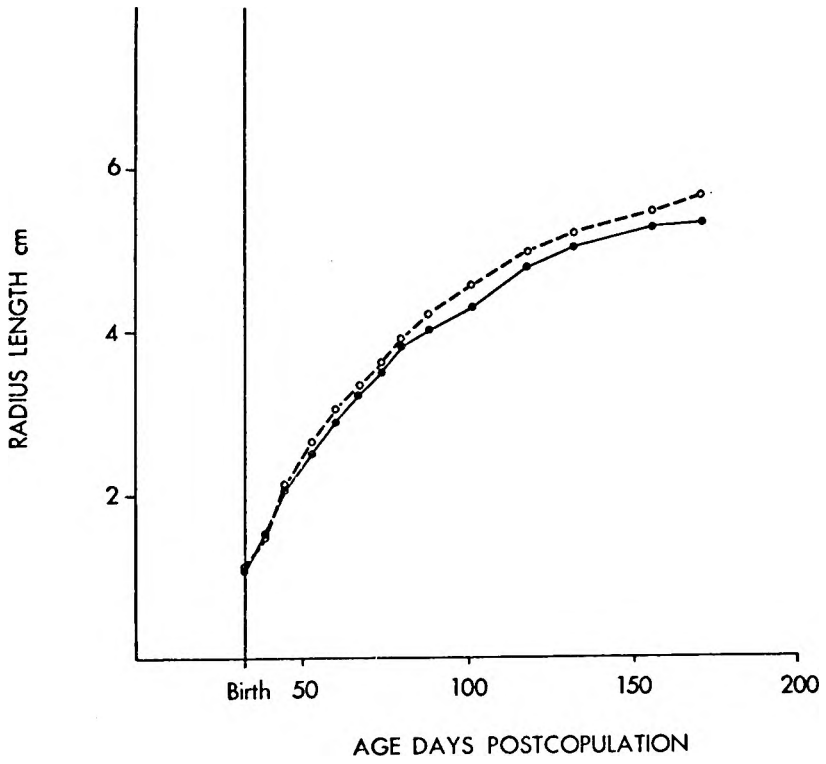


Figure 28. Radius length (right) of male (●—●) and female (o--o) rabbits.

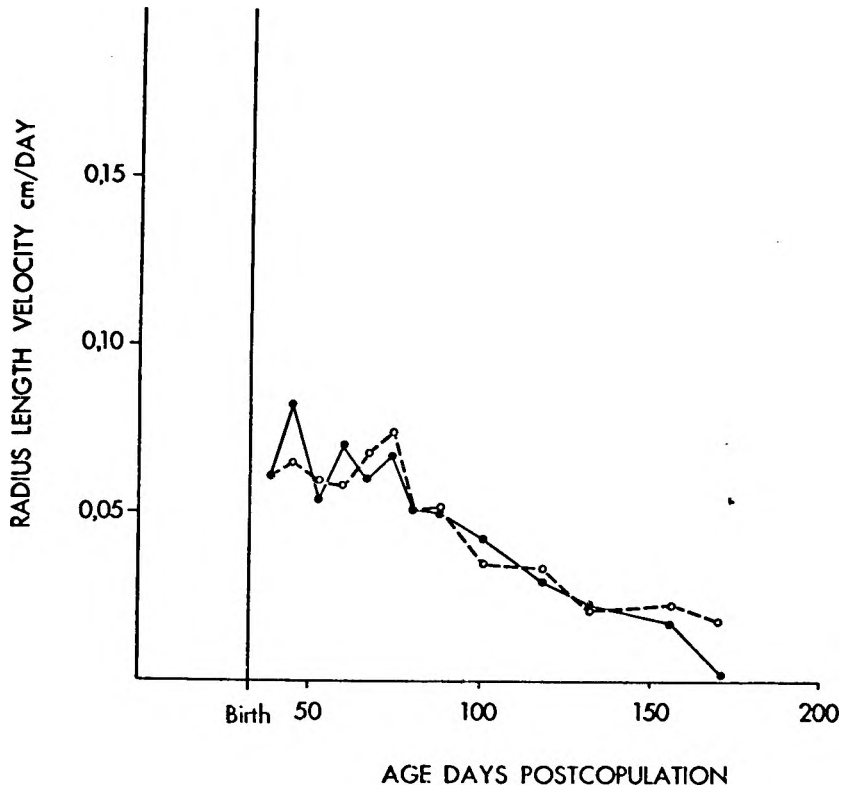


Figure 29. Radius length velocity of male (●—●) and female (o--o) rabbits.

TABLE 13. Right radius length distance and velocity data (means with standard deviations below at each age).

AGE (days pc)	DISTANCE (cm)				VELOCITY (cm/day)	
	n	MALE	n	FEMALE	MALE	FEMALE
33	32	1,09 0,69	23	1,14 0,69	-	-
39	33	1,52 0,78	29	1,49 0,73	0,061 0,032	0,051 0,030
45	37	2,02 0,91	31	2,13 0,86	0,082 0,021	0,065 0,029
53	37	2,52 1,07	31	2,66 1,00	0,054 0,029	0,060 0,022
60	37	2,86 1,18	31	3,01 1,10	0,070 0,028	0,068 0,037
67	35	3,20 1,34	30	3,30 1,20	0,060 0,030	0,068 0,032
74	34	3,49 1,45	32	3,62 1,32	0,067 0,041	0,075 0,049
80	36	3,80 1,58	32	3,93 1,40	0,051 0,036	0,051 0,050
88	37	3,98 1,66	32	4,19 1,51	0,050 0,124	0,052 0,028
101	36	4,32 1,78	32	4,30 1,65	0,043 0,053	0,035 0,013
118	36	4,82 2,22	32	4,99 1,84	0,030 0,051	0,034 0,024
132	36	5,07 2,37	32	5,22 1,93	0,023 0,015	0,021 0,018
156	37	5,34 2,21	31	5,51 2,02	0,018 0,012	0,023 0,010
171	36	5,36 2,31	31	5,57 2,04	0,002 0,015	0,019 0,017

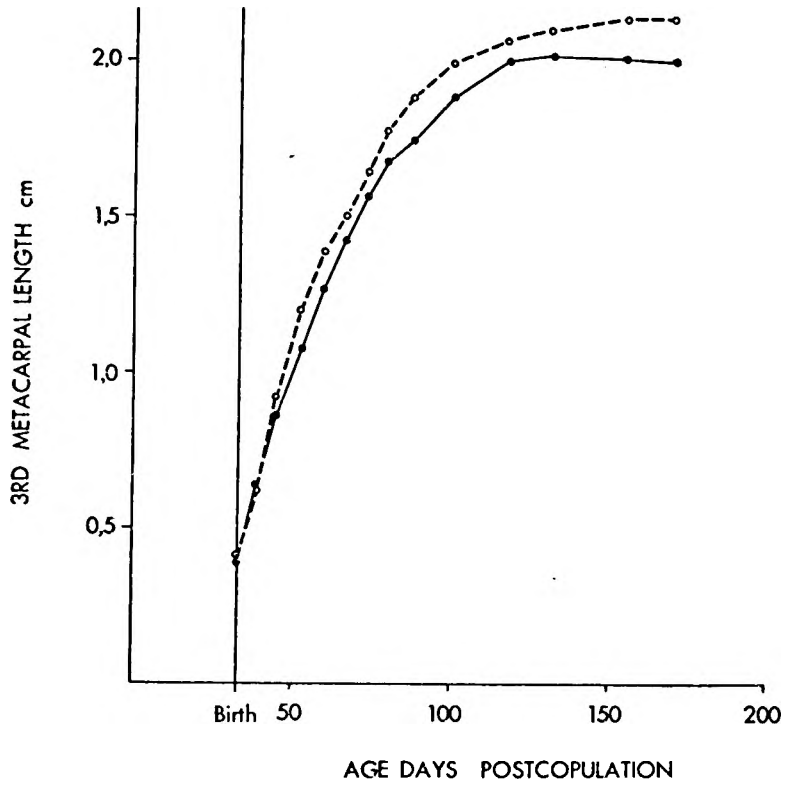


Figure 30. 3rd metacarpal length (right) of male (●—●) and female (o--o) rabbits.

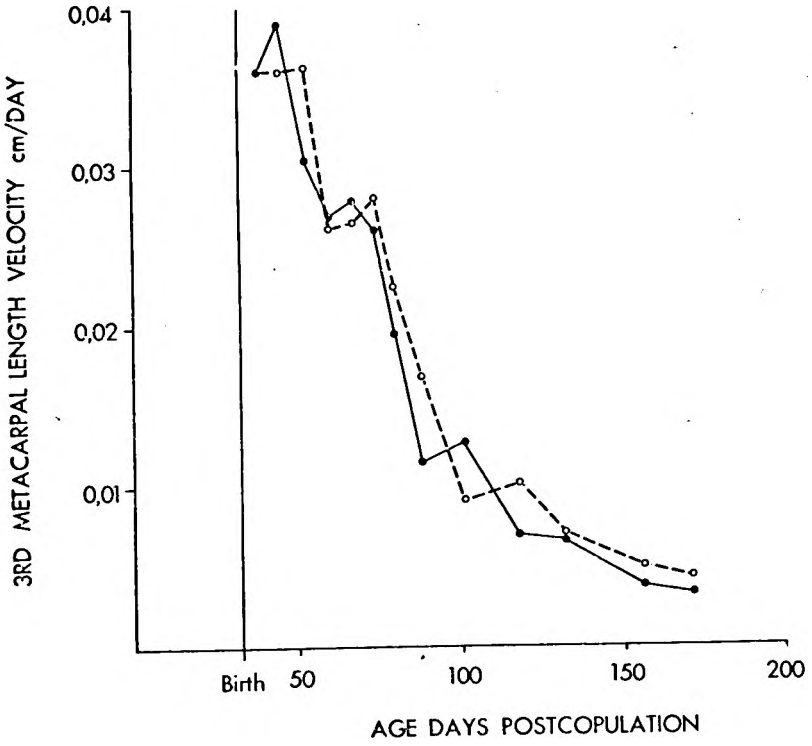


Figure 31. 3rd metacarpal length velocity of male (●—●) and female (o--o) rabbits.

TABLE 14. Right 3rd metacarpal length distance and velocity data (means with standard deviations below at each age)

AGE (days pc)	DISTANCE (cm) n	MALE	n	FEMALE	VELOCITY (cm/day) MALE	FEMALE
33	28	0,39 0,50	16	0,41 0,56	-	-
39	33	0,64 0,57	19	0,62 0,58	0,036 0,021	0,036 0,020
45	36	0,86 0,58	28	0,93 0,55	0,039 0,017	0,036 0,019
53	36	1,11 0,66	32	1,20 0,59	0,031 0,013	0,036 0,045
60	36	1,28 0,71	32	1,39 0,63	0,027 0,010	0,026 0,017
67	36	1,43 0,78	31	1,51 0,68	0,028 0,012	0,027 0,014
74	36	1,57 0,84	30	1,65 0,73	0,026 0,010	0,028 0,016
80	36	1,68 0,89	30	1,78 0,77	0,019 0,010	0,022 0,010
88	36	1,75 0,93	32	1,89 0,84	0,012 0,006	0,017 0,011
101	36	1,89 0,98	32	2,00 0,91	0,013 0,018	0,014 0,005
118	36	2,00 1,01	32	2,08 0,94	0,007 0,015	0,071 0,003
132	36	2,04 1,02	31	2,11 0,94	0,007 0,007	0,053 0,004
156	35	2,02 1,07	30	2,16 0,96	0,003 0,004	0,003 0,003
171	35	2,00 1,10	30	2,16 0,95	0,004 0,003	0,004 0,005

Femur length (Figures 32, 33; Table 15)

Females are larger than males throughout, although not statistically so ($p > 0,05$), and there is no clear change in velocity at the time of puberty.

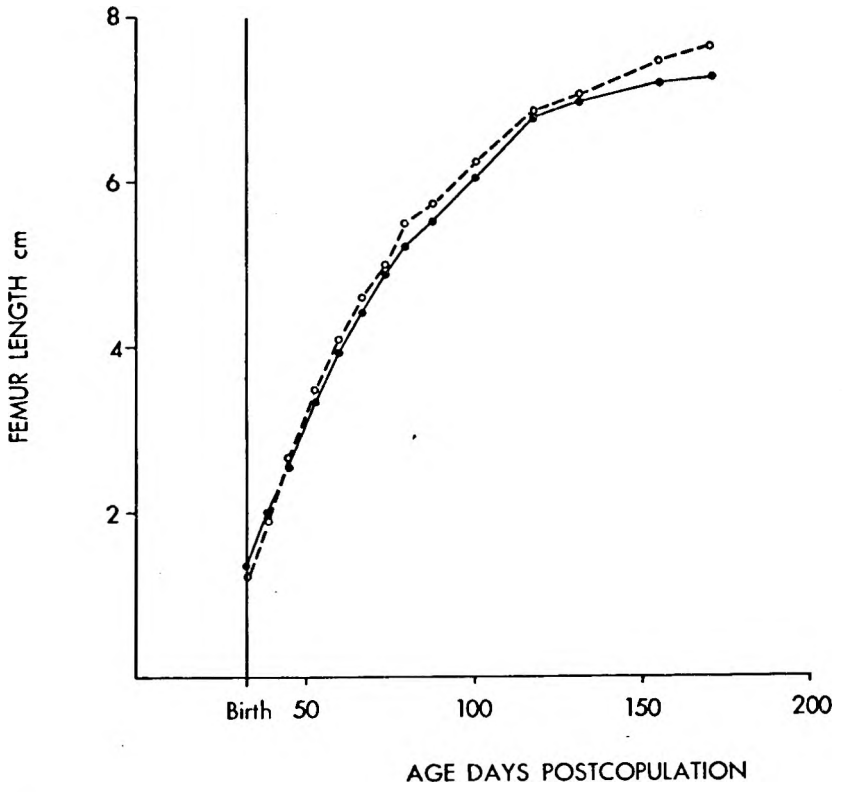


Figure 32. Femur length (right) of male (●—●) and female (o--o) rabbits.

Tibia length (Figures 34, 35; Table 16)

Females are larger than males, but not statistically so ($p > 0,05$), and between 80 and 100 days PC the fall in velocity declines in both sexes.

TABLE 15. Right femur length distance and velocity data (means with standard deviations below at each age).

AGE (days pc)	DISTANCE (cm)				VELOCITY (cm/day)	
	n	MALE	n	FEMALE	MALE	FEMALE
33	31	1,32 0,80	27	1,24 0,78	-	-
39	35	2,00 0,95	30	1,92 0,87	0,105 0,043	0,104 0,041
45	37	2,57 1,09	32	2,67 1,00	0,104 0,027	0,103 0,053
53	37	3,34 1,32	32	3,51 1,22	0,092 0,030	0,103 0,033
60	37	3,94 1,50	32	4,11 1,36	0,110 0,023	0,098 0,041
67	37	4,42 1,73	31	4,62 1,53	0,105 0,038	0,101 0,037
74	36	4,87 1,90	31	5,04 1,66	0,090 0,035	0,090 0,028
80	37	5,21 2,05	32	5,52 1,79	0,067 0,025	0,074 0,027
88	37	5,57 2,21	32	5,77 1,96	0,055 0,025	0,056 0,028
101	37	6,04 2,36	32	6,26 2,21	0,044 0,037	0,048 0,027
118	37	6,81 2,73	31	6,89 2,38	0,041 0,038	0,031 0,019
132	36	7,10 2,87	31	7,10 2,46	0,023 0,017	0,026 0,014
156	36	7,24 2,92	32	7,53 2,58	0,014 0,008	0,019 0,014
171	36	7,30 3,07	32	7,67 2,62	0,013 0,010	0,018 0,016

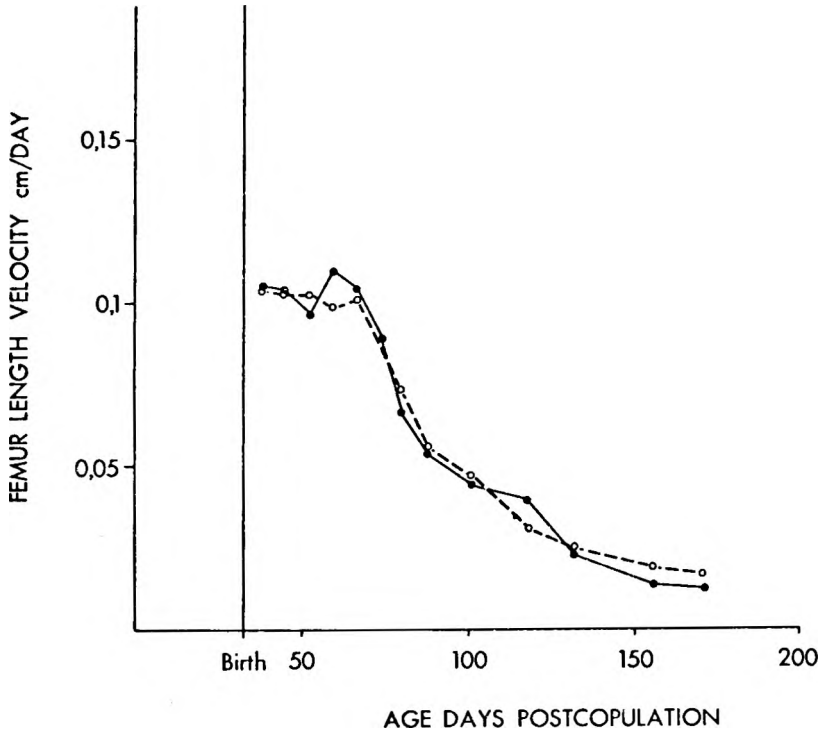


Figure 33. Femur length velocity of male (●—●) and female (o--o) rabbits.

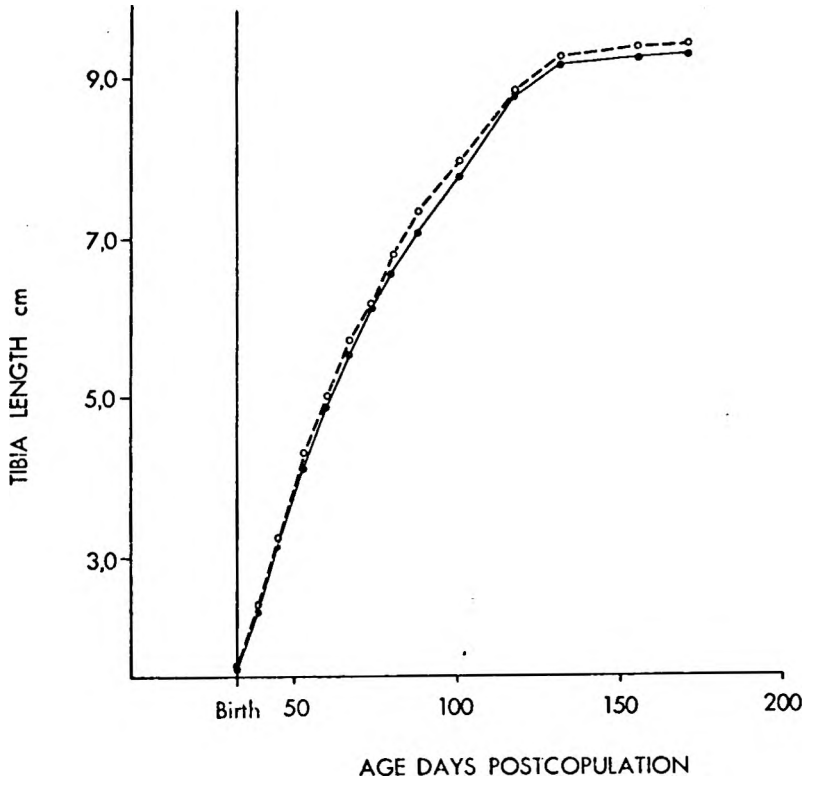


Figure 34. Tibia length (right) of male (●—●) and female (o--o) rabbits.

TABLE 16. Distance and velocity data for right tibia
(means with standard deviations below at each age)

AGE (days pc)	n	LENGTH (cm) MALE	n	FEMALE	LENGTH VELOCITY (cm/day) MALE	FEMALE
33	32	1,58 0,89	27	1,60 0,81	-	-
39	35	2,30 1,03	32	2,35 0,90	0,113 0,031	0,100 0,025
45	37	3,13 1,26	32	3,27 1,15	0,112 0,018	0,109 0,020
53	37	4,14 1,56	32	4,30 1,41	0,126 0,016	0,118 0,021
60	37	4,89 1,79	32	5,07 1,61	0,110 0,016	0,114 0,024
67	37	5,52 2,07	31	5,74 1,81	0,110 0,030	0,115 0,030
74	37	6,13 2,31	31	6,32 2,01	0,105 0,021	0,098 0,024
80	37	6,56 2,49	32	6,80 2,14	0,081 0,020	0,079 0,017
88	36	7,07 2,71	32	7,39 2,43	0,069 0,017	0,078* 0,029
101	36	7,79 2,97	32	8,04 2,73	0,066 0,062	0,055 0,020
118	37	8,81 3,56	31	8,87 3,00	0,044 0,057	0,038 0,013
132	36	9,19 3,72	31	9,29 3,12	0,032 0,011	0,030 0,010
156	37	9,28 3,64	31	9,76 3,26	0,014 0,010	0,016 0,008
171	36	9,34 3,83	32	9,88 3,31	0,011 0,014	0,011 0,009

* female mean significantly different from male mean at 5%

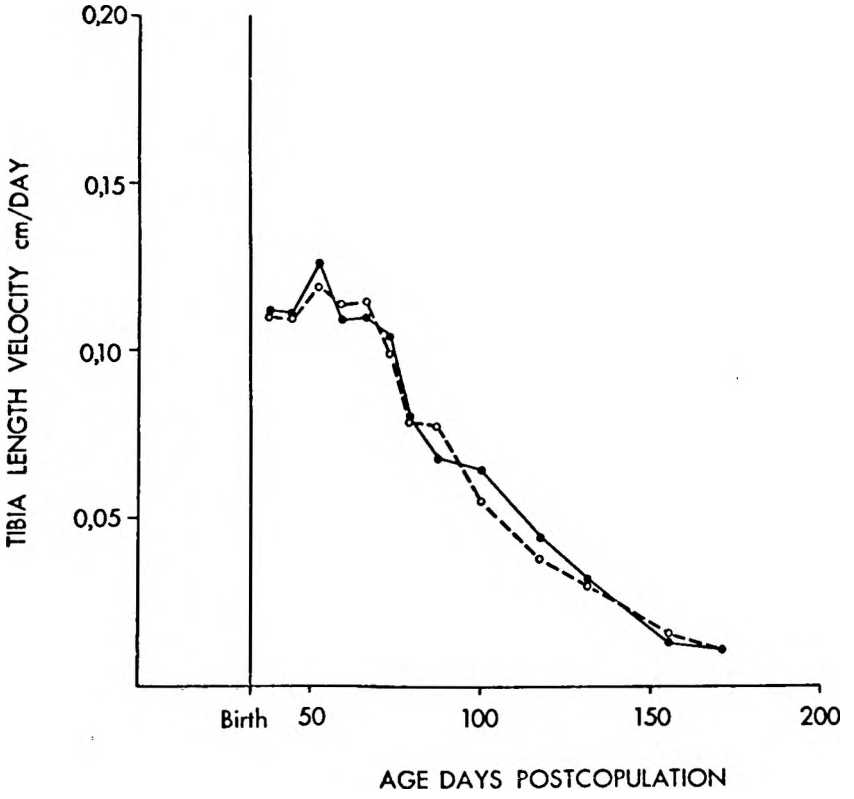


Figure 35. Tibia length velocity of male (●—●) and female (o--o) rabbits.

Calcaneus length (Figures 36, 37; Table 17)

The female is larger than the male from birth onwards, but there is no statistically significant difference ($p > 0,05$) and growth ceases after 120 days PC. Velocity declines rapidly soon after birth and no pubertal phenomenon is observed.

Shape changes

Shape changes developing during growth may be shown by plotting the mean distance values of pairs of measurements against each other at successive ages (Hiernaux 1968). Using this technique sex differences in shape in addition to those already shown for size have been sought (see Figure 38 for an example). For all of

the following plots (not shown) no changes in relationship have been demonstrated: skull length: body length, humerus length: radius length, radius length: 3rd metacarpal length, femur length: tibia length, femur length: humerus length, ilium length: ischium length, ilium length: body length, iliac length: bi-iliac width, bi-iliac width: bi-ischial width.

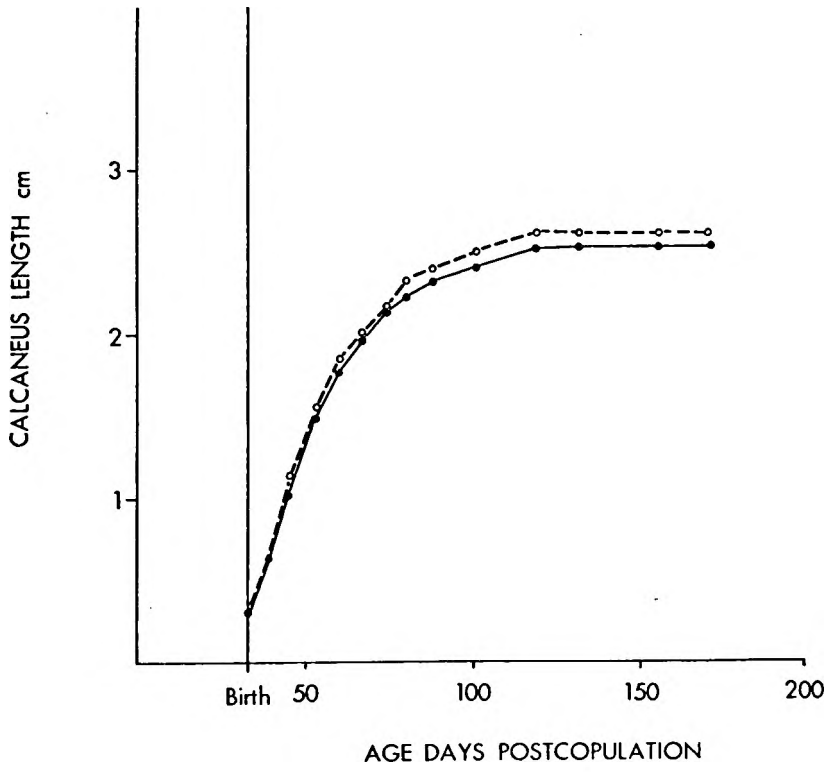


Figure 36. Calcaneus length (left) of male (●—●) and female (o--o) rabbits.

8.5. Discussion

Methods.

The techniques of anaesthesia, body length measurement and radiography developed by Hughes and Tanner for the rat (1970a) have been successfully modified for the rabbit in this study.

TABLE 17. Distance and velocity data for left calcaneus length (means with standard deviations below at each age)

AGE (days pc)	LENGTH (cm) n	MALE	n	FEMALE	LENGTH VELOCITY (cm/day) MALE	FEMALE
33	33	0,33 0,43	28	0,33 0,42	-	-
39	34	0,64 0,51	29	0,64 0,47	0,067 0,016	0,063 0,019
45	37	1,03 0,62	31	1,14 0,61	0,068 0,017	0,071 0,018
53	37	1,49 0,76	32	1,56 0,69	0,053 0,009	0,057 0,009
60	37	1,77 0,84	32	1,85 0,77	0,037 0,010	0,039 0,013
67	37	1,96 0,94	31	2,03 0,83	0,033 0,013	0,033 0,015
74	36	2,13 1,02	31	2,17 0,88	0,025 0,013	0,031 0,010
80	35	2,22 1,06	31	2,34 0,93	0,019 0,012	0,023 0,015
88	35	2,32 1,12	31	2,41 1,02	0,018 0,014	0,019 0,013
101	34	2,41 1,14	32	2,51 1,11	0,013 0,011	0,010 0,007
118	34	2,52 1,19	31	2,62 1,11	0,009 0,009	0,009 0,006
132	34	2,53 1,20	32	2,61 1,11	0,006 0,005	0,005 0,004
156	36	2,53 1,23	32	2,62 1,11	0,001 0,007	0,001 0,004
171	36	2,54 1,31	31	2,61 1,29	0,001 0,007	0,001 0,006

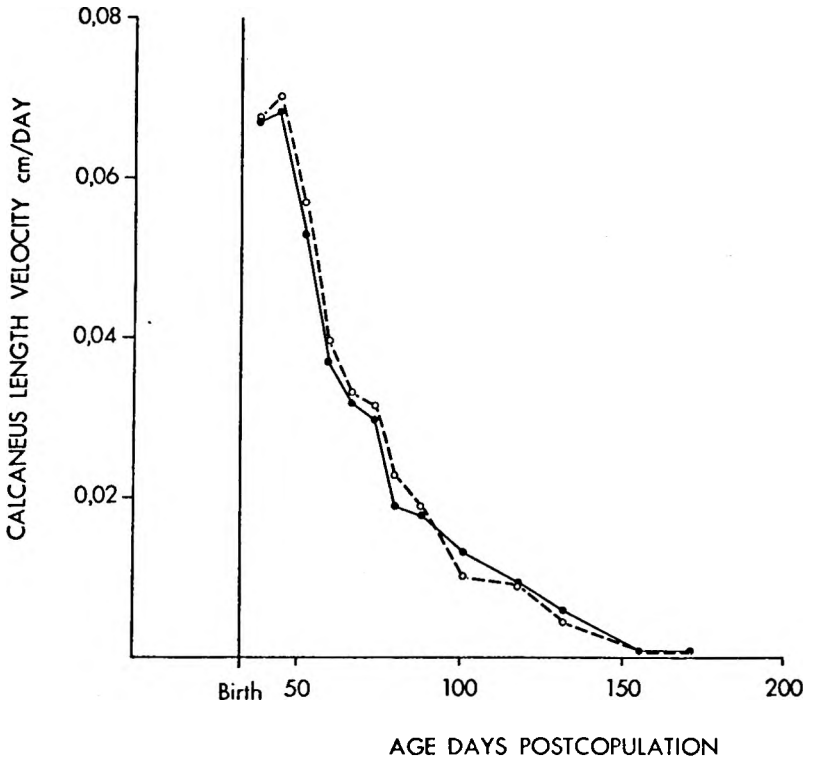


Figure 37. Calcaneus length velocity of male (●—●) and female (o--o) rabbits.

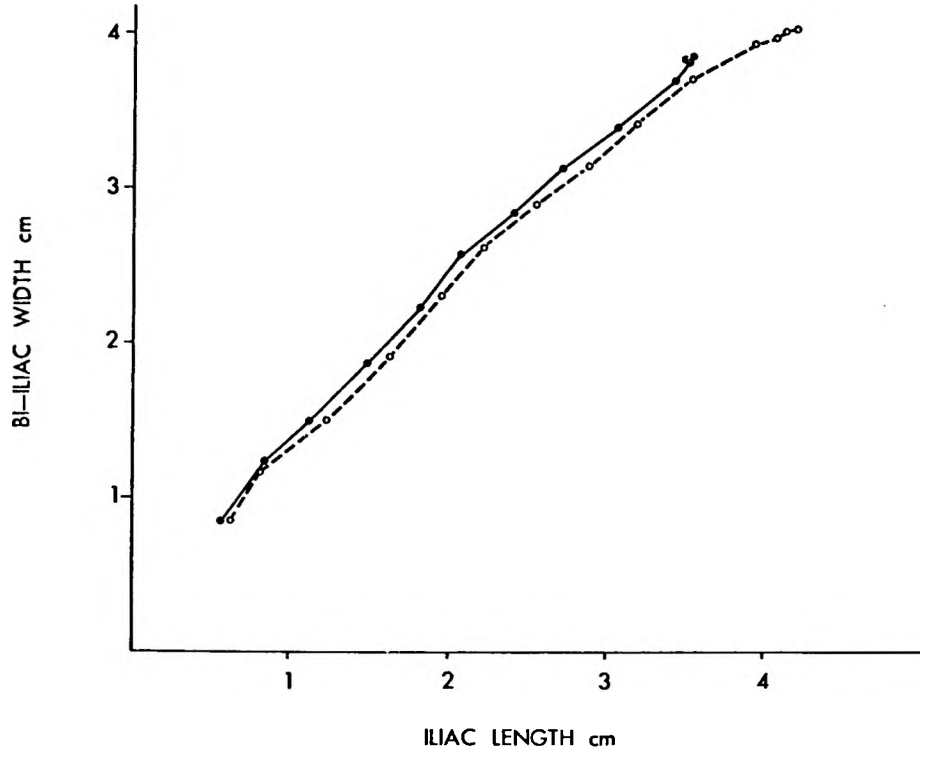


Figure 38. Bi-iliac width plotted against iliac length for male (●—●) and female (o--o) rabbits.

Ether anaesthesia is remarkably safe, except in neonatal rabbits, with a 6,4% mortality and 2,6% morbidity in the 78 rabbits that underwent a total of 1064 anaesthetics. Comparison of the body weight velocities of the pure and mixed longitudinal groups reveals no differences between them. This is taken as evidence that anaesthesia, to which only the mixed longitudinal group was exposed, has no effect on growth. A similar observation has been made by Hughes in rats (1970, personal communication). Anaesthesia permits precise body measurement and simplifies radiography as the restraining methods used by other authors are unnecessary (Crary & Sawin 1949; Selman & Sarnat 1953). The within- and between observer errors in body length measurement are low and compare favourably with those reported for the rat (Hughes & Tanner 1970a).

Body weight at birth varies considerably with length of gestation and litter number in the rabbit. This variability is greatly reduced by using only litters born on day 33 of gestation and numbering four to seven animals. These two manoeuvres reduce variability more than expressing ages in days to permit the use of litters irrespective of gestation length and artificially reducing litter size as has been done by others (Crary & Sawin 1949; Hughes & Tanner 1970a). The procedures used in the animals in this study gives a coefficient of variation of 11% for birth weight of both sexes combined, compared to that of 18% for all other litters born in the colony.

Radiographic definition was adequate to allow measurement of all the skeletal dimensions described except in a few neonatal x-rays. However, definition was not adequate to accurately describe shape changes in epiphyseal centres and the construction of a bone age scale was not possible.

Data analysis had to overcome the problem of unequally spaced observations at varying ages. Most classical interpolation methods require equally spaced data observations and therefore could not

be used. The other classical formulae which admit unequally spaced data are cumbersome from a computational point of view; therefore, a spline fit interpolation formula was used.

By inspection the velocity curves of body weight and body length obtained from the first derivative of the interpolation equation appear to be identical with those obtained by the more conventional method of increment analysis (Figures 3 & 4); strongly suggesting that the first derivative method offers a reliable description of the growth velocity curve. This is further supported by the comparison of the body weight velocities of this study with those in the literature (Figures 39 & 40).

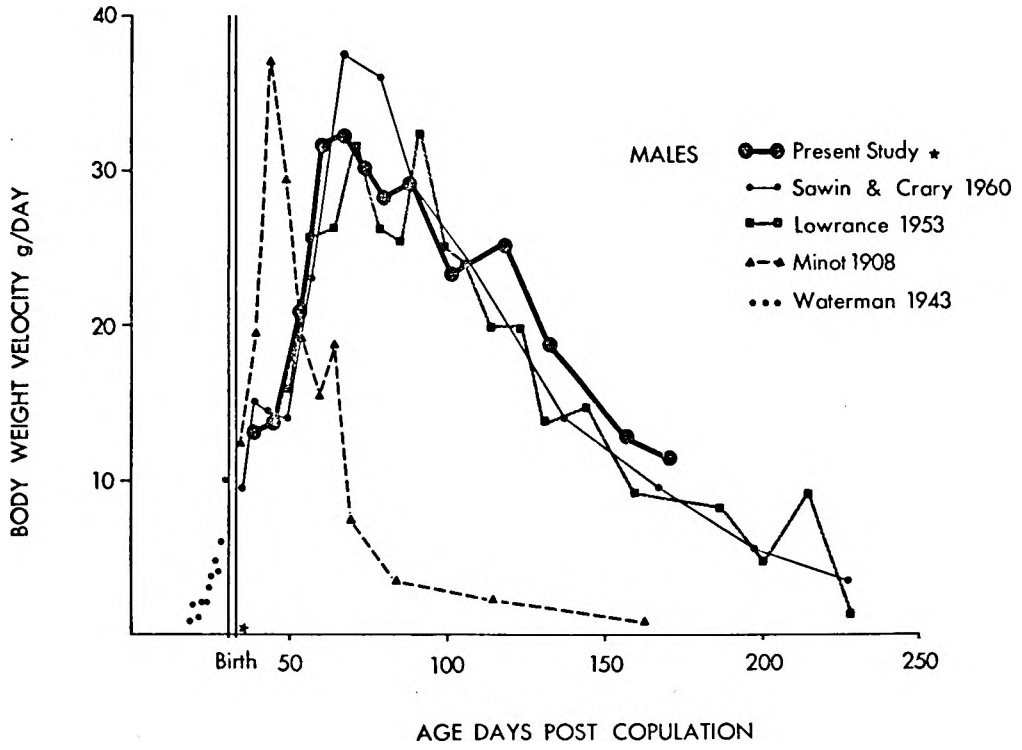


Figure 39. Body weight velocity of male rabbits from the present study compared with 3 other studies.

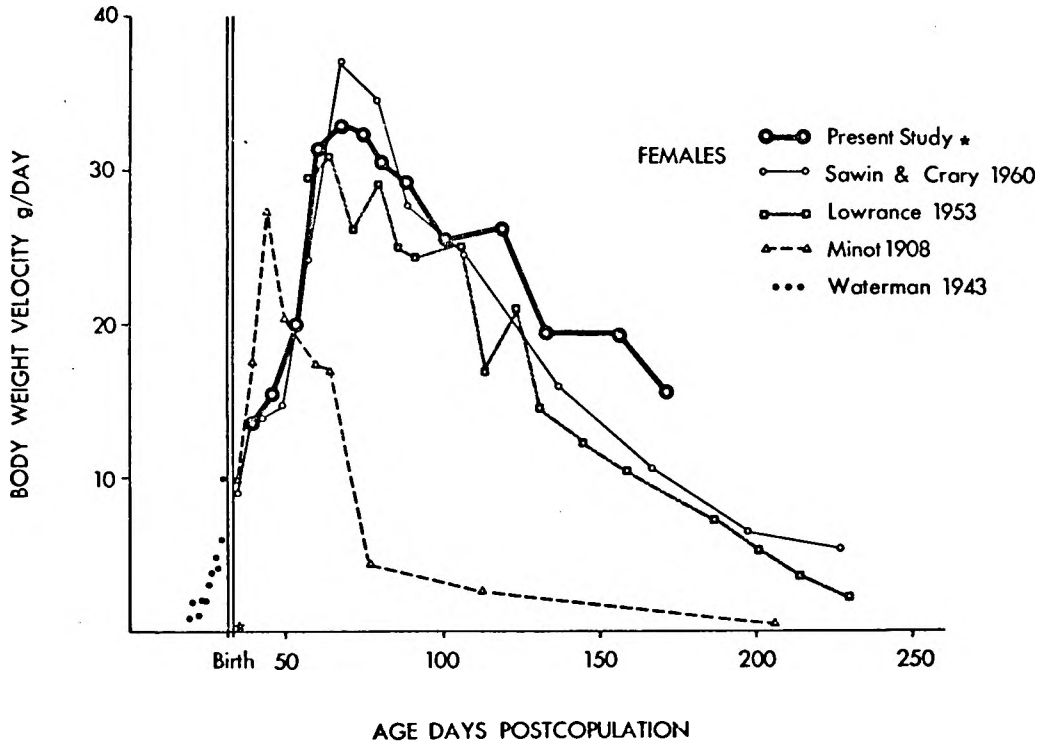


Figure 40. Body weight velocity of female rabbits from the present study compared with 3 other studies.

The curves for Minot's unspecified strain and Lowrance's mixed Himalayan strain were derived from the published distance means and therefore are not mean constant curves (Merrell 1931, discussed in Chapter 3). The curves of Sawin and Crary are true mean-constant curves from a pure longitudinal series of New Zealand White rabbits and therefore are strictly comparable with those of the present study. Waterman's foetal weight velocities have been calculated from his cross-sectional data for "average"-sized foetuses. The male curves and the female curves of the present study are identical in shape and duration to those of Sawin and Crary, but of somewhat lower peak intensity.

Results

The important features of the rabbit growth curve may be summarized by smoothing a selection of curves of various linear dimensions. Figure 41 shows the smoothed velocity curves in males of body length, skull length, pelvis (i.e. ilium plus ischium) length, 11th rib length and tibia length.

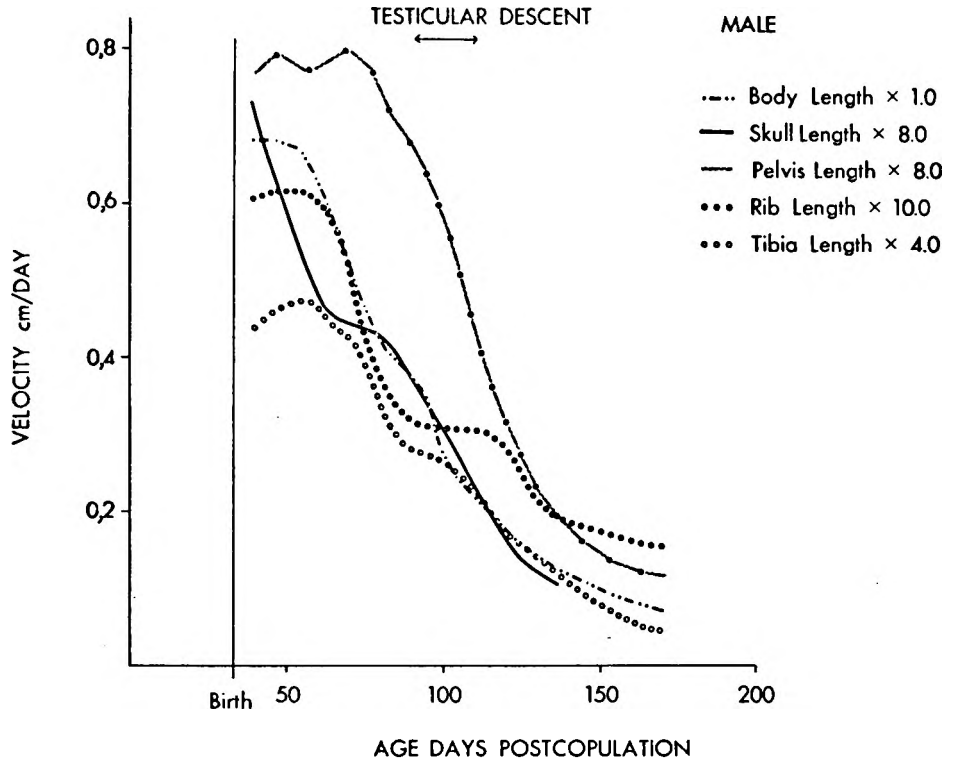


Figure 41. The smoothed velocity curves of male body length, skull length, pelvis length, 11th rib length and tibia length.

The basic pattern is a rise in velocity from some time before birth to a peak in the weeks following birth, and then a gradual decline. The peak velocity is reached at different ages by different parts of the body. As in other animals the skull peak appears to occur at or shortly after birth. There is no clear cephalocaudal gradient for the other skeletal dimensions, either

within the limbs, between the limbs or between the axial and appendicular skeletons. A marked difference does exist, however, between body length with a well-defined peak between 40 and 50 days PC and body weight between 60 and 70 days PC. The female peak intensity is greater than the male in body weight, skull length, lumbar vertebra length, rib length, ilium length and humerus length. This greater peak plus generally greater velocities in the females contributes to the overall larger size in the female.

The declining slope of the growth curve is halted temporarily at a time related to testicular descent, which is the only external indicator of puberty in the rabbit. The dangers of drawing conclusions about the host of changes associated with puberty from only one event are well recognized (Marshall 1970), but there can be little doubt that the relative growth spurts observed in many of the bodily dimensions of the rabbit are a pubertal phenomenon. In man and other primates this growth spurt occurs at a time when the preceding velocity is relatively lower, and the spurt appears as a marked rise in velocity (Tanner 1962). In the rabbit, as in the rat, (Hughes & Tanner 1970a; Swanson & van den Werff ten Bosch 1963), the spurt is usually relative, being a slowing in the decline of velocity. In some dimensions in the rabbit a true spurt is seen, for example, skull length in both sexes (Figure 11) and 11th rib length in females (Figure 15). Where this spurt can be unmistakably discerned there is little difference in intensity between males and females, except in the 11th rib where that of the female is significantly greater. In respect of the timing of the spurt there also appears to be no difference between males and females.

Sex differences, including those in the pelvis, are purely in size and not in shape. This contrasts sharply with the situation in man (Tanner 1962), but is similar to that in the rat (Hughes & Tanner 1973).

It is noteworthy that at no age do differences in size achieve statistical significance and on very few occasions do differences in velocity achieve statistical significance. This contrasts with the observations in the rat where the sex difference (male larger in this case) becomes significant in all dimensions after puberty (Hughes & Tanner 1970a). This difference between the two studies is probably caused by the larger number of rats studied and the fact that the growth statistics were calculated directly from the data without need to resort to interpolation. Lack of statistical significance of differences in this situation does not preclude biological significance being attributed to the differences. For a given dimension of the rabbit the pattern of sex difference is highly consistent, females always being larger than males, and where changes in difference occur these are smooth and progressive. Both of these facts strongly suggest biological significance must be attributed to the observed sex differences, even in the face of lack of statistical significance.

CHAPTER 9

RABBIT SERUM SOMATOMEDIN

Somatomedin activity in human serum has been widely investigated and there have been several reports on rat serum SM (reviewed in Chapter 5). Van den Brande and co-workers (1974b) have investigated SM in other species including monkey, pig, horse, cow, dog, turtle, pigeon and fish. Only in man has a systematic study been made of age-related changes in serum SM (Van den Brande & Du Caju 1973).

This chapter reports the results of investigation of serum SM in the rabbit at all ages from foetal to adult life. The serum activity bioassayed is shown to be growth hormone-dependent by both porcine and chick cartilage assay systems. The effects of fasting on serum SM are also described.

9.1. Assay techniques

Porcine cartilage assay. The method described by Van den Brande and Du Caju (1974) was used. A brief outline of this method follows:

The costal cartilages and adjacent sternum of a freshly slaughtered pig were obtained from the Johannesburg Abbatoir. Cartilage was selected personally from a pig weighing 30 to 45kg, after slaughter and removal of the viscera. The criteria of suitability were a smooth, shiny cut surface and marked resilience on bending. Like Phillips et al. (1974) I have observed that cartilage that is macroscopically calcified and has a roughened cut surface, has an irregular and shallow dose-response curve.

The excised tissue was placed in saline at ambient temperature and transported to the laboratory. Muscle was cleaned off the 4th to 8th cartilages and each cartilage dissected free. The 2cm zone adjoining the rib of each cartilage was discarded. Cartilages were then sliced into 2mm transverse sections on a commercial meat slicer. From each section between one and five cylinders 2mm in diameter were punched by means of the stainless steel punch illustrated in Figure 42.

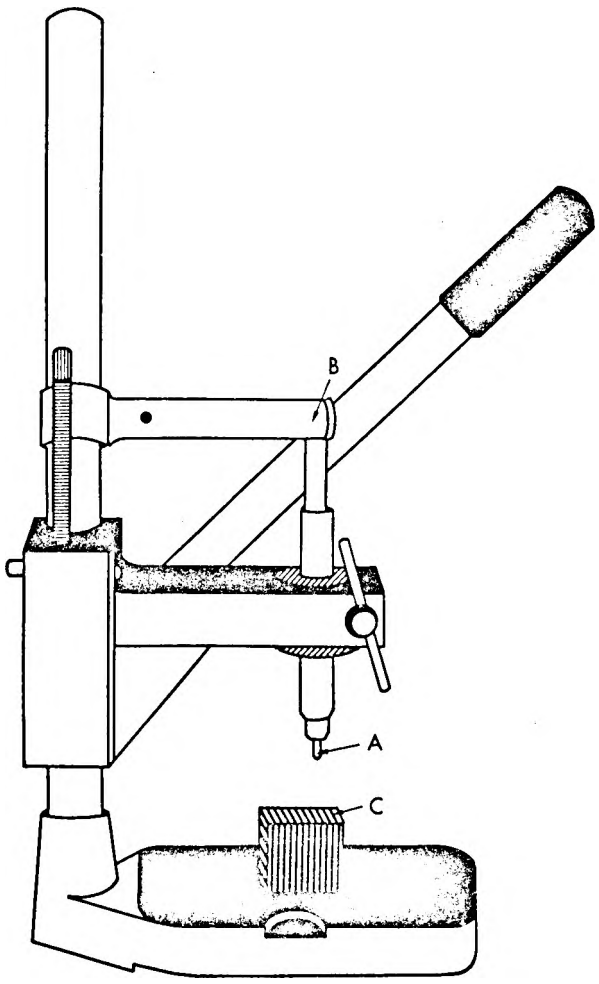


Figure 42: Line drawing of the cartilage punch. The mounting and lever portions are a Bosch electric drill holder. The punch (A) is machined from hardened stainless steel and has an externally tapered end of 2mm internal diameter. A stainless steel rod (not shown) lies inside the punch to expel the cartilage cylinder as the handle is raised against the stop (B). The hatched cube (C) is of Teflon.

Cylinders were taken from the central and peripheral portions of the section, but not closer to the margin than 2mm.

The cartilage cylinders, about 230 in number, were placed in a beaker containing 50ml of incubation medium in a shaking water-bath at 37°C for 20 hours (overnight). The incubation medium was that described by Daughaday and Reeder (1966) and contained:

1. amino acids: cystine 0,05mM, methionine 0,03mM, phenylalanine 0,05mM, tyrosine 0,06mM, arginine 0,1mM, lysine 0,2mM, histidine 0,08mM, tryptophan 0,05mM, isoleucine 0,07mM, leucine 0,13mM, threonine 0,12mM, valine 0,25mM, glutamine 0,57mM, serine 0,11mM and glycine 0,21mM. (All amino acids L-isomers from BDH, Johannesburg and Merck, Darmstadt).

2. Krebs phospho-saline buffer (Krebs & Eggleston 1940) adjusted to give Na 158,6mM, K 4,56mM, Mg 0,73mM, Cl 118,7mM, HPO_4 22,2mM and SO_4 0,73mM. (All salts AR, BDH, Johannesburg).

3. Dextrose (BDH, Johannesburg) 10mM.

4. Antibiotics: streptomycin sulphate (Novo, Johannesburg) 100µg/ml, Na benzylpenicillin (Novo, Johannesburg) 100 units/ml and kanamycin (Bristol, Johannesburg) 100µg/ml.

Incubation of the cartilage segments was performed in a specially designed rack, shown in Figure 43. The standard and unknown sera were diluted with the incubation medium to give 40, 20, 10, 5 and 2,5% dilutions and 1ml of each dilution was added in triplicate to the pots of the rack. Two cylinders of cartilage were placed in the bottom of each tube and the supporting frame positioned. The covered rack was placed in an incubator (Gallenkamp) for 46 hours without shaking.

In these 10-point assays six unknowns and one reference standard filled 105 places in the rack. The remaining three places were filled with incubation medium only.

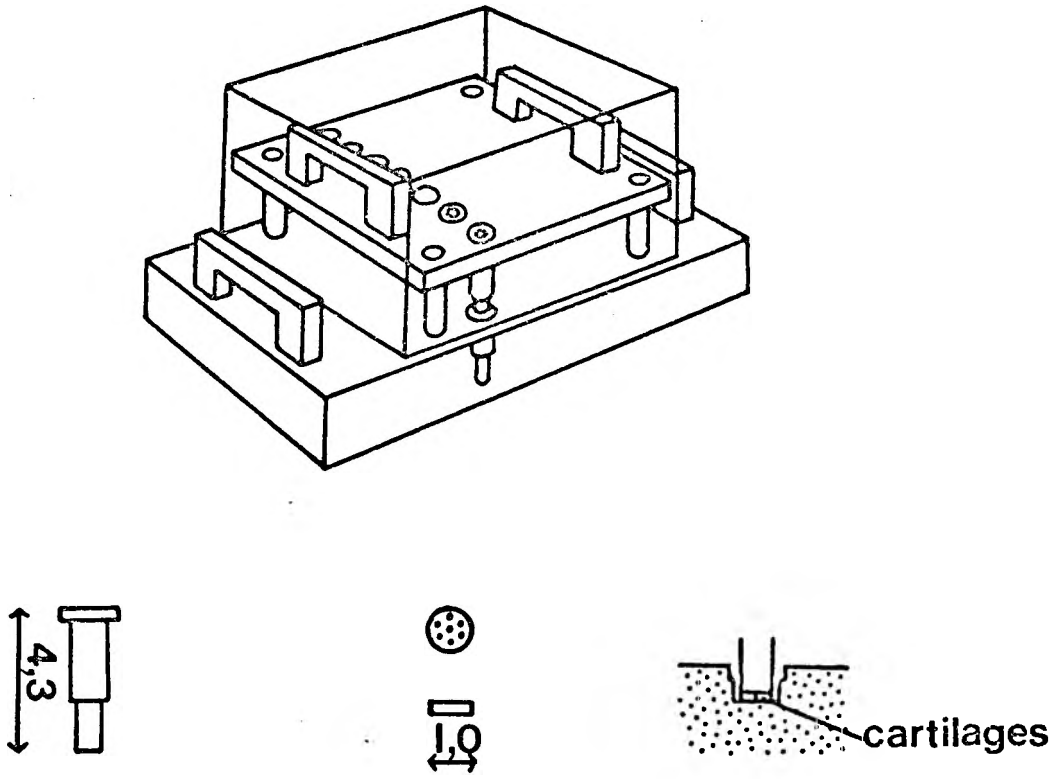


Figure 43: Line drawing of the incubation rack. The base consists of a perspex block into which 108 holes were machined. Teflon pots, 1,2cm internal diameter x 1,3cm depth were pressed into these holes. The supporting perspex frame (A) holes 108 Teflon tubes, 1,0cm internal diameter x 4,3cm depth which have 9 1mm perforations in the base. In position the base of each tube lies 1mm above the base of the corresponding pot. Individual tubes can be removed from the frame for cleaning. The whole rack is covered with a 27cm x 23cm x 15cm lid.

After 46 hours the frame was removed, lowered into a bath of normal saline so that the bottom third of the tubes was immersed, agitated briefly, removed and shaken. Next it was placed in a perspex bath, 22cm x 17cm x 2,5cm, containing 2 μCi/ml of $Na_2{}^{35}SO_4$ (carrier free, Amersham) and 2 μCi/ml ^{3}H-methyl-thymidine (17 Ci/mmole, Amersham (^{3}HTdr) in incubation medium. Again the depth was such that the base of each tube was 1mm from the bottom of the bath and the cartilages completely immersed. Incubation was continued for 4 hours at 37°C.

Next the frame was transferred to a siphon bath. The tube bases were washed once with water at ambient temperature and then heated in water at 80°C for 10 minutes. Thereafter the water level was raised and lowered in the bath, with complete emptying of the bath after each cycle, for 20 minutes to wash off all unincorporated isotope. Measurement of the activity in the effluent was checked in a series of assays and found to be at background levels after 6 to 7 washings. In the 20 minute washing period at least 15 washings occurred.

The cartilage pairs were then added to labelled glass isotope-counting vials containing 0,5ml 23N formic acid (Protea, Johannesburg). Polyethylene caps were lightly screwed onto the vials and the vials heated at 80°C in a water bath for 30 to 60 minutes until complete solution had occurred. After cooling 10ml Instagel (Packard) was added to each vial.

Activity was determined in a Packard 3390 liquid scintillation counter with automatic activity analyser (AAA). The AAA setting was adjusted weekly using ^{3}H and ^{14}C standards.

Somatomedin potency ratios were calculated on a Hewlett - Packard 9100 B calculator with plotter and extended memory. Semi-log transformation of ^{3}HTdr data and log-log transformation of $^{35}SO_4$ data yielded linear dose-response curves. The computation programme, generously provided by Dr. J.L. Van den Brande, permits visual selection of the linear portion of the curve. Linearity is then statistically checked at the 95% level, regression analysis performed and the index of precision calculated. The standard and unknown responses are analysed for linearity, preparation differences, regression, parallelism and heteroscedasticity. Finney's g is calculated and finally the potency ratio with 95% confidence limits is calculated. The statistics are those developed for classical bioassays (Finney 1964).

Chick cartilage assay. The method described by Hall (1970) was used to check the validity of the porcine cartilage assay for rabbit serum (see 9.2. below). The method used was slightly modified from Hall's technique as follows.

Pelvic leaflets of 11-day old White leghorn chick embryos were dissected and cleaned by gentle rubbing with gauze. The incubation medium was the same as that used for the porcine assay.

The leaflets were preincubated for 2 hours in medium at 37°C. Three pairs of leaflets were then added separately to 4ml of each of the 4 dilutions of the standard and unknown sera (40, 20, 10 and 5%) in 10ml Erlenmeyer flasks. Each dilution contained 2 μCi/ml $Na_2{}^{35}SO_4$. Incubation was continued in a shaking water-bath (Gallenkamp) at 50 cycles/minute for 6 hours at 37°C.

Incubation was stopped by rinsing out the flasks with a saturated Na_2SO_4 solution and then heating them at 80°C for 10 minutes. After standing overnight in a saturated Na_2SO_4 solution cartilage pairs were dessicated over P_2O_5, weighed to 0,00001g, digested in 23N formic acid and counted in the same way as the porcine cartilage.

Reference serum. Three fasting healthy adult males each donated 500ml of serum. The pool of sera was aliquotted into 4ml lots and frozen at -30°C. Two pools were used in the course of the work. The potency ratio of pool 2 compared to pool 1 was 0,98, with 95% confidence limits of 0,89 to 1,07. Correction for this difference was made.

Serum SO_4 concentrations. Although a post-incubation schedule was used for the porcine assay, the serum SO_4 concentration of the rabbits was investigated.

Serum inorganic SO_4 was measured by the benzidine colorimetric method of Kleeman et al. (1956). The method was shown to have a

linear response between 10 and 40 µg/ml. Rabbit sera were diluted 2- or 3-fold with DD H_2O before assay. Table 18 shows the results of these assays. No sex difference was demonstrated. The overall mean concentration was 70,4 µg/ml, and incubation medium concentration was therefore adjusted to this concentration, i.e. 0,73mM.

TABLE 18. Serum inorganic SO_4 concentrations in the rabbit

Age (days PC)	Number of sera assayed	Inorganic SO_4 (µg/ml) mean	(& range)
30	1 (pooled)	74,8	-
37	2	68,4	(61,8 - 75,0)
48	4	73,0	(64,7 - 81,3)
53	3	64,4	(61,7 - 70,2)
88	4	69,8	(65,0 - 73,4)
114	4	77,2	(64,3 - 91,4)
400	5	68,4	(59,3 - 86,9)

Serum amino acid concentrations. Because amino acid concentrations are critically important in the SM bioassay, serum amino acids in the rabbit were measured and compared to those in the human reference pool and published values in the literature. Amino acid concentrations were determined, after 5% TCA precipitation of proteins, in a Beckman auto-analyser. Determinations were made on fasting serum from three adult rabbits (two female, one male) and three young rabbits aged 43 days PC (two males, one female).

Table 19 shows the results of these determinations and compares them with published concentrations for human serum (Albritton 1952), fasting rabbit serum (Bromer & Chance 1969) and the incubation medium (Daughaday & Reeder 1966).

TABLE 19. Amino acid concentrations (mM) in human and rabbit serum

	Human Serum		Incubation	Rabbit Serum		
	Reference Pool- Present study	Normal Range (Albritton 1952)	Medium (Daughaday & Reeder 1966)	Adult n = 3	43 days PC n = 3	Adult (Bromer & Chance 1969) n = 8
Arginine	0,07	0,06 - 0,2	0,10	0,20	0,19	0,26
Cystine	0,06	0,03 - 0,08	0,05	0,11	0,14	-
Glutamine	0,60	0,41 - 1,16	0,57	0,48	0,63	-
Histidine	0,06	0,07 - 0,12	0,08	0,09	0,12	0,14
Isoleucine	0,04	0,07 - 0,17	0,07	0,09	0,08	0,11
Glycine	0,19	0,17 - 0,31	0,21	1,14	1,19	1,27
Leucine	0,15	0,10 - 0,19	0,13	0,10	0,13	0,17
Lysine	0,19	0,14 - 0,26	0,20	0,24	0,30	0,26
Methionine	0,03	0,02 - 0,04	0,03	0,05	0,05	0,07
Phenylalanine	0,04	0,03 - 0,13	0,05	0,10	0,08	0,09
Serine	0,09	0,11	0,11	0,36	0,29	0,32
Tyrosine	0,07	0,04 - 0,12	0,06	0,14	0,20	0,15
Valine	0,27	0,19 - 0,30	0,25	0,26	0,20	0,29
Tryptophan	0,04	0,03 - 0,07	0,05	0,04	0,03	0,05

To determine whether altering the normal (i.e. those described by Daughaday and Reeder) amino acid concentrations in the incubation medium to match those of the mean concentrations of all six rabbit sera measured affected SM activity in the bioassay, a porcine cartilage assay was conducted with the results shown in Table 20.

TABLE 20. Effect on the bioassay of altering amino acid concentrations in the medium

Specimen	Incubation Medium amino acid composition	Potency Ratio (± 95% limits)	Index of precision
Reference Pool	normal*	standard = 1,00	0,16
Reference Pool	rabbit means	1,06 (0,88 - 1,19)	0,18
Adult rabbit A	normal	0,67 (0,53 - 0,82)	0,22
Adult rabbit A	rabbit means	0,73 (0,59 - 0,86)	0,11
Adult rabbit B	normal	0,54 (0,47 - 0,60)	0,14
Adult rabbit B	rabbit means	0,55 (0,43 - 0,65)	0,21
40 day PC rabbit pool	normal	0,34 (0,27 - 0,45)	0,23
40 day PC rabbit pool	rabbit means	0,31 (0,26 - 0,39)	0,17

* i.e. those described by Daughaday and Reeder (1966).

Thus alteration in amino acid concentrations, chiefly in glycine and serine, had no measurable effect on SM activity in the bioassay. Therefore, in the present study the amino acid concentrations used in the incubation medium were those described by Daughaday and Reeder (1966).

Collection of serum. In post-natal rabbits blood was collected

by cardiac puncture during light ether anaesthesia between 06h30 and 10h00 after a 4 - 6 hour fast. In foetal rabbits blood was collected from the neck after decapitation. Foetuses were delivered by hysterotomy under ether anaesthesia after a 6 hour fast.

Blood taken by cardiac puncture was usually venous in colour, but occasionally arterial or mixed samples were obtained.

Possible effects of ether anaesthesia and possible differences between venous and arterial serum SM were investigated. Three adult female rabbits were restrained and venous blood taken from the ear. Ether anaesthesia was induced and after 10 minutes blood was taken from the ear vein and femoral artery and by cardiac puncture, simultaneously. All three cardiac punctures yielded venous colour blood. The results of SM assays on these sera are shown in Table 21.

TABLE 21. Somatomedin assay of sera before and during ether anaesthesia and of venous and arterial sera

Specimen n = 3	Potency ratio mean	(& range)	Index of precision
Ear vein-unanaesthetized	0,59	(0,51 - 0,72)	0,19
Ear vein-anaesthetized	0,69	(0,60 - 0,77)	0,17
Femoral artery-anaesthetized	0,65	(0,60 - 0,69)	0,23
Cardiac puncture-anaesthetized	0,70	(0,58 - 0,79)	0,20

Removal of serum somatomedin inhibitors. Putative SM inhibitors were removed from sera for some experiments (Chapters 9, 12 and 13). The pH was adjusted to 5,5 with 1N H Cl and the solution heated at 80°C for 15 minutes. The precipitate was removed by centrifugation, the supernatant lyophilized and reconstituted to the original volume (Salmon 1972; Cohen et al. 1975).

9.2. Specificity of the porcine assay.

Hypophysectomy and GH treatment. By definition serum SM activity is at least partially GH-dependent (Van Wyk et al. 1974; Hall & Van Wyk 1974). The GH-dependence of the activity assayed in rabbit serum by the porcine cartilage system was shown by treating hypophysectomised rabbits with human GH as follows.

Eleven 80 to 90 days PC rabbits from three litters which had been gaining body weight normally from birth were subjected to hypophysectomy by the transbuccal approach (White 1933). Under ether anaesthesia the soft palate was incised and separated with a small intra-oral spring retractor. The now visible nasal mucosa was infiltrated with a 2% adrenalin solution and compressed until completely blanched, at which time the cavernous foramen in the basi-sphenoid became visible. The mucosa was incised in the midline from the foramen for a distance of 3 or 4mm rostrally. Bone wax was pushed into the foramen. Using a 2mm dental burr the bone from the rostral edge of the foramen was carefully removed for a distance of 3mm. The underlying fibrous sheet was slit open precisely in the midline with an iridectomy knife and the pituitary gland bulged into sight. The gland was removed by negative pressure through a fine glass pipette. The cavity and burr-hole were plugged with bone wax and the mucosal incision pulled together but not sutured. The soft palate incision was closed with interrupted sutures. Post-operatively animals were given 25mg of hydrocortisone intramuscularly and supplied with 10% dextrose drinking water.

The peri-operative mortality was high with six animals dying within 6 hours of operation. Three further animals died 3 to 5 days after operation. The remaining two rabbits made excellent recoveries and their body weight velocity declined compared with their sham-operated litter mates (Table 22).

Ten days post-operatively each rabbit was given 1mg of human GH

(van Rooyen et al. 1974) intramuscularly on two successive days and sacrificed 12 hours after the second injection. Scrapings of the sella turcica were examined histologically (haematoxylin and eosin) and no pituitary tissue was observed. Three littermates were given control saline injections and also sacrificed by cardiac exsanguination.

Table 22 shows the body weights and serum SM assays (sulphation) obtained during this specificity experiment.

TABLE 22. Effects of hypophysectomy and GH-treatment on rabbit serum somatomedin activity by porcine assay (λ is the index of precision)

	BODY WEIGHT			
	10 days postop. % of controls	Pre-op. λ	10 days postop. λ	After GH-treatment λ
Littermate control mean	100	0,46 (0,16)	0,40 (0,19)	0,49 (0,11)
Hypox rabbit A	79	0,36 (0,24-0,44) (0,14)	0,21 (0,16-0,28) (0,14	0,34 (0,26-0,43) (0,21)
Hypox rabbit B	84	0,48 (0,35-0,61) (0,13)	0,14 (0,08-0,23) (0,22)	0,39 (0,30-0,46) (0,13)

Rabbit serum SM, as determined by the porcine cartilage assay, is clearly GH-dependent.

Comparison of porcine and chick assay results. These results were confirmed by assaying in the chick cartilage system. There was insufficient serum available for a full comparison and so pooled sera were used. In addition sera from normal rabbits of different ages were also assayed. The excellent correlation between the porcine and chick cartilage results is shown in Figure 44.

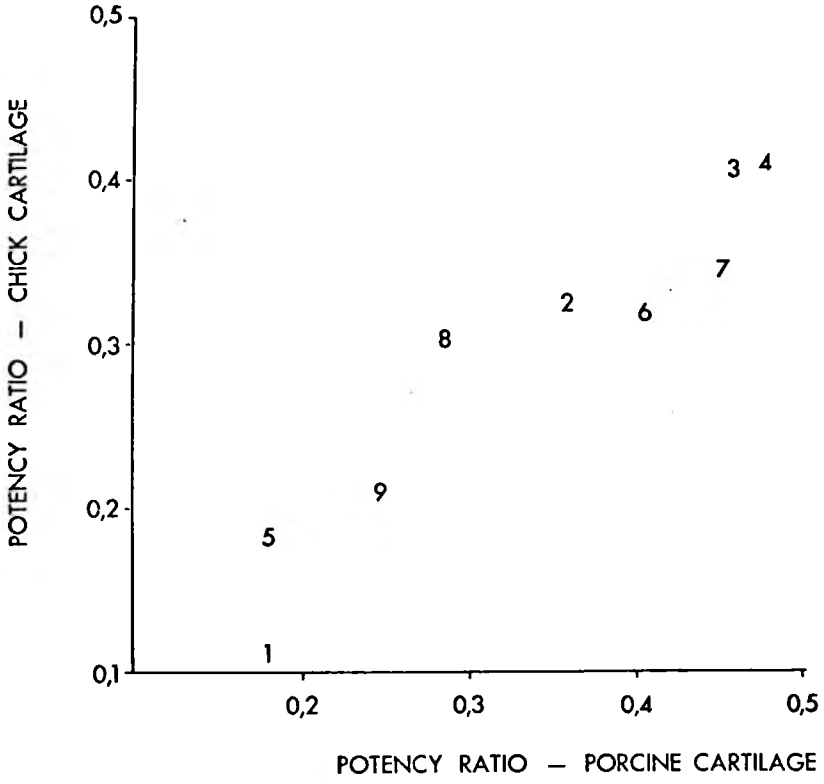


Figure 44: Comparison of somatomedin activity in the same serum determined by both the porcine cartilage and chick cartilage methods. Numerals refer to the following sera: 1: - hypox pool 10 days postoperative; 2: - GH-treated pool; 3: - littermate control pre-operative pool; 4: - littermate control GH-treated pool; 5: - 36 days PC normal pool; 6 and 7 adult normal pools, male and female; 8: - 84 days PC normal pool; 9: - 63 days PC normal pool.

Reproducibility of the porcine assay results. The reproducibility of the porcine assay results and the stability of rabbit somatomedin at -30°C (relative to the human reference standard) was investigated by pooling 5 adult male rabbit sera, aliquotting the pool and assaying an aliquot in each of six assays at approximately monthly intervals (Table 23).

The method is thus highly reproducible with the variation in the six month period falling well within the confidence limits of the assay.

TABLE 23. Reproducibility of rabbit serum somatomedin assay (sulphation)

	Assay date		
Date of collection	2.6.74	3.8.74	19.9.74
Potency Ratio (± 95% limits)	0,63 (0,54 - 0,74)	0,50 (0,46 - 0,55)	0,59 (0,51 - 0,66)
	28.9.74	15.10.74	14.11.74
	0,55 (0,49 - 0,60)	0,61 (0,53 - 0,70)	0,60 (0,54 - 0,67)

9.3. Results

Effects of fasting on somatomedin. Twelve adult male rabbits were fasted, six for 12 hours and six for 48 hours. Access to water was allowed. At the end of the fasting period and again after 24 hours free access to food blood was obtained by cardiac puncture under ether anaesthesia. Half of each serum specimen was heated and acidified (see above) and the resulting four specimens from each rabbit were assayed in the same porcine assay (sulphation).

Prolonged (48 hour) fasting consistently depresses serum SM but overnight (12 hour) fasting has no effect (Table 24).

Heating and acidification consistently depresses the 12 hour fasting and all fed levels. The effect on the 48 hour fasting samples is variable; three remaining unchanged, two being depressed and one being elevated (Table 24). Similar heating and acidification treatment of the reference pool serum depresses its activity to 76% of the untreated level.

Variation of somatomedin with age. Forty eight sera or serum pools were assayed for SM activity. In the younger age groups the volume was not sufficient for assay of individual sera and pooled sera from the same litter were used. Serum

TABLE 24. Effects of fasting on somatomedin

Rabbit	12 hour fast			48 hour fast			Fed state		
	normal	heated & acidified	(% change)	normal	heated & acidified	(% change)	normal	heated & acidified	(% change)
A				0,26	0,27	(+ 4)	0,43	0,39	(- 9)
B				0,74	0,63	(- 15)	1,13	0,87	(-23)
C				0,62	0,50	(- 19)	0,95	0,81	(-15)
D				0,30	0,32	(+ 7)	0,57	0,43	(-25)
E				0,42	0,44	(- 5)	0,49	0,39	(-20)
F				0,36	0,96	(+167)	0,54	0,48	(-11)
							0,69	0,56	(-19)
G	0,63	0,47	(-25)				0,71	0,67	(- 6)
H	0,52	0,44	(-19)				0,59	0,48	(-19)
I	0,78	0,61	(-22)				0,61	0,57	(- 7)
J	0,88	0,70	(-20)				0,73	0,58	(-21)
K	0,64	0,58	(- 9)				0,62	0,53	(-15)
L	0,70	0,62	(-11)				0,71	0,63	(-13)
							0,66	0,58	(-18)
Mean	0,69	0,57	(-16)	0,45	0,52	(+ 16)	0,67	0,57	(-18)

from litters used for the experiments in Chapter 10 was always assayed as a pool. Only sera from rabbits gaining body weight at the normal rate were assayed. The mean indices of precision of the reference standards in the 17 assays were 0,14 for sulphation and 0,23 for thymidine. For the unknowns the index means and ranges were 0,19 (0,09 - 0,28, n = 48) and 0,28 (0,14 - 0,40, n = 47), respectively.

Figures 45 and 46 show the variation with age of SM assayed by its sulphation-and thymidine uptake- stimulating potencies respectively. By both parameters SM rises progressively from low foetal levels to a plateau reached about 150 days PC. There is no difference between males and females at any age. The degree of variation of the SM level at a given age increases with increasing age so that by the time adulthood is reached the range is 0,36 to 1,01. It is important to note that SM in serum pools, consisting of variable amounts of serum from six or more rabbits of both sexes, falls within the range of SM of the individual rabbits at all ages. Neither birth (33 days PC) nor puberty (approximately 100 days PC) is associated with any clearcut change in SM level.

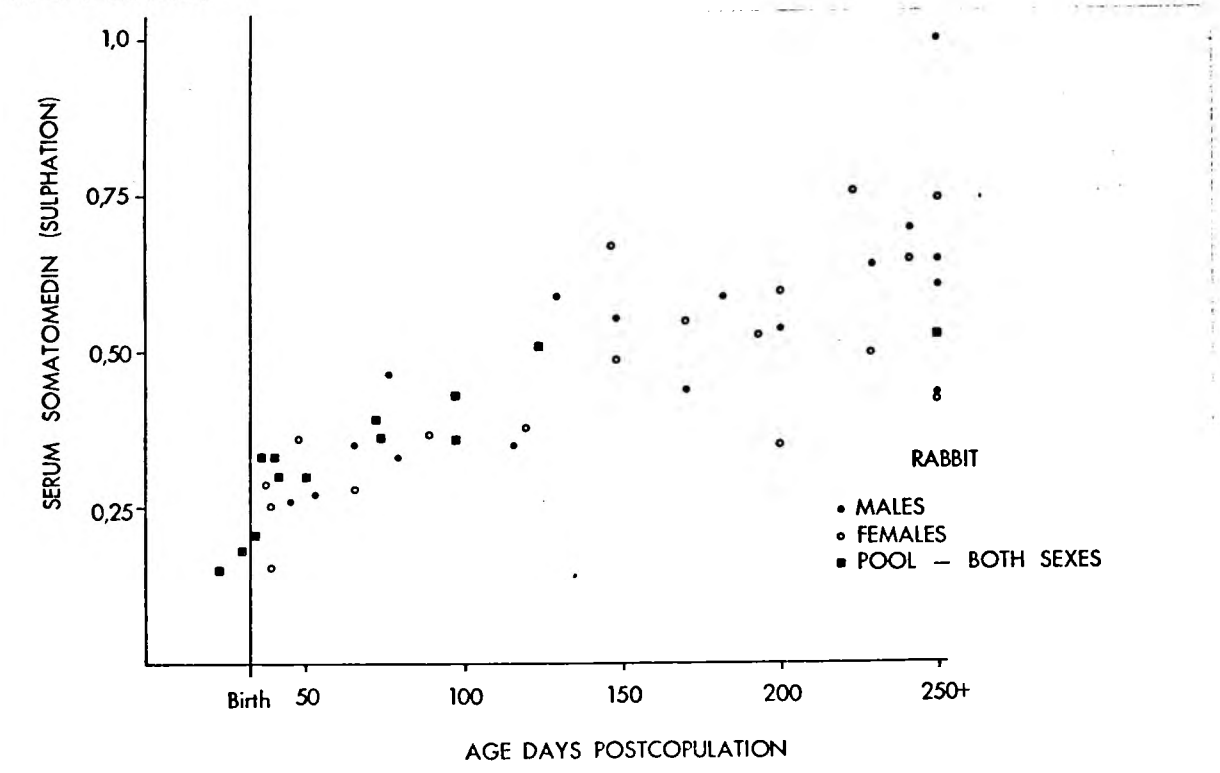


Figure 45: Serum somatomedin (sulphation) variation with age.

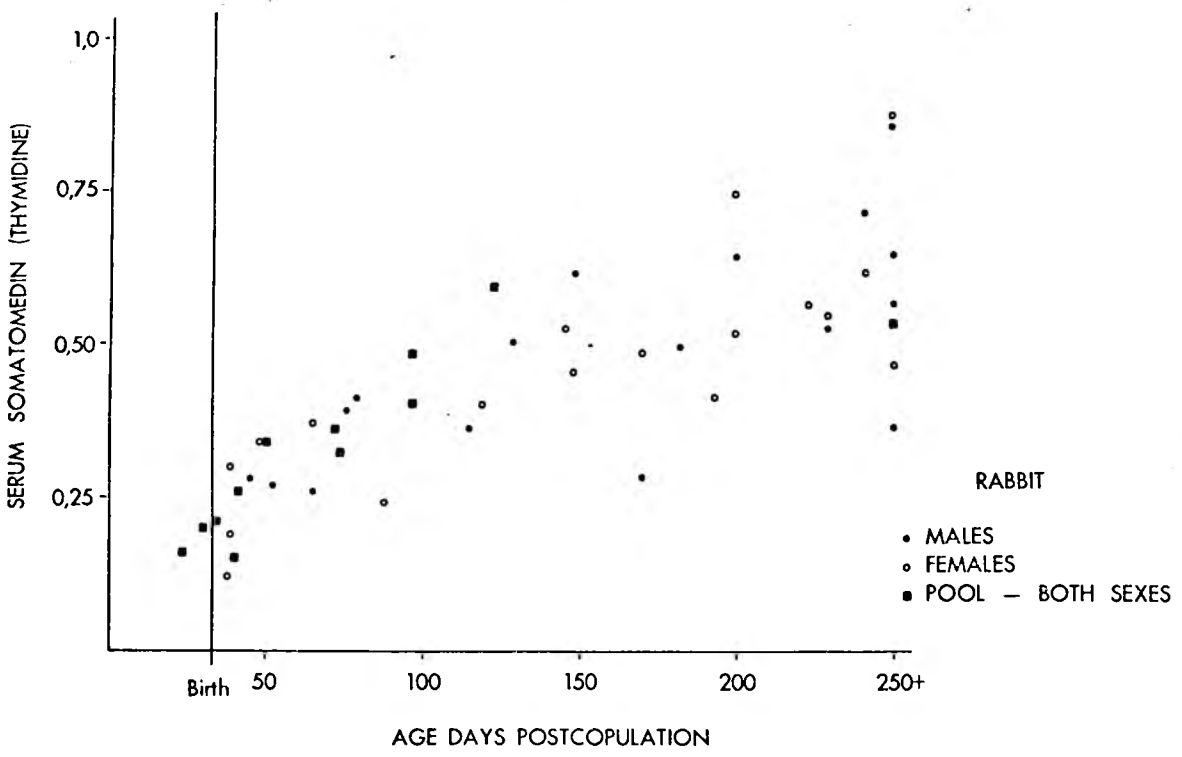


Figure 46: Serum somatomedin (thymidine uptake) variation with age.

There is an excellent correlation between the potencies estimated by the two different isotopes (r = 0,87).

A plot of the body weight velocity, over the 5 or 6 days prior to the serum assay for the individual rabbits and for the groups of rabbits used as the source of costal cartilage in the experiments in Chapter 10, against serum somatomedin shows no correlation, r =-0,39 (sulphation) and r =-0,36 (thymidine), (Figure 47).

The statistical design of the bioassay permits the calculation of a potency ratio only when the standard and unknown dose-response curves are parallel. Non-parallelism occurred with both isotopes in seven samples and with ^{3}HTdr in one additional sample. The age distribution of these non-parallel samples is of interest (Table 25).

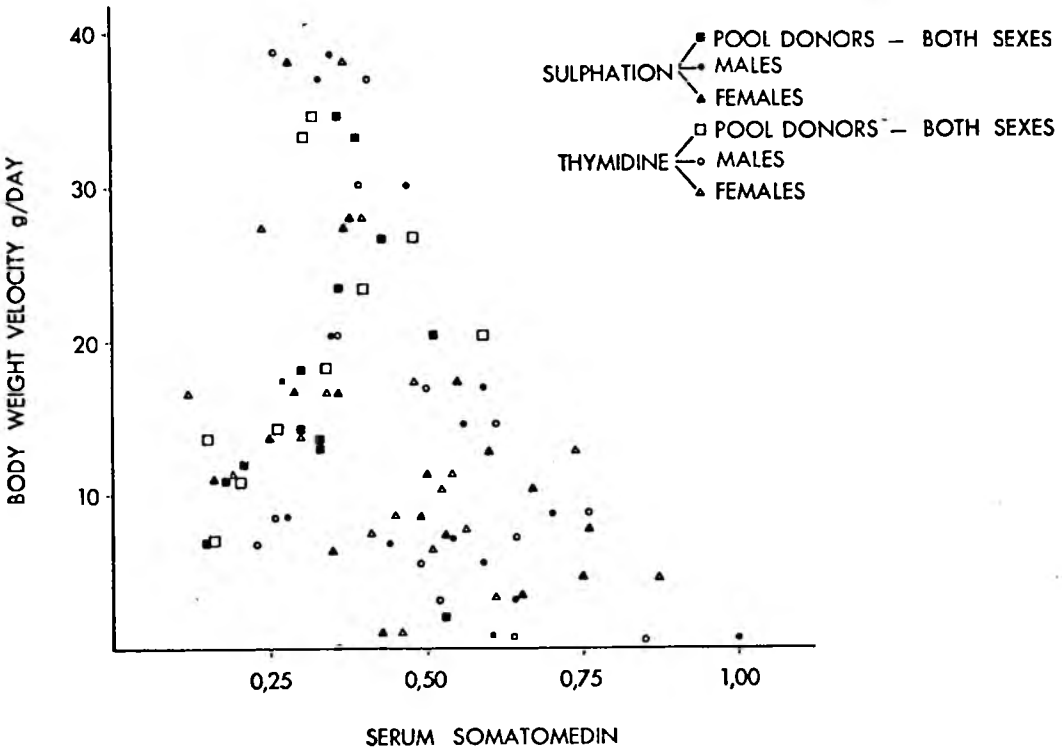


Figure 47: Relationship between serum somatomedin and body weight velocity in the preceding 5 or 6 days. The velocities described by Waterman (1943) have been used for the foetal rabbits - see Figures 39 and 40.

TABLE 25. Age distribution of sera giving non-parallelism in the assay

Age (days PC)	Number
30	1
33 - 37	4
49 - 56	3

9.4. Discussion.

Assay technique and specificity. GH-dependence of the serum activity assayed in any cartilage incubation system is a sine qua non for the stimulating effects of the serum to be ascribed to SM (Van Wyk et al. 1974). The specificity of the SM activity

in rabbit serum was unequivocally proven in both the porcine and chick cartilage assays. Treatment of two GH-deficient children with GH (Crescormon, Kabi), the details of which are shown in Chapter 13, also resulted in a marked rise in SM assayed in the porcine system.

The excellent indices of precision (λ) for both sulphate and thymidine uptakes confirms the high degree of precision for this assay reported by other authors (Van den Brande 1973). As others have also observed the thymidine precision is considerably lower than that of sulphate-uptake. Nevertheless λ was less than 0,40 for all thymidine potency ratios. The good correlation between the sulphate and thymidine potency ratios has not been reported before. This correlation is further evidence, in addition to the purification data reported in Chapter 5, that these two separate metabolic actions of SM on cartilage are resident in the same or very similar serum fractions.

Serum inorganic SO_4 concentrations have not been reported in the rabbit. The results indicate no difference between males and females and no change with age. This latter observation contrasts with the finding of a decrease in sulphate in the rat with increasing age (Dziewiatowski 1954). Although the post-incubation schedule in a common bath containing the isotopes in medium of fixed composition obviates the problem of a variable sulphate pool, the SO_4 concentration in the medium was lowered to match that of the rabbit serum. This was nevertheless some 3-fold greater than the human reference pool SO_4 concentration of 0,19mM.

Serum thymidine estimation was not attempted as this can only be achieved accurately by radioimmunoassay (Hughes et al. 1973). Human and mouse serum thymidine concentrations are in the order of 10^{-6}M.

The possibility that the different serum amino acid

composition of rabbit serum may significantly alter the bioassay incubation medium requirements was investigated. In spite of major differences in the glycine and serine concentrations and other smaller differences, no difference in response was obtained when amino acid concentrations were changed from a normal human to a normal rabbit profile.

Few authors have reported the times and conditions of collection of samples for assay. This work has shown no difference between venous and arterial serum and no effect of acute ether anaesthesia. It is possible that minor differences do exist, but the precision of the assay is not sufficient to detect them. These findings are consistent with the concept of stability of serum SM acting as a modulator of fluctuating GH levels (Daughaday 1971b). Somatomedin does appear to rise in response to chronic stress such as repeated venesection (Bozović & Boström 1969) and as with other hormone measurements it would be wise to define and standardize the conditions under which samples are collected.

Finally, the porcine assay provides highly reproducible results over a period of time and the stability of rabbit SM appears to match that of human origin in the deep-frozen state.

Serum somatomedin variations. As in the rat (Salmon 1972) prolonged fasting causes a fall in serum SM in the rabbit. The mechanism of this decrease is not entirely clear, but at least in one rabbit (F) it was unequivocally due to a thermolabile inhibitory factor. In the other five rabbits the decline may also have been due to inhibitors, but their removal was either not as dramatic as in the case of rabbit F or an equivalent portion of the stimulatory fraction was also destroyed by the heat-acid treatment, resulting in little net change. This latter explanation is favoured because in the other sera, viz. 12 hour fast, fed state and reference standard, in which inhibitors were

presumably not present, the treatment uniformly resulted in a modest fall in SM activity.

The role of this inhibitor in the fasting state remains unresolved. Since inhibitors, but not necessarily the same ones, have also been demonstrated in serum from patients with protein-energy malnutrition it is possible that they play a protein-sparing role by blocking the insulin-like action of SM.

The changes with age of SM in the rabbit are similar to those seen in man (Van den Brande & Du Caju 1973). These serum SM levels give no indication of the rate of turnover of the hormone. In view of the observation that SM is either removed from or inactivated in the serum (Daughaday et al. 1968) it is possible that the low levels in early life reflect a high rate of utilization by rapidly growing tissues. As the growth rate slows so the serum level would then rise to a steady plateau when growth ceases. This hypothesis is attractive and warrants further investigation.

The failure to detect a change in SM levels around birth, as has been observed in man (Tato et al. 1975), and during puberty may indicate a true lack of importance of these events for SM or may again be due to inadequate precision of the assay.

The lack of correlation between serum SM and body weight velocity contrasts, at first impression, with the clear correlation in children between somatomedin A and stature velocity. Even when the correlation coefficient is recalculated only for rabbits under 100 days PC, i.e. those growing rapidly, no correlation is found, r = 0,41 (sulphation), r = 0,33 (thymidine). Hall & Filipsson (1975) only studied children between the ages of 7 and 16 years and their correlation was with stature, a predominantly skeletal dimension, rather than body weight which has many components.

INTRODUCTION TO CHAPTERS 10, 11 AND 12

The velocity of growth of a tissue is dependent on both the micro-environmental stimuli and the responsiveness of the tissue to these stimuli. To quantitate the relative contribution of each of these two factors they must be experimentally separated and each studied with the other being held constant, insofar as is possible. This can only be achieved *in vitro* where the known variables can be controlled. The growth of cartilage is particularly suited to this type of *in vitro* study (Daughaday and Reeder 1966, Daughaday 1971a).

In Chapter 9 one aspect of the micro-environmental stimuli to cartilage growth, namely serum SM, was investigated. In Chapters 10, 11 and 12 aspects of the cartilage response to serum *in vitro* are described.

In Chapter 10 two indices of cartilage metabolism, the uptake of thymidine and of inorganic sulphate, by rabbit costal cartilage are shown to be age-dependent and closely correlated with the velocity of growth of the cartilage. By varying the serum SM source, the serum concentration and the duration of incubation it will be shown that both basal metabolism and dose-response characteristics are a function of cartilage age, not the SM stimulus.

Chapter 11 describes experiments that attempt to bridge the gap between the *in vitro* situations in Chapters 9 (serum stimulus) and 10 (cartilage response). The experiments consist in short-term homotransplantation of rat costal cartilages. By both age-matching the donor and recipient rats and transplanting cartilages from rats of different ages into adult rats the stimulus-response

conditions are varied in a controlled manner.

Chapter 12 describes the isolation of rabbit costal chondrocytes from the matrix and the age-dependency of thymidine uptake by the chondrocytes is examined. These cell cultures remove a further variable, namely the possible role of the matrix in modifying the response of chondrocytes to SM.

CHAPTER 10

CHARACTERISTICS OF THE RESPONSE OF RABBIT COSTAL CARTILAGE IN VITRO

Few studies have been made of the variables that influence the response of cartilage to serum stimuli *in vitro*, and most work has concentrated on somatomedin and the host of factors that modify its action on cells (see Chapter 5). What little is known of the role of age, sex, anatomical site and species in the *in vitro* response of cartilage is discussed in Chapter 6.

In this chapter costal cartilage from rabbits over a wide range of ages (15 days PC to adults) is studied. The velocity of growth in both length and weight of the fourth to seventh costal cartilages over this age range has been calculated. The uptake of thymidine as ^{3}HTdr and of sulphate as $Na_2{}^{35}SO_4$ *in vitro* is quantitated. Aspects of the experimental technique are examined and the putative serum SM to which the cartilage responds is shown to be GH-dependent. The correlation between *in vitro* cartilage responsiveness and growth velocity is described and discussed.

10.1. Methods

Rabbit litters of standard size (see Chapter 8) were sacrificed between 07h00 and 08h00 under light ether anaesthesia by exsanguination. The serum from rabbits of each litter or group of litters was pooled and stored at 4°C for use later in the day.

The 4th to 7th ribs and adjoining vertebrae and sternum were dissected free. The lengths of the bony and cartilaginous portions

of each rib were measured by placing a monofilament cotton thread along the greatest curvature of the rib, cutting the thread to give lengths representing the bony and cartilaginous portions, and then laying the threads along a ruler graduated to 0,5mm. The cartilaginous portion was separated from the sternum and from the bony portion through the costochondral junction and stripped of adhering soft tissue and perichondrium. Cartilages from all rabbits assigned to the experiment were pooled by number, i.e. all 4th cartilages together, and when allocations were made each assay flask received an equal proportion of 4th, 5th, 6th and 7th cartilages. Cartilages were placed in incubation medium at room temperature for 30 to 90 minutes prior to allocation.

Cartilages were incubated in groups in stoppered 25ml Erlenmeyer flasks at 37°C in a shaking water bath (Gallenkamp) at 30 cycles/minute. The solutions were dilutions of serum (either standard human pool or cartilage donor pool) in incubation medium (described in Chapter 9). After either 4 or 20 hours the cartilages were removed, blotted dry and transferred for a further 4 hours to medium containing 2 μCi/ml each of $^{35}SO_4$ and ^{3}HTdr. At all ages 2ml of solution per cartilage was provided. Incubation was stopped by washing the flasks through with a boiling saturated solution of Na_2SO_4 for 5 minutes. Cartilages then stood overnight at ambient temperature in saturated solutions of Na_2SO_4.

After rinsing in distilled water and being blotted dry each cartilage was divided into costal, middle and sternal thirds (zones), each of which was weighed to 0,1mg. Each zone was dissolved in 0,5ml 23N formic acid at 80°C for 30 - 40 minutes. After cooling 10ml Instagel was added and activity of both isotopes determined by liquid scintillation counting as described in Chapter 9. In cartilages from rabbits older than 100 days PC

each zone had to be divided into half for solution in the formic acid.

10.1.1. Calculation of velocity of growth. To obtain an estimate of the mean length velocity of cartilage growth the following procedure was adopted. The lengths of all measured ribs (4th to 7th inclusive) of all rabbits of a given age were summed and a mean length for bony and for cartilaginous portions calculated. From the means at each age mean velocities for the intervals were calculated. By linear interpolation between the mid-points of these intervals, velocity at each of the ages studied was derived. Velocity at the first age studied, viz. 15 days PC, was obtained by linear extrapolation from the two adjacent velocities.

The same method was applied to obtain velocity estimates for cartilage weight.

10.1.2. Autoradiography methods. Thymidine incorporation into cartilage was also studied by autoradiographic techniques.

Costal cartilages were dissected out as before, but not stripped of perichondrium. They were incubated in 2ml medium/rib containing 10 μCi ^{3}HTdr/ml at 37^{o}C for 3 hours. Half of the cartilages from each rabbit were processed for liquid scintillation counting as before and the other half processed for autoradiography.

The cartilages for autoradiography were washed in distilled water and fixed in fresh Carnoy's solution (3 : 1, 80% alcohol : acetic acid) for 2 hours. Dehydration and embedding in paraplast was performed following Humason (1962). Sections of 4 μm were cut and mounted on glass slides.

The slides were dipped in Ilford GS nuclear emulsion and stored in absolute darkness for 14 days at 4^{o}C. Thereafter

Kodak Microdol-X was used for development and the slides were then lightly stained with haematoxylin. The details of the autoradiographic technique are those described by Rogers (1972).

Rabbits aged 34, 40, 47 and 54 days were studied. At each age both 4th and 6th ribs were examined in two animals. At each of the following sites 800 to 1000 cells were counted and the proportion of definitely labelled nuclei calculated : 1mm from the costochondral junction, the centre of the middle zone, 1mm from the sternal articulation. Perichondrial cells were not included in the counts.

10.2. Results

The mean lengths and weights and respective velocities for the 4th to 7th ribs are shown in Tables 26 and 27. For both dimensions the velocity reaches its maximum value just after birth (34 days PC), declining progressively thereafter. Length growth slows earlier than weight growth, tending to flatten off around 70 days PC (Figure 48). The ratio bone velocity: cartilage velocity remains close to unity throughout the growth period.

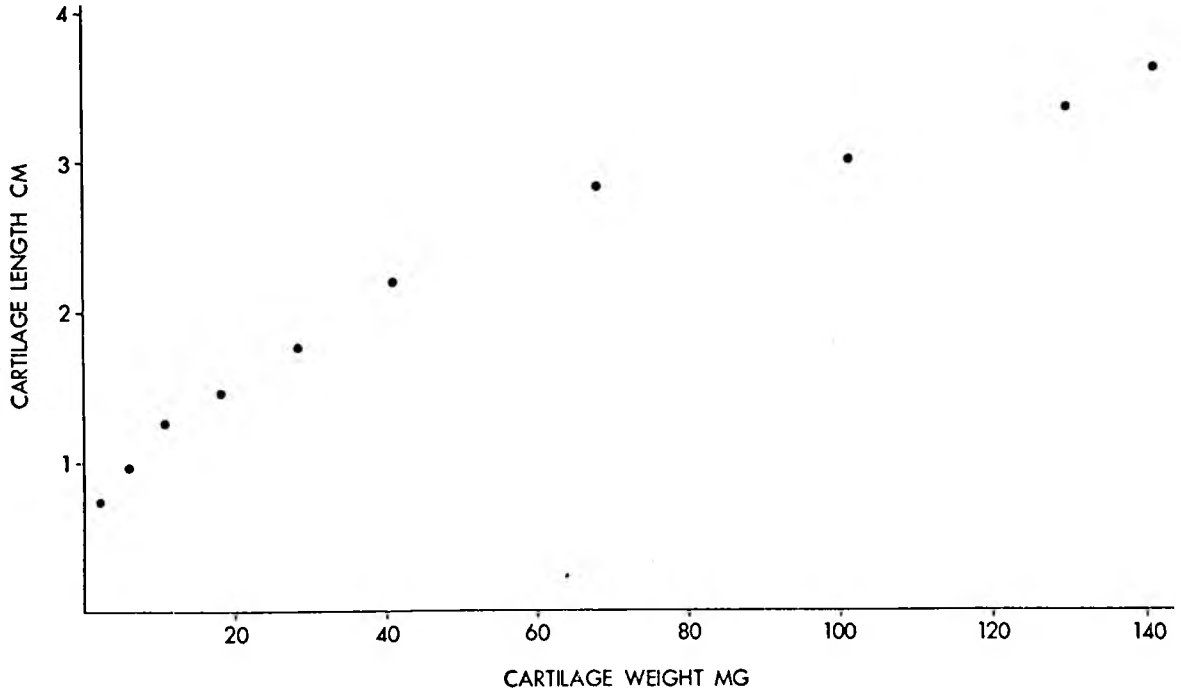


Figure 48. Rabbit costal cartilages (4th to 7th inclusive) : mean length plotted against mean weight.

TABLE 26. Mean length and mean length velocity of the cartilaginous and bony sections of the 4th, 5th, 6th and 7th ribs in the rabbit. "Ratio" indicates the bone : cartilage ratio Velocity is derived by interpolation - see text.

AGE (days pc)	BODY WEIGHT (mean)	n	LENGTH (cm)			LENGTH VELOCITY (cm/day)		
	g		Bone	Cartilage	Ratio	Bone	Cartilage	Ratio
15	9	12	0,88	0,75	1,17	0,021	0,023	0,91
23	19	40	1,12	0,95	1,18	0,041	0,036	1,14
30	41	40	1,49	1,26	1,18	0,055	0,048	1,15
34	62	40	1,73	1,46	1,18	0,057	0,051	1,12
40	144	34	2,07	1,76	1,27	0,055	0,048	1,15
50	381	34	2,61	2,20	1,19	0,047	0,040	1,18
72	841	40	3,29	2,84	1,16	0,019	0,019	1,00
97	1630	28	3,47	2,98	1,16	0,011	0,011	1,00
123	2224	24	3,86	3,34	1,16	0,014	0,014	1,00
365+	3147	24	4,71	3,63	1,30	0,001	0,001	-

TABLE 27. Mean weight and mean weight velocity of the cartilaginous sections of the 4th, 5th, 6th and 7th ribs in the rabbit. Velocity is derived by interpolation - see text.

AGE (days pc)	n	WEIGHT (mg)	WEIGHT VELOCITY (mg/day)
15	12	2,25	0,32
23	80	6,09	0,64
30	56	11,44	1,45
34	52	18,12	1,68
40	74	28,43	1,56
50	40	40,96	1,27
72	74	67,88	1,33
97	32	103,07	1,22
123	72	129,92	0,90
365+	24	141,65	<0,01

TABLE 28. Experiment to demonstrate the lack of influence of the volume of incubation fluid on isotope uptake. Cartilages were incubated in 20% standard pool serum with volumes of 1ml, 5ml and 10ml/100mg wet cartilage for 4 hours, followed by 4 hours in isotope - containing medium at the same volume. "C/S" indicates the costal zone - to - sternal zone ratio of isotope uptake.

VOLUME PER 100mg OF RIB	n*	dpm/mg (± SD)	AGE OF RABBITS 44 days PC	C/S	113 days PC	C/S
1 ml	12	^{3}H	9630 (673)	2,7	2171 (298)	15,7
		^{35}S	967 (111)	1,3	484 (49)	1,4
5 ml	12	^{3}H	8651 (910)	2,9	2214 (331)	16,1
		^{35}S	839 (214)	1,4	458 (74)	1,4
10ml	12	^{3}H	9113 (1001)	2,4	2052 (357)	14,4
		^{35}S	908 (167)	1,2	427 (94)	1,7

*n denotes the number of cartilage segments assayed at each volume

The possible influence of variable stimulus, or variable substrate supply, per unit mass of cartilage, as a result of keeping solution volume constant per cartilage irrespective of size, was investigated. Cartilages from two sizes of rabbits, 44 and 113 days PC, were incubated in solutions of 1, 5 and 10ml/rib cartilage and isotope uptake measured (Table 28). The results indicate no significant difference ($p > 0,05$) for either isotope at any of the three volumes studied. A volume of 2ml/rib was therefore used to ensure that the larger cartilages were adequately immersed during incubation.

10.2.1. GH-dependence of SM effect on rabbit costal cartilage. To demonstrate that the serum-stimulus to cartilage is, at least in part, somatomedin-like in that it is GH-dependent the following experiment was performed. Serum from a 4 year old child with isolated GH deficiency (basal GH 1,0 ng/ml and no response to adequate insulin hypoglycaemia; SM on porcine bioassay 0,14) and serum from a 32 year adult with untreated acromegaly (fasting GH 56 ng/ml and no suppression with glucose loading; SM on porcine bioassay 1,9) was obtained. The response of cartilage from 34 day PC rabbits to normal human serum (standard pool), the GH deficient serum and the acromegalic serum at 5% and 20% concentration was assessed (Table 29). In incubation medium and in normal serum responses were quantitatively similar to those obtained for 34 day PC cartilage in later experiments (see Tables 30 and 31). Stimulation occurred at both 5% and 20% concentrations. Uptake in the SM-poor serum was significantly lower than that in normal serum ($p < 0,05$) and no stimulation occurred. On the other hand uptake in the SM-rich serum was significantly greater than in normal serum ($p < 0,05$) and marked stimulation occurred.

TABLE 29. The effect of normal adult (standard pool), hypopituitary and acromegalic human serum on the uptake of sulphate and thymidine by rabbit costal cartilage, aged 34 days PC.

Serum	n**	Sulphate uptake moles x 10^{-10}/mg ($\pm$SD)	Thymidine uptake moles x 10^{-14}/mg ($\pm$SD)
Incubation medium	8	6,3 (2,7)	9,8 (2,3)
Normal adult serum			
5%	8	8,4 (2,6)	13,7 (2,9)
20%	8	11,7 (2,7)	17,9 (3,1)
Hypopituitary serum			
5%	8	5,9 (2,4)*	9,9 (2,6)*
20%	8	6,3 (3,1)*	10,4 (2,8)*
Acromegalic serum			
5%	8	9,9 (2,4)	14,8 (3,1)
20%	8	17,6 (2,7)*	23,7 (4,4)*

** n indicates the number of cartilage segments assayed in each solution

* indicates that uptake is significantly different from the uptake at the corresponding concentration of normal adult serum, at the 5% level.

TABLE 30. Uptake of sulphate, moles x 10^{-10}/mg wet cartilage (±SD), by rabbit costal cartilage in incubation medium, 20% standard pool serum, and 20% serum from the cartilage donor rabbits.

AGE (days PC)	BODY WEIGHT (mean) g	n*	4 HOURS INCUBATION			20 HOURS INCUBATION		
			Medium	20% Standard Serum	20% Donor Serum	Medium	20% Standard Serum	20% Donor Serum
15	9	16(2)**	4,1(1,7)	5,8(2,3)	-	2,9(1,8)	3,4 (2,7)	-
23	19	12(1)	4,2(2,6)	7,7(2,9)	6,8(2,7)+	3,1(2,0)	5,0(2,6)	-
30a)		8(1)	7,8(1,9)	10,3(2,3)	9,7(2,6)	5,3(2,9)	7,2(3,9)	6,2(4,1)
b)		8(1)	9,0(2,3)	13,4(3,4)	8,5(3,1)	-	-	-
mean	41		8,4(2,2)	11,8(3,2)	9,1(2,9)			
34	62	8(1)	7,2(3,0)	9,6(3,0)	9,3(2,1)	6,8(4,1)	8,0(2,9)	7,2(4,3)
40a)		8(1)	2,1(0,9)	3,3(2,0)	3,2(2,1)	1,8(1,1)	3,1(1,3)	2,8(0,9)
b)		12(1)	3,8(1,4)	7,5(1,9)	4,9(2,0)	-	-	-
mean	144		3,0(1,2)	5,4(2,0)	4,1(2,0)			
50	381	8(1)	3,8(2,3)	5,0(1,8)	4,7(3,2)	3,1(1,7)	3,8(2,7)	3,2(2,7)
72	841	12(2)	1,9(1,2)	2,8(0,9)	2,7(1,3)	-	-	-
97a)		8(1)	1,8(0,6)	1,9(0,7)	1,1(1,7)	1,3(1,4)	1,8(1,0)	1,0(1,2)
b)		8(1)	2,9(0,8)	3,7(1,2)	3,4(2,1)	-	-	-
mean	1630		2,4(0,8)	2,8(0,9)	2,3(1,3)			
123	2224	8(1)	2,0(1,1)	2,1(1,2)	1,8(0,9)	1,2(1,0)	0,7(0,9)	1,0(1,3)
365+	3147	8(1)	0,09(1,3)	0,2(0,9)	-	-	-	-
Boiled 44		6(1)	0,008	0,007	-	-	-	-

*n indicates the number of cartilage segments in each group, e.g. 4 hours incubation in medium

** figure in brackets indicates the number of litters from which cartilage was obtained

+ for this experiment maternal serum was used

10.2.2. Variation in cartilage response with age and velocity of growth. The *in vitro* response of cartilage from rabbits aged 15, 23, 30, 34, 40, 50, 72, 97, 123 and more than 365 days PC is shown in Tables 30 and 31. Stimulation was provided by 20% solutions of standard pool and 20% donor pool serum. There was insufficient donor serum in the younger foetal rabbits to incubate cartilage in.

At three ages, 30, 40 and 97 days PC, two assays were performed on separate occasions. For both sulphate and thymidine uptake there was reasonably close agreement at each age with no statistically significant difference between any of the pairs ($p > 0,05$).

Sulphate (Table 30). Irrespective of duration of incubation, 4 or 20 hours, or of incubating solution, medium, 20% standard serum or 20% donor serum, a general age-related pattern of uptake emerges. Uptake rises from 15 days PC to a peak at 30 days PC just before birth. It then falls more or less regularly to very low levels in adulthood. Cartilage boiled for 10 minutes prior to incubation shows no active uptake.

Up to and including 72 days PC 20% serum stimulates uptake significantly ($p < 0,05$). This is true for both standard pool and donor sera, except at 30 days PC with 20% donor serum in the second litter (b) studied. In all cases, however, the stimulation by the donor serum is less than that caused by the standard pool serum. This ranges from a maximum difference after 4 hours incubation at 30 days PC of 75% to a minimum at several ages of 98%.

At 23 days PC a 20% solution of maternal serum was used as there was insufficient foetal serum. No difference in uptake compared to that occurring in 20% standard serum was noted.

The remaining feature of the sulphate uptake is its dimunition after 20 hours compared to that after 4 hours incubation.

This dimunition is consistent for all ages and all incubating solutions, ranging from 33% to 97%.

Thymidine (Table 31). As with sulphate there is a general age-related trend for thymidine incorporation to rise from 15 days PC, but in this case the peak is reached just after birth at 34 days PC. Incorporation then falls steadily reaching negligible levels in adulthood. Boiled cartilage shows no active incorporation.

Again 20% serum, both standard and donor stimulates uptake significantly ($p < 0,05$) at all ages up to and including 50 days PC. Thereafter although some stimulation does occur, it is not significant.

As with sulphate, the incorporation of thymidine is generally less in donor serum compared to standard serum. At 23 days PC uptake in 20% maternal serum is not significantly different from uptake in 20% standard serum ($p > 0,05$).

Thymidine, like sulphate, incorporation is also lower at 20 hours compared to 4 hours except for 20% standard serum at 97 days PC. This difference ranges from 30% to 89% with a tendency for it to be larger at the younger ages when greater uptake is occurring.

The correlation between sulphate uptake (Figure 49) and thymidine uptake (Figure 50), stimulated by 20% standard pool serum, and velocity of growth in length is excellent. The correlations between length velocity and uptake under other conditions are good, but the correlations between uptake and weight velocity are poor (Table 32).

TABLE 31. Uptake of thymidine, moles x 10^{-14}/mg wet cartilage (±SD), by rabbit costal cartilage in incubation medium, 20% standard pool serum, and 20% serum from the cartilage donor rabbits.

AGE (days PC)	BODY WEIGHT (mean)	n*	4 HOURS INCUBATION			20 HOURS INCUBATION		
			Medium	20% Standard Serum	20% Donor Serum	Medium	20% Standard Serum	20% Donor Serum
15	9	16(2)**	4,8(1,2)	11,2(1,1)	-	3,6(1,1)	10,6(2,0)	-
23	19	12(1)	6,3(2,1)	16,9(2,1)	14,8(3,2)+	6,0(1,7)	14,3(3,1)	-
30a)		8(1)	9,3(2,0)	18,7(1,6)	12,9(2,3)	9,0(1,8)	18,9(3,0)	
b)		8(1)	8,6(1,9)	21,5(1,9)	16,3(2,9)	-	-	
mean	41		9,0(2,0)	20,1(2,0)	14,6(2,7)			
34	62	8(1)	12,5(1,9)	20,4(2,4)	16,7(3,0)	9,6(1,6)	17,7(4,6)	14,8(5,3)
40a)		8(1)	8,6(1,8)	14,2(1,9)	13,6(2,0)	6,4(2,1)	11,4(2,7)	9,2(4,0)
b)		12(1)	11,2(2,6)	18,4(2,3)	14,7(2,8)	-	-	-
mean	144		9,9(2,1)	16,3(1,7)	14,2(2,6)			
50	381	8(1)	10,4(2,7)	14,0(1,8)	13,3(2,0)	8,3(3,1)	11,3(3,8)	12,4(4,6)
72	841	12(2)	5,6(3,0)	6,1(1,0)	6,2(1,3)	3,9(1,8)	3,5(2,7)	-
97a)		8(1)	2,9(1,1)	3,3(0,8)	2,8(1,5)	2,8(1,8)	3,6(1,7)	2,3(1,8)
b)		8(1)	3,8(1,3)	5,1(1,2)	5,2(2,3)	-	-	-
mean	1630		3,4(1,3)	4,2(1,0)	4,0(1,8)			
123	2224	8(1)	2,6(1,9)	2,7(1,1)	2,7(0,9)	1,9(1,8)	1,8(1,3)	1,4(0,9)
365+	3147	8(1)	0,03(0,4)	0,03(0,4)	-	-	-	-
Boiled 44		6(1)	0,002	0,003	-	-	-	

*n indicates the number of cartilage segments in each group, e.g. 4 hours incubation in medium
** figure in brackets indicates the number of litters from which cartilage was obtained
+ for this experiment maternal serum was used

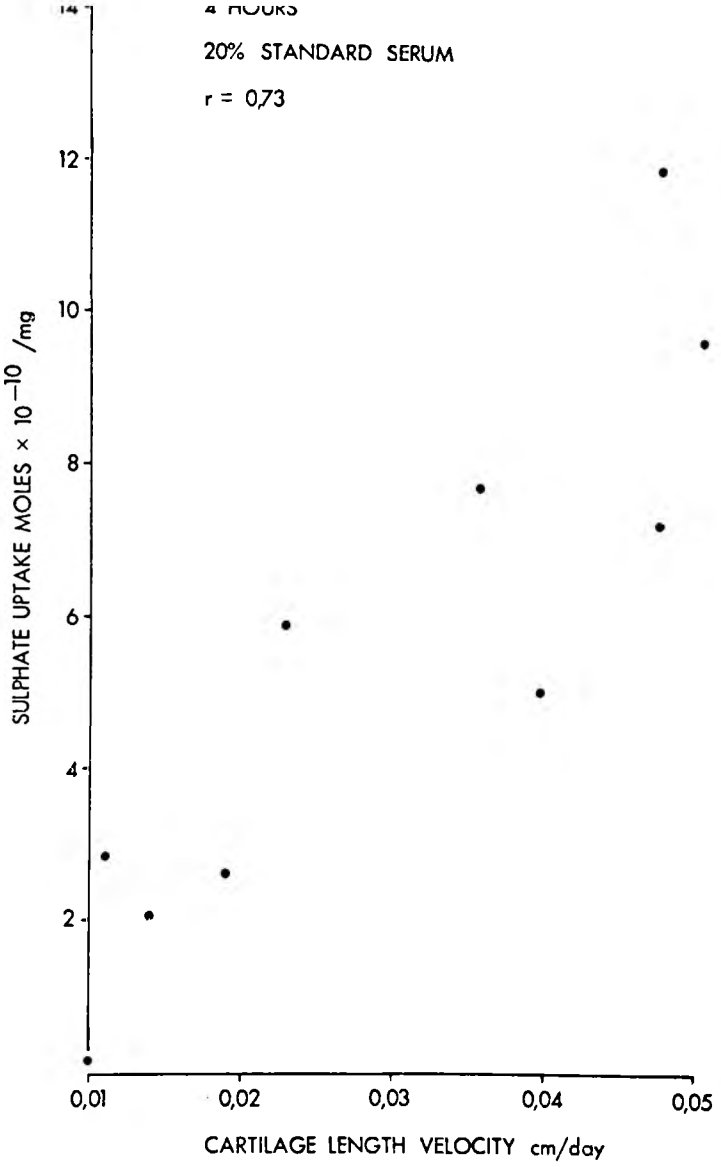


Figure 49. Correlation between velocity of growth in cartilage length and sulphate uptake.

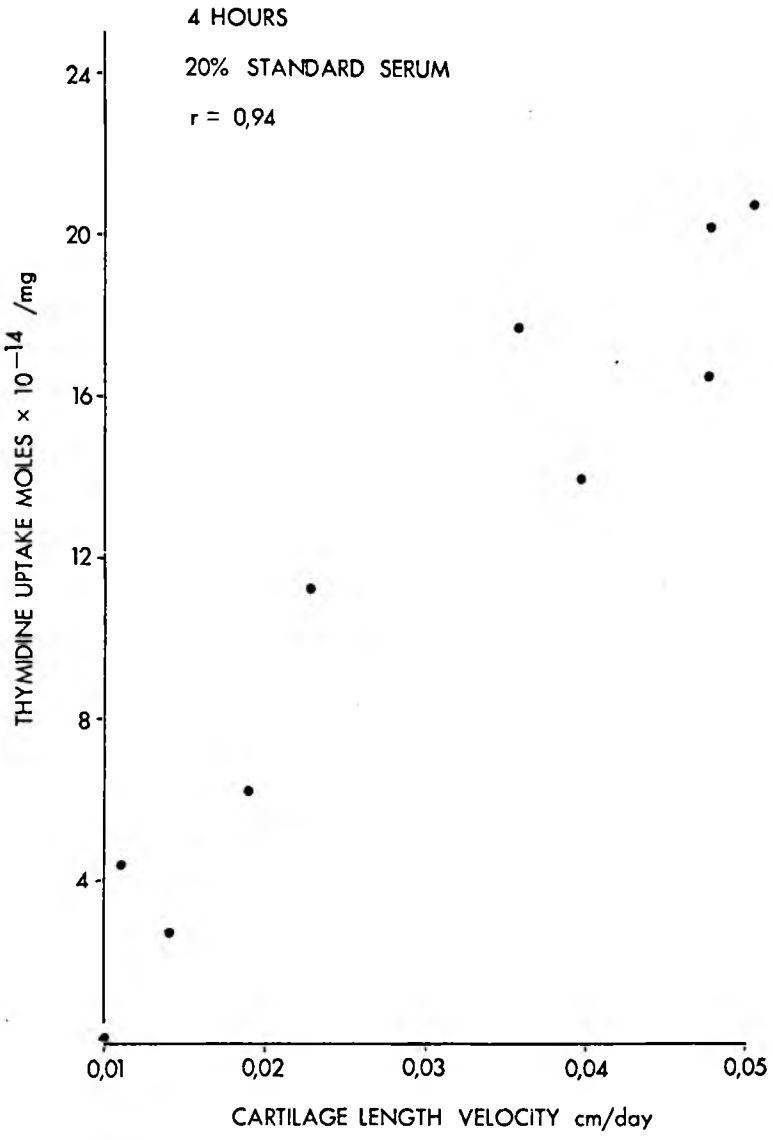


Figure 50. Correlation between velocity of growth in cartilage length and thymidine uptake.

TABLE 32. Coefficients of correlation between both length and weight velocity and uptake of sulphate (^{35}S) and thymidine (^{3}H) of rabbit costal cartilage

			Length Velocity	Weight Velocity
4 Hours Incubation	Medium	^{35}S	0,65*	0,27
		^{3}H	0,91***	0,60
	20% std. serum	^{35}S	0,73*	0,23
		^{3}H	0,94***	0,30
	20% donor serum	^{35}S	0,69*	0,53
		^{3}H	0,97***	0,72
20 Hours Incubation	Medium	^{35}S	0,64*	0,16
		^{3}H	0,87**	0,31
	20% std. serum	^{35}S	0,65*	0,16
		^{3}H	0,80**	0,05
	20% donor serum	^{35}S	0,69*	0,60
		^{3}H	0,86**	0,64

Note: asterisks indicate that the correlation is significant at $*p<0,05$ $**p<0,01$ $***p<0,001$

10.2.3. Dose-response characteristics of cartilage.

Cartilage from rabbits aged 23, 42 and 90 days PC was incubated in serial dilutions of standard pool serum from 2,5% to 20% (Figures 51, 52 and 53).

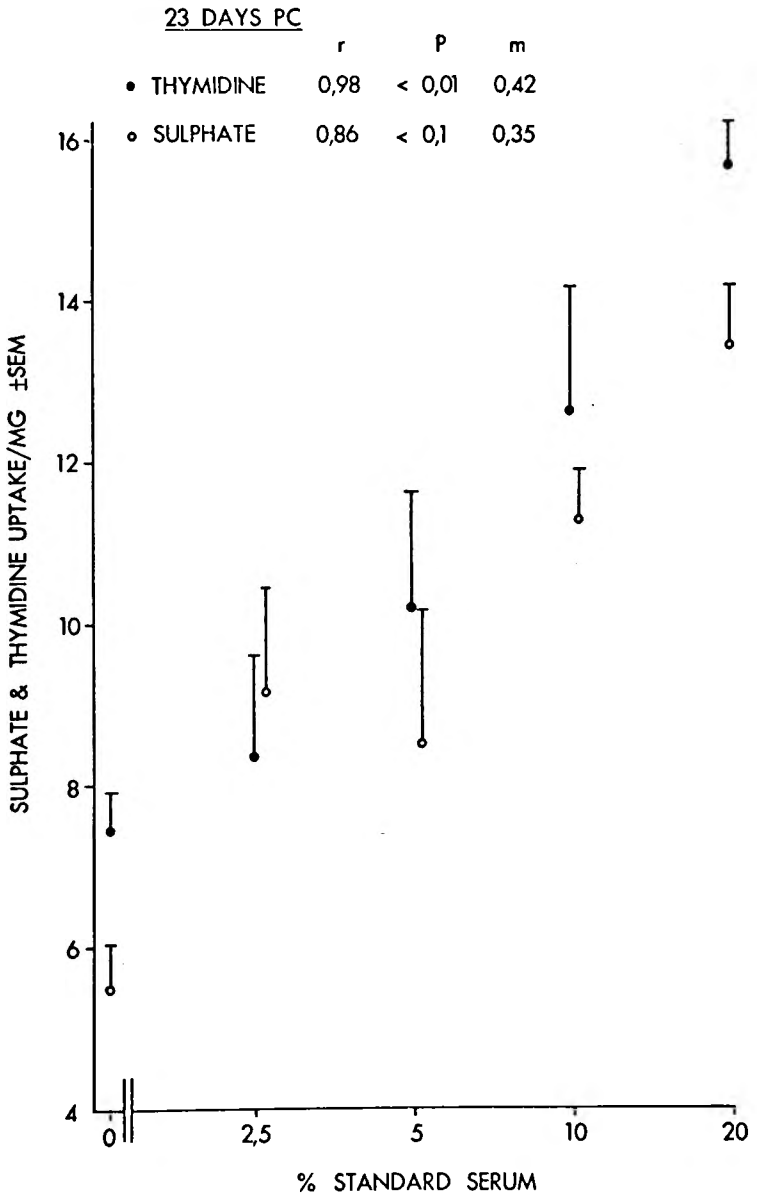


Figure 51. Response of cartilage from 23 day PC rabbits to increasing concentrations of standard pool serum. Sulphate uptake in this and Figures 52 and 53 expressed as moles x 10^{-10}/mg wet cartilage and thymidine uptake as moles x 10^{-14}/mg wet cartilage. The serum concentration in these three figures is shown on a logarithmic scale.

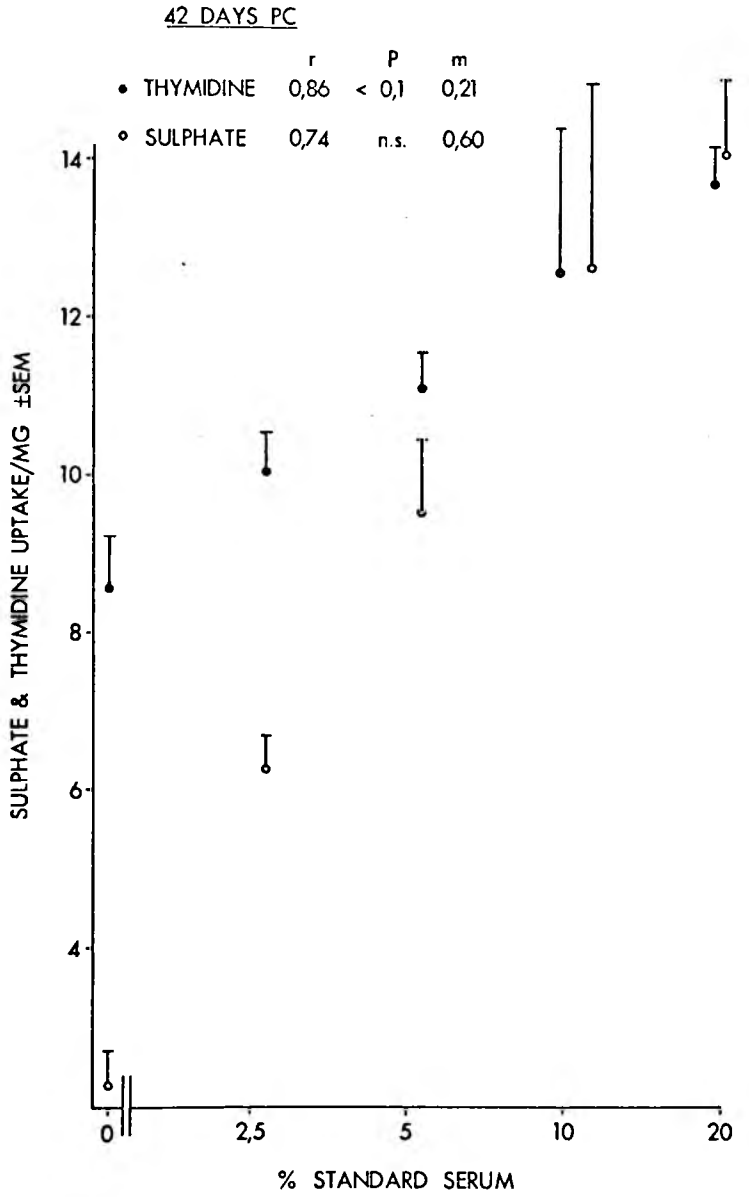


Figure 52. Response of cartilage from 42 day PC rabbits to increasing concentrations of standard pool serum.

Positive correlations were obtained for both sulphate and thymidine uptake at all three ages.

With increasing age the slope of the dose-response curve tends to decline for both isotopes.

10.2.4. Investigation of sex differences in cartilage response. Cartilage from rabbits aged 41, 88 and 114 days PC was incubated for 4 hours with male and female segments kept separate.

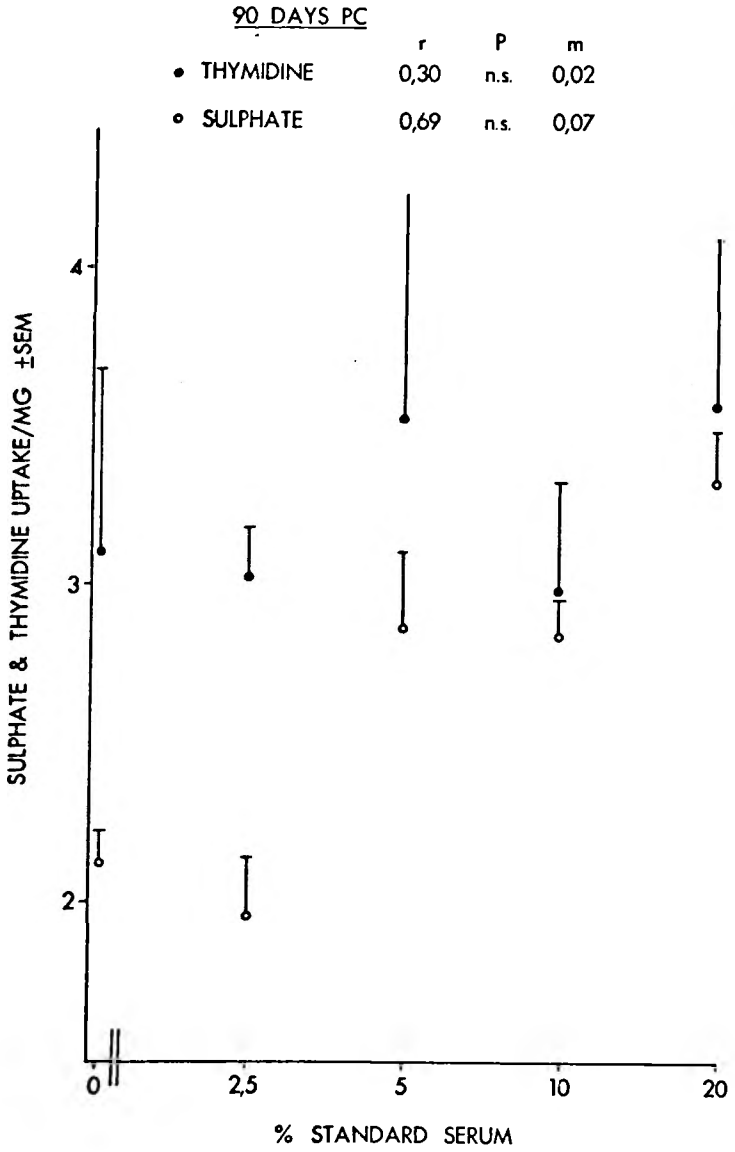


Figure 53. Response of cartilage from 90 day PC rabbits to increasing concentrations of standard pool serum.

Incubation medium only, 20% standard pool serum, and at 88 days PC 20% donor serum were used. No sexual differences were noted at any age in any of the solutions (Table 33).

10.2.5. Ratio calculations. For both sulphate uptake and thymidine uptake after 4 hours incubation the costal zone : sternal zone ratios are shown in Figure 54. For sulphate the ratio

TABLE 33. Experiment to demonstrate the lack of any sexual differences in sulphate and thymidine uptake by rabbit costal cartilage.

AGE (days PC)	Sex	Serum	n[1]	Sulphate uptake moles x 10^{-10}/mg	(±SD)	Thymidine uptake moles x 10^{-14}/mg	(±SD)
41	male	medium[2]	8	2,7	(0,8)	9,9	(2,3)
	female		12	2,9	(0,9)	9,6	(2,0)
	male	20%[3]	12	4,6	(2,1)	17,7	(2,3)
	female		8	5,1	(1,8)	16,8	(2,9)
88	male	medium	8	2,2	(0,7)	4,3	(1,8)
	female		8	1,9	(0,8)	4,9	(1,3)
	male	20%	8	3,3	(0,9)	5,2	(1,6)
	female		8	3,1	(0,9)	5,4	(1,7)
	male	20%[4] donor	8	2,9	(1,1)	5,0	(1,3)
	female		8	3,1	(0,9)	4,8	(1,1)
114	male	medium	12	2,0	(1,0)	2,0	(1,1)
	female		8	1,8	(0,9)	2,2	(0,9)
	male	20%	8	2,4	(0,8)	2,9	(1,3)
	female		12	2,0	(1,2)	3,0	(1,5)

Note: (1)n indicates the number of cartilage segments in each group
(2) incubation medium only (3) solution of 20% normal adult human serum (standard pool)
(4) solution of 20% serum from the cartilage donor rabbits

remains near unity throughout the age range studied. For thymidine, however, the ratio rises from unity before birth to between 10 to 15 around 100 days PC. Thereafter it declines reaching unity again in adulthood. The ratio in incubation medium only remains consistently lower than the ratio in serum. No pattern of difference between standard pool and donor serum emerges.

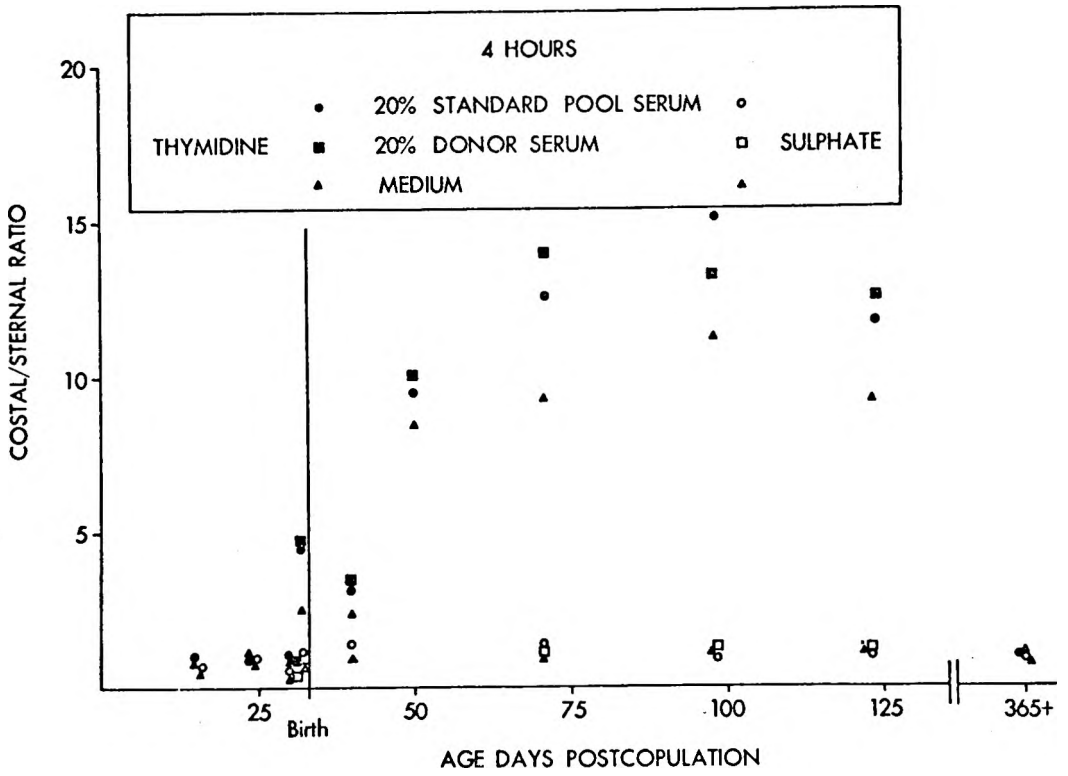


Figure 54. Ratios of uptake of sulphate and thymidine in costal zone of the rib cartilage to uptake in the sternal zone.

The thymidine-sulphate molar ratio exhibits one feature of interest (Figure 55). Ranging between one and three before birth and in the immediate peri-natal period, it then rises slightly at 40 days PC before gradually falling towards unity at 123 days PC and in adulthood.

10.2.6. Thymidine incorporation by autoradiography. The results of these experiments are expressed as the number of definitely labelled nuclei per 1000 cells counted (Table 34).

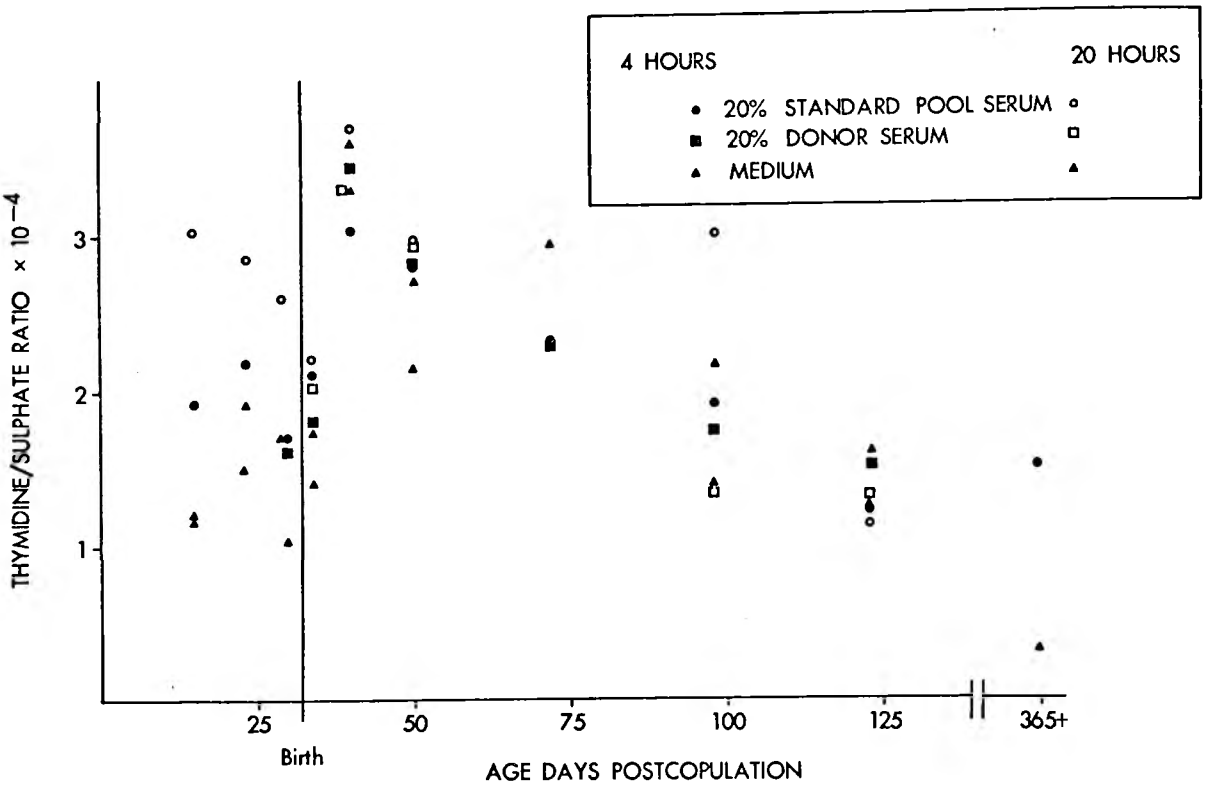


Figure 55. Ratios of uptake of thymidine by the whole cartilage to uptake of sulphate on a molar basis per unit mass of cartilage.

TABLE 34. Autoradiographic measurement of thymidine uptake by rabbit costal cartilage. The C/S ratio indicates the ratio of the number of labelled cells/1000 cells in the sternal zone.

AGE (days PC)		MEAN NUMBER OF LABELLED CELLS/1000 CELLS				
		Costal Zone	Middle Zone	Sternal Zone	Whole Cartilage	C/S ratio
34	4th	26,0	12,9	17,7	18,9	1,45
	6th	26,1	16,8	16,7	19,9	1,56
40	4th	31,7	3,6	12,4	15,9	2,56
	6th	27,7	4,5	13,3	15,2	2,08
47	4th	32,0	5,0	11,1	16,0	2,90
	6th	26,8	6,7	5,4	13,0	4,90
54	4th	21,0	2,6	2,2	8,6	9,50
	6th	21,2	2,4	2,0	8,5	10,60

These results show an age-related trend in thymidine incorporation in chondrocytes. From 34 days PC there is a fall in incorporation with age, so that three weeks after birth (54 days PC) the number of labelled cells is less than half the number immediately after birth. As conditions of incubation for autoradiography differed from those for liquid scintillation counting quantitative comparisons cannot be made between the results obtained by the two methods. Qualitatively, however, the two methods show a similar decline in incorporation from 34 days PC onwards, (cf. Table 31).

The costo-sternal ratio for thymidine incorporation shows a progressive rise from 34 days PC onwards, starting just above unity and reaching the region of ten at 54 days PC. In this situation direct comparison with the liquid scintillation is possible, and almost identical results were obtained (cf. Figure 54).

10.3. Discussion

The *in vitro* method of assessing cartilage responsiveness has unique advantages over *in vivo* methods. These advantages outweigh the technical difficulties and artificial conditions of the method (Chapter 6; Daughaday and Reeder 1966) and include controlled exposure to nutrients, stimuli and radio-isotopic labels.

The method used in these experiments is a modified version of that of Heins *et al*. (1970). The modifications improve on the method of these authors in several respects. The possible influence of variable volume of incubation fluid relative to the cartilage mass has been shown to be negligible (Table 28). The serum stimulus to cartilage has been shown to be GH-dependent (Table 29) and therefore, by inference, to be SM or SM-like (see Chapter 5.11). Direct proof of the exact nature of these serum fractions must await the availability of purified somatomedin. Further, the uptake of both sulphate and thymidine has been shown

to be active since boiled cartilage exhibited no uptake (Tables 30 and 31). The final advantages of the present method are the range of sera used, the use of both a standard serum and a donor serum pool for stimulation and the use of the two time periods for incubation, namely 4 hours and 20 hours. The biological fate of the $^{35}SO_4$ and the $^{3}HTdr$ has not been examined in this present work. Previous authors have amply investigated this question and shown that chondroitin sulphate and nuclear DNA are the respective end-products of these molecules in cartilage (evidence summarized in Chapters 5 and 9). The autoradiographic observation that the labelled thymidine is incorporated in cell nuclei and the similarity of qualitative results for thymidine incorporation by the liquid scintillation and autoradiographic methods do offer some evidence from the present work that DNA synthesis is stimulated by SM.

A major problem that has largely been overlooked in the literature on end-organ responsiveness is the relationship of the responsiveness to growth velocity. Authors have used either chronological age or distance parameters, such as body weight or length, as yardsticks. Logically it is the velocity of growth of the tissue being studied that should be the yardstick. In this study an attempt has been made to estimate the velocity of growth of the costal cartilages. By measuring a large number of cartilages at each age the errors resulting from the derivation of velocity by linear interpolation may have been offset to a degree. The absence of longitudinal data for costal cartilages precludes validation of the method used here. However, comparison of the length and length velocity obtained by longitudinal study of the bony portion of the 11th rib (Table 9 in Chapter 8) with that of the mean of the bony portion 4th to 7th ribs (Table 26 in this chapter) shows values of a similar order. This similarity suggests that the cross-sectional method used in this study is a valid one.

At this point it is worth noting why the 1st to 3rd and 8th to 12th ribs were not used for assay purposes. The small size of the cranial ribs and the extreme thinness of the caudal ribs presented many technical difficulties that made rapid preparation for incubation impossible. This delay and the additional trauma to which small cartilages were subject greatly increased the variability of uptake in early experiments and they were therefore excluded from the work reported here. In these early experiments where all 12 cartilages were included no evidence of a cranio-caudal gradient was found, in contrast to the observations of Herbai in the mouse (1970a).

The most important result of this work is the demonstration of a significant correlation between the responsiveness of cartilage and its velocity of growth in length (Figures 49 and 50 and Table 32). This significant correlation occurred irrespective of whether cartilage was incubated for 4 or 20 hours in medium only, in 20% standard serum or in 20% donor serum. The correlation coefficients were consistently higher for thymidine ($p < 0,001$ or $p < 0,01$) than for sulphate ($p < 0,05$). The correlation with weight velocity did not achieve significance under any conditions. These results may be interpreted to mean that the major determinant of growth in length is cell multiplication and that chondroitin sulphate synthesis is of lesser importance. The fact that neither DNA synthesis nor sulphation correlate with the growth in weight suggests that other metabolic processes are more important in this respect. Studies of the rate of collagen and chondromucoprotein synthesis are more likely to yield significant results since these constituents contribute the greatest bulk in cartilage. The thymidine-sulphate ratio does, however, provide one item of evidence suggesting that sulphation may contribute substantially to weight growth. This ratio falls progressively after birth (Figure 55) indicating that

mitotic activity slows down more rapidly than chondroitin sulphate synthesis. The start of this decline and the point at which growth in weight continues after growth in length has all but ceased (Figure 48) are similar (70 days PC). The origin of the SM stimulus does not appear to influence the outcome of the stimulus-tissue interaction since there is no difference in the correlation coefficients for standard and donor sera.

The slopes of the dose-response curves also provide evidence that the responsiveness of the cartilage varies as a function of age (and growth velocity) and not of stimulus. Over this wide range of serum concentrations consistent age-specific trends in both sulphate and thymidine uptakes were observed (Figures 51-53). As cartilage ages and velocity of growth declines the response to stimulation diminishes until at 90 days PC the slopes are very shallow indeed. This suggests that the number of growing cells is diminishing with age and this is corroborated, at least for thymidine, by the results obtained by autoradiography (Table 34), and for both sulphate and thymidine by the progressive rise with age in the costo-sternal ratio (Figure 54). This rise suggests a progressive localization of the growth zone towards the costal end of the cartilage. In adulthood when no growth is occurring the ratio returns to unity, a situation obtaining in the foetal stages when the whole cartilage is actively growing. This costal end growth zone is clearly similar to the one described in the rat (Evans et al. 1948) and analogous to the epiphyseal plate of long bones.

The failure to demonstrate a sexual dimorphism in cartilage responsiveness in vitro (Table 33) and the lack of any such dimorphism in serum SM (Chapter 9) suggest that either no dimorphism exists in the growth rate of the costal cartilages studied or that additional unidentified factors operate in vivo.

Rabbit foetal cartilage metabolism is undoubtedly SM-dependent

since 20% serum results in significant increases ($p < 0,05$) in both sulphate and thymidine uptake at all three foetal ages studied (Tables 30 and 31 and Figure 51). This observation contrasts with that of Heins et al. (1970) in the rat, but as shown in Chapter 11 their findings may not be generally applicable to the rat, or to any other species.

Finally, the fall in both sulphate and thymidine uptake after 20 hours incubation compared to that at 4 hours deserves brief comment. In the light of observations by other workers (Daughaday and Reeder 1966; Van den Brande 1973) this fall, especially for thymidine, is surprising and unexplained. The consistency of its occurrence at all ages suggests that a common factor is responsible. This factor may be suboptimal in vitro supply of nutrients or physical conditions and warrants further study.

10.4. Conclusions

Several important conclusions may be drawn from the work described in this chapter.

The GH-dependence of a serum factor(s) that stimulates rabbit cartilage sulphation and DNA synthesis has been demonstrated.

While cartilage from growing rabbits of all ages is stimulated by serum, the basal uptake and slope of the dose-response curve appear to be functions of the age and growth velocity of the cartilage, and not of the serum stimulus.

A sexual dimorphism has not been shown in cartilage responsiveness, nor in serum SM, and functional basis of the larger size of the female rabbit has not been explained.

CHAPTER 11

COSTAL CARTILAGE TRANSPLANTS IN THE RAT

This chapter describes the response of transplanted costal cartilage from rats of different ages in both adults and animals of the same age.

The technique was used to complement the *in vitro* and *in vivo* studies of Chapter 10. *In vitro* incubations were also performed on age-matched cartilages.

11.1. Methods and Materials

Albino Sprague-Dawley rats were supplied by the University Central Animal Unit, as described in Chapter 7. Cartilage donors were drawn from two or more litters with equal numbers of males and females in each donor group.

The cartilage donors were killed by rapid exsanguination under ether anaesthesia. The 4th to 7th bony and cartilaginous ribs of both sides and the intervening sternum were dissected out. The lengths of the bony and cartilaginous portions were measured by placing a monofilament cotton thread along the greatest curvature of the rib, cutting it to exact length and then laying the thread along a ruler graduated to 0,5mm. The cartilaginous portion was separated from the bony through the costochondral junction and stripped of adhering soft tissue and perichondrium. The sternum was split down the middle leaving the cluster of four cartilages joined together. Cartilage clusters were pooled and then allocated to one of four protocols:

1: One cluster of cartilages was transplanted into an adult

male rat. The recipient was lightly anaesthetized with ether. Through a mid-line interscapular incision the cluster was positioned in a cleft, opened by blunt dissection, in the muscles between the scapula and the thoracic wall. A control cluster, boiled for 10 minutes in normal saline, was positioned on the opposite side of the same recipient. The wound was closed in layers.

One hour later the recipient was weighed and given 0,5 μCi/g $Na_2{}^{35}SO_4$ and 1,0 μCi/g ^{3}HTdr by intra-peritoneal injection.

Twenty-four hours later the recipient was sacrificed. The transplanted cartilages and the recipient's 4th to 7th costal cartilages were removed, cleaned, washed and left overnight in a saturated solution of Na_2SO_4. Each costal cartilage, except in the case of foetal cartilages, was divided into sternal, middle and costal thirds, blotted dry and weighed to 0,01mg. The foetal specimens were divided into thirds without separation of the individual cartilages. The segments were dissolved in 0,5ml 23N HCOOH at 80°C, 10ml Instagel added and counted.

2: The same method was followed except that the recipients were males of the same age as the donors. Boiled controls were not used. Foetal and neonatal recipients could not be used.

3: The same method was followed except that prior to transplantation the clusters were pre-incubated for 24 hours in incubation medium (Chapter 9) at 37°C. Boiled controls were not used.

4: For comparison <u>in vitro</u> uptake was also assessed by the methods used in Chapter 10, as follows.

Cartilages were incubated with shaking at 37°C in solutions of medium and 20% and 40% dilutions of the standard human serum pool. After 20 hours the cartilages were transferred for 4 hours to medium only containing 2 μCi/ml each of $^{35}SO_4$ and ^{3}HTdr.

Throughout 2ml of solution per cartilage was provided. Incubation was then stopped and uptake measured as before.

Isotope uptake was measured in a group of costal cartilages of different lengths. To obtain an estimate of the mean length velocity of the cartilage growth the following procedure was adopted: the mean length of all cartilages measured at a given age was calculated. From these means mean velocities for the intervals were calculated. By linear interpolation between the mid-points of these intervals velocity at each of the ages studied was derived. Velocity at the first age studied, viz. 18 days PC, was obtained by linear extrapolation from the two adjacent velocities. The same method was used to obtain velocity estimates for cartilage weight.

11.2. Results

The weights and lengths and respective velocities of the 4th to 7th ribs are shown in Tables 35 and 36 and Figure 56.

TABLE 35. Mean weight and mean weight velocity of the cartilaginous sections of the 4th, 5th, 6th and 7th ribs in the rat. Velocity is derived by interpolation - see text

AGE (days PC)	n	WEIGHT (mg)	WEIGHT VELOCITY (mg/day)
18	96	1,31	0,83
20	80	2,90	0,83
22	96	4,63	0,83
26	96	7,75	0,78
30	96	11,29	0,75
37	96	16,03	0,62
47	96	21,33	0,52
70	80	32,37	0,38
105	80	40,77	0,12
300+	76	46,80	-

For both dimensions the velocity declines progressively from the peri-natal period, the decline being somewhat more rapid for length. (Figure 56). The ratio bone velocity : cartilage velocity is

very close to unity until the age of 70 days PC when it increases to 1,71 and it remains elevated at 105 days PC.

TABLE 36. Mean length and mean length velocity of the cartilaginous and bony sections of the 4th, 5th, 6th and 7th ribs in the rat. "Ratio" indicates the bone : cartilage ratio. Velocity is derived by interpolation - see text

AGE (days PC)	BODY WEIGHT (mean)	n	LENGTH (cm)			LENGTH VELOCITY (cm/day)		
			Bone	Cartilage	Ratio	Bone	Cartilage	Ratio
18	1,0	32	0,57	0,55	1,04	0,074	0,072	1,03
20	2,6	28	0,70	0,69	1,01	0,068	0,065	1,05
22	5,9	32	0,93	0,81	1,14	0,058	0,059	0,98
26	10,6	28	1,03	1,06	0,97	0,050	0,053	0,94
30	18,4	32	1,18	1,27	0,93	0,057	0,048	1,19
37	25,6	40	1,42	1,35	1,05	0,021	0,026	0,81
47	48,3	32	1,51	1,60	1,06	0,015	0,014	1,07
70	123,4	32	1,96	1,69	1,16	0,012	0,007	1,71
105	152,0	32	2,21	1,87	1,18	0,005	0,003	1,67
300+	473,1	28	2,47	2,03	1,22	-	-	-

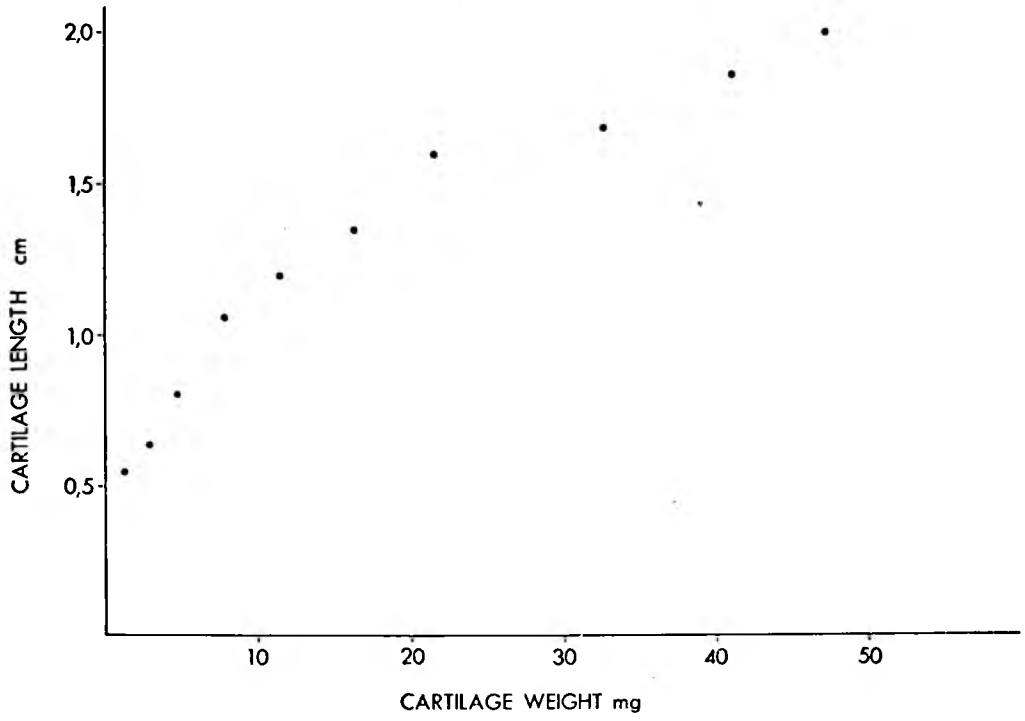


Figure 56. Rat costal cartilages (4th to 7th inclusive): mean length plotted against mean weight.

The uptake by the costal cartilages transplanted into adults declined progressively from 18 to 105 days PC (Table 37). The decline was similar for both isotopes. The decline occurred irrespective of whether the cartilage was transplanted immediately or pre-incubated for 24 hours before transplantation.

Pre-incubation caused a variable, but consistent, decrease in uptake at a given age. The uptake for ^{3}H-thymidine was 56-98% and for $^{35}SO_4$ 48-88% of the value obtained without pre-incubation. Costo-sternal differences were reduced by pre-incubation with the ratio for both isotopes falling after incubation at all ages except 105 days PC.

Although recipient uptake was not measured in all experiments it was fairly constant and in all 4 instances lower than the uptake of 105 day PC recipients (Figure 57) Boiled cartilage showed very low counts.

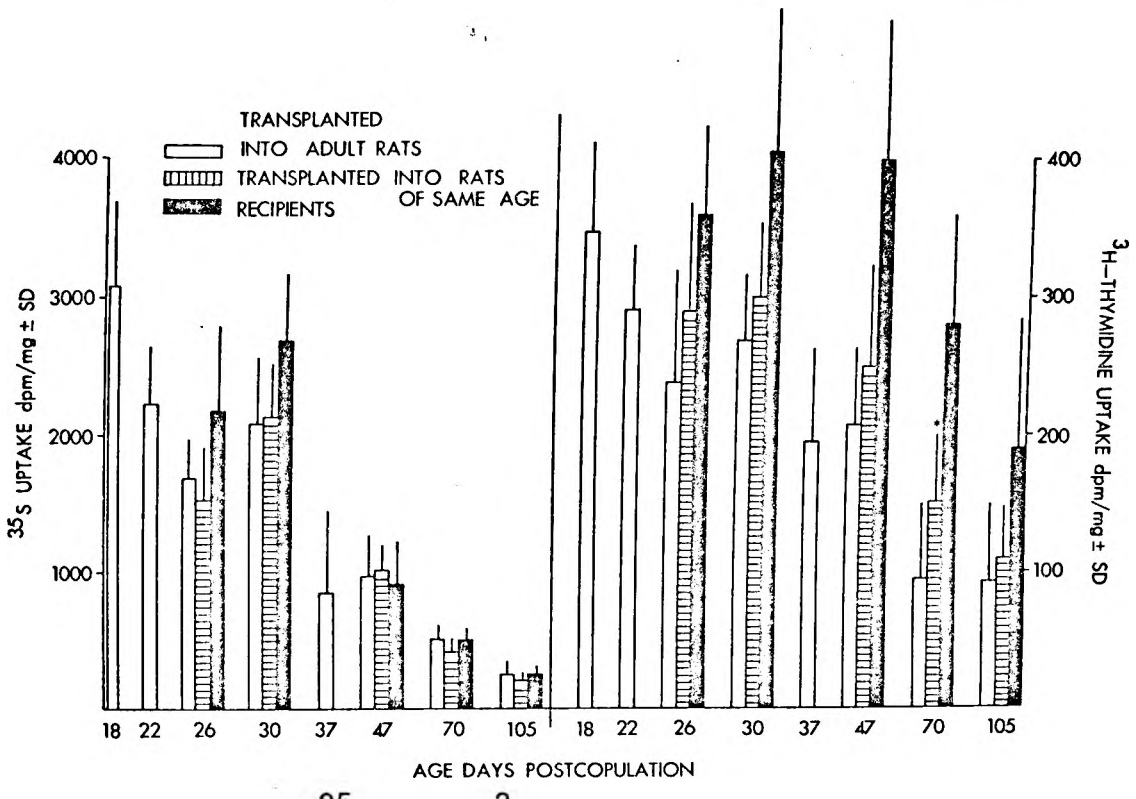


Figure 57: Uptake of $^{35}SO_4$ and ^{3}H-thymidine by costal cartilage transplanted into both adult rats and rats of the same age as the donor. The uptake by the costal cartilage of the recipients of the same age is also shown. The asterisk indicates that the difference between the cartilages transplanted into adults and rats of the same age as the donor is significant at the 5% level.

TABLE 37. Costal cartilage transplants into adult rats. Results are expressed as dpm/mg (± SD) for the whole cartilage. The ratio of costal zone to sternal zone uptake (C/S) is also shown.

AGE (days PC)	n	UPTAKE OF TRANSPLANTS INTO ADULTS immediate ^{3}H	immediate ^{35}S	pre-incubated ^{3}H	pre-incubated ^{35}S	n	UPTAKE OF BOILED CONTROLS ^{3}H	^{35}S	n	UPTAKE OF ADULT RECIPIENTS ^{3}H	^{35}S
18	16	347±68 (2,01)	3079±608 (1,11)	-	-		-	-	8	47±16 (0,89)	108±48 (1,09)
22	24	290±48 (2,14)	2231±471 (1,27)	202±69* (1,28)	1520±406* (1,08)		-	-	8	68±27 (0,89)	108±67 (1,01)
26	16	238±81 (2,66)	1693±287 (1,59)	-	-	8	28±17 (1,06)	27±13 (1,03)		-	-
30	24	268±49 (2,17)	2068±506 (1,67)	207±38* (1,23)	1817±604 (0,98)		-	-		-	-
37	22	195±68 (2,31)	853±204 (1,23)	110±78* (0,91)	548±157* (1,03)	8	23±16 (1,00)	27±26 (0,86)		73±31 (0,96)	98±78 (0,86)
47	24	208±58 (1,56)	983±301 (1,77)	165±77 (1,07)	743±160* (0,98)		-	-		-	-
70	16	95±58 (1,03)	524±102 (1,07)	84±63 (0,98)	302±76* (0,98)	8	28±26 (0,87)	13±21 (0,98)		-	-
105	24	93±47 (0,90)	250±98 (0,87)	91±38 (0,98)	120±69* (0,90)		-	-		40±11 (0,99)	26±13 (1,11)

*indicates that the pre-incubated transplant uptake is significantly lower than the immediate transplant uptake at the 5% or lower level.

Transplantation with donor and recipient being of the same age, without any pre-incubation, gave similar results to those obtained with adult recipients. Uptake of both isotopes increased from 26 to 30 days PC and then steadily fell until the low levels of adulthood were approached at 105 days PC (Figure 57). Uptake of $^{35}SO_4$ in the transplanted cartilage was lower than that in the recipients of the same age (70 - 81%), except at 47 days PC where it was slightly higher (111%). Transplanted uptake for ^{3}HTdr was lower at all ages (54 - 86%). There is a good correlation (r=0,9) between ^{3}HTdr and $^{35}SO_4$ uptake in the transplanted cartilages (Figure 58).

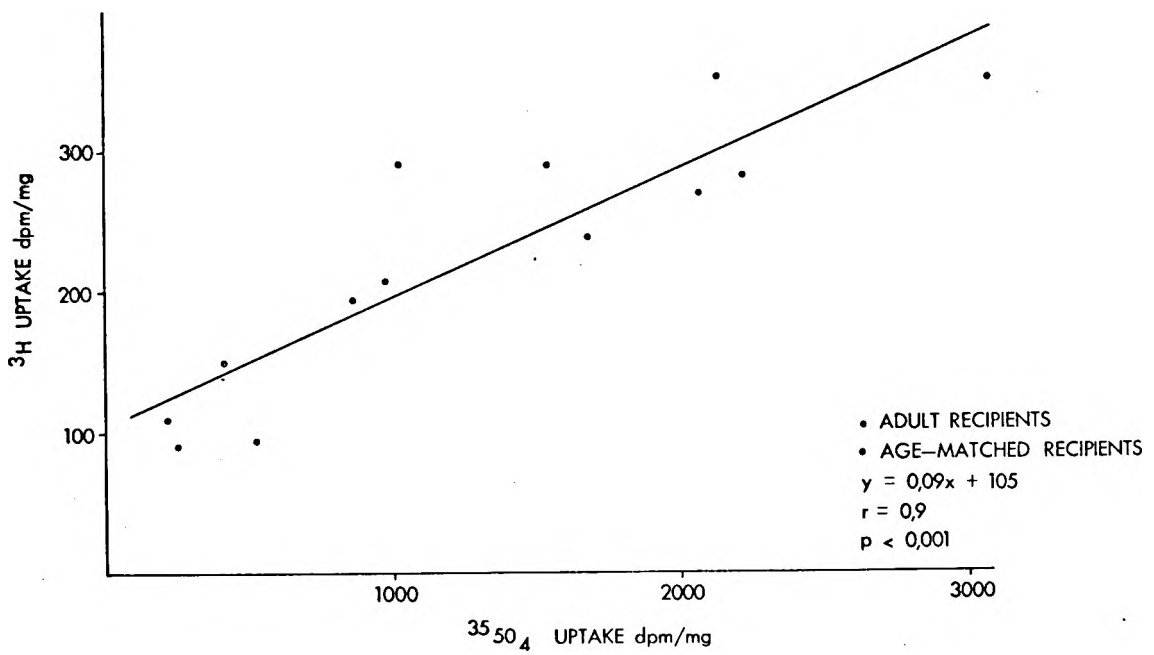


Figure 58. Correlation between the uptake of ^{3}HTdr and $^{35}SO_4$ by transplanted rat costal cartilage.

Thymidine uptake in the costal third of the cartilage of 18 to 37 day PC rats transplanted without pre-incubation was at least twice as great as that in the sternal third in both adult (Table 37) and same age recipients (not shown). From 47 days PC onwards the

costo-sternal ratio falls to approximate unity. Except at 26 and 30 days PC the ratio for $^{35}SO_4$ was close to unity.

Pre-incubation lowered the costo-sternal ratio considerably and in all cases it approached unity (Table 37).

In vitro incubation yielded a qualitatively similar picture to that for transplanted cartilages. It should be noted that the ages of the rats studied in this section differ in some respects to those in the transplantation section. Rats aged 20 and 304 days PC were included and no 30 day olds were studied. The uptakes and slopes of the dose-response curves are shown (Table 38).

There are several notable features of the in vitro incubations. The uptake of both isotopes was maximal at 20 days PC, and then fell progressively to very low levels in adulthood. The cartilage of the younger of the two foetal ages, 18 days PC, was relatively refractory to stimulation by SM; a significant increase ($p < 0,05$) in uptake of both ^{3}HTdr and $^{35}SO_4$ only occurring with 40% serum,whereas at 20 days PC 20% and 40% caused significant stimulation. Furthermore, the slope of the dose-response curve at 18 days PC was more shallow than that at 20 days PC. Significant dose-related stimulation occurred for ^{3}HTdr up to 70 days PC and for $^{35}SO_4$ up to 105 days PC. At ages greater than these the cartilage could not be stimulated.

In addition to basal uptake being greatest at 20 days PC the slope of the dose-response curve for $^{35}SO_4$ was also greatest at this age. Thereafter it declined progressively becoming zero at 304 days PC. The slope for ^{3}HTdr was greatest at 22 days PC and declined more rapidly than that for $^{35}SO_4$ approaching zero at 70 days PC.

The costo-sternal ratio was not calculated in the foetal rats. For $^{35}SO_4$ the ratio remained close to unity with a slight, but not consistent, trend to be higher up to 37 days PC. Serum stimulation

TABLE 38. Uptake of ^{3}H-thymidine and $^{35}SO_4$ by rat costal cartilage in vitro. Results are expressed as uptake/mg** of wet cartilage ± SD. The ratio of costal to sternal zone uptake is shown in brackets. The slope of the linear regression of the dose-response curve (m) is also shown.

AGE days PC	n	^{3}H ($g \times 10^{-12}$/mg) MEDIUM	20% SERUM	40% SERUM	m
18	16	20,2 ± 4,8 -	26,6 ± 5,8 -	42,4 ± 9,9* -	0,56
20	16	48,3 ± 2,2 -	56,3 ± 3,4* -	78,1 ± 4,8* -	0,75
22	16	27,9 ± 5,4 (1,49)	52,4 ± 8,9* (1,97)	71,5 ± 8,4* (2,31)	1,09
26	16	18,2 ± 4,0 (2,43)	27,4 ± 5,0* (2,01)	41,9 ± 8,1* (2,68)	0,59
37	12	5,7 ± 1,1 (3,67)	8,6 ± 2,3* (3,96)	11,0 ± 2,9* (4,70)	0,13
47	16	2,1 ± 0,7 (3,16)	3,7 ± 1,0* (3,01)	4,2 ± 0,7* (3,68)	0,05
70	16	0,2 ± 0,08 (2,03)	0,4 ± 0,2* (2,17)	0,3 ± 0,2* (2,86)	0,004
105	16	0,2 ± 0,1 (1,04)	0,2 ± 0,1 (0,99)	0,2 ± 0,1 (0,89)	-
304	16	0,2 ± 0,04 (1,06)	0,2 ± 0,1 (0,96)	0,1 ± 0,05 (1,06)	-

* indicates that the response is greater than that in medium at the 5% level of significance

continued overleaf

TABLE 38. continued

Uptake of $^{35}SO_4$ by rat costal cartilage in vitro.

AGE days PC	n	^{35}S (g x 10^{-4}/mg) MEDIUM	20% SERUM	40% SERUM	m
18	16	3,4 ± 0,8	3,8 ± 0,5	4,3 ± 0,7*	0,02
20	16	5,4 ± 0,3	10,1 ± 0,5*	13,7 ± 0,7*	0,21
22	16	4,0 ± 0,5 (1,60)	6,9 ± 1,1* (0,96)	9,0 ± 1,7* (1,12)	0,13
26	16	4,0 ± 1,1 (1,11)	4,7 ± 0,8* (1,30)	6,8 ± 1,0* (1,09)	0,07
37	12	1,6 ± 0,8 (1,31)	3,5 ± 1,1* (1,49)	5,7 ± 2,0* (1,68)	0,10
47	16	2,1 ± 0,4 (1,23)	2,5 ± 0,5* (0,98)	5,1 ± 0,8* (1,00)	0,09
70	16	3,7 ± 0,8 (1,09)	3,8 ± 0,8 (0,89)	4,9 ± 1,1* (1,13)	0,03
105	16	2,6 ± 0,7 (0,96)	3,3 ± 1,1* (1,01)	3,5 ± 1,2* (0,71)	0,02
304	16	0,7 ± 0,8 (0,86)	0,8 ± 0,2 (0,94)	0,7 ± 0,1 (0,98)	

** uptake is expressed as µg/100mg to facilitate comparison with Heins et al (1970)

did not increase the ratio for $^{35}SO_4$. In contrast the ratio for ^{3}HTdr rose to approximately four at 37 days PC and then fell to unity by 105 days PC. Under conditions of stimulated thymidine uptake, i.e. with 20% and 40% serum compared to medium, the ratio rose at all ages between 22 and 70 days PC.

The relationships between the cartilage responsiveness, both in vivo and in vitro, and the velocity of growth, both in length and weight, is shown as correlation coeffients in Table 39.

TABLE 39. Correlation coefficients showing the relationship between various measures of rat costal cartilage responsiveness and the velocity of growth of the cartilage.

	Length velocity	Weight velocity
Immediate transplant uptake - into adults		
^{3}H	0,94	0,92
^{35}S	0,94	0,87
Immediate transplant uptake - ages matched		
^{3}H	0,92	0,81
^{35}S	0,92	0,89
Age-matched recipient uptake		
^{3}H	0,82	0,79
^{35}S	0,97	0,88
In vitro incubation dose-response curve slope		
^{3}H	0,84	0,87
^{35}S	0,43*	0,40*

* not significant. All other values are significant at the 1% level.

11.3 Discussion

The technique of cartilage transplantation has been used before, but only in studies on the origin of osteogenic cells (Holtrop 1966, for example). Crelin (1969) has shown that transplanted mouse pelves take on morphological characteristics that are host sex-specific. That is to say, transplants from young female mice into adult males attain male shape, a qualitative feature. To my knowledge there has been no investigation of the quantitative aspects of the response of transplanted cartilage.

The results suggest that for the duration of the transplant the cartilage is metabolically active. The lower uptake by the transplants compared to the age-matched recipients (Figure 57) may be caused by several factors. These include the trauma of dissection, the immediate tissue reaction of the host site and a less effective blood supply. Uptake by the transplanted cartilage was undoubtedly an active process as the boiled cartilage showed very low activity. Time course studies were not done in this study, but Holtrop's (1966) work suggests that osteogenesis does not begin for several days after transplantation. Although her paper gives few details it seems reasonable to assume that the cartilage in this study did not undergo significant osteogenic differentiation in the 24 hour period of transplantation.

Transplant and recipient uptakes are expressed in terms of dpm/mg cartilage wet weight. Absolute uptake could not be expressed as the endogenous pool sizes of thymidine and inorganic sulphate were unknown. The inter-animal variability appears to be reasonably small as judged by the coefficients of variation for uptake. For thymidine the coefficient ranges between 16 and 28% up to 47 days PC and for sulphate between 17 and 31% up to 70 days PC (from Table 37). At the older ages the coefficients are greater but because of the low uptakes this is not considered to detract from the results. Other authors who also made no

correction for variations in pool size in young rats of equivalent ages found similar coefficients of variation (Collins & Baker 1960; Chesley 1963). Where correction has been made for variations in pool size by measurement of the inorganic sulphate pool (Herbai 1970b, 1970c) or by the addition of carrier sulphate to the radioactive material (Tessler & Salmon 1975) the coefficients of variation are no smaller than those in this study or in the others (Collins & Baker 1960; Chesley 1963), in which no attempt was made to compensate for pool size variability.

The calculation of the velocity of growth in length and weight of the cartilage in these rats is beset by the same problems and limitations as those encountered in the rabbits (Chapter 10). The consistency of the trends within the results suggests that the method is valid. The velocities of growth in length of the bony and cartilaginous portions of the rib are very similar during the active growth phase. As growth slows, from 70 days PC onwards, the bone velocity exceeds the cartilage velocity (Table 35). These observations are in close agreement with the general decline in growth velocity in the rat that occurs about 70 days PC (Hughes & Tanner 1970a). Because the velocities are very low at 70 and 105 days PC the interpretation of the bone: cartilage ratios must be made with caution. The fact that the ratio at these ages is considerably greater than at earlier stages suggests that during this period the rate of ossification exceeds that of cartilage proliferation in the growth zone at the costo-chondral junction (Evans et al. 1948). This type of differential growth rate is the mechanism by which long bone epiphyses are closed and the same process occurs in the rib (Bloom & Fawcett 1962).

All foetal costal cartilages in this study had clearly defined ossified segments. This finding is in agreement with the observations of others (Strong 1925; Dawson & Spark 1928) who have shown that ossification begins at 17 days PC in the 6th to 9th

ribs and is present in all ribs by 18,5 days PC.

Plotting cartilage length against weight reveals a sinusoidal relationship (Figure 56). While little length is gained after 70 days PC (see Table 35) weight continues to increase. This gain in weight must be attributed to subperiosteal appositional growth and to accretional growth (see Chapter 2.2) by subperiosteal matrix deposition and interstitial calcification (Strong 1925). The persistence of sulphation stimulation up to 105 days PC when the thymidine response ceases at 70 days PC (Table 38) suggests that matrix deposition contributes more to the gain in weight after 70 days PC than does appositional growth. This interpretation is strengthened by the fact that pre-incubation reduces sulphate uptake, but not thymidine uptake after 47 days PC (Table 37). Pre-incubation for 24 hours reduces cartilage metabolic activity to basal levels by exhausting any residual stimulus (Van den Brande 1973).

The in vitro response of the foetal cartilage to stimulation (Table 38) contrasts with the apparent refractoriness of the cartilage studied by Heins et al. (1970). Both 18 and 20 day PC cartilage responded to serum, but the younger cartilage did require 40% serum to obtain a significant response. The experimental conditions of the two studies were not identical, most notably in the concentrations of serum used. Heins et al. used only 10% serum whereas 20% and 40% were used in this study. In respect of other ages of rats investigated the results of the two studies are in agreement except that in the present study adult cartilage did not respond to stimulation.

The cartilage zone adjacent to the costochondral junction is the site of the growth centre of the rib, analagous to the epiphyseal plate of long bones (Bloom & Fawcett 1962). During the rapid phase of length growth (up to 47 days PC) the costo-sternal ratio for both ^{3}H and $^{35}SO_4$ is greater than unity (Table 37),

indicating that the regional localization of the isotopes is functionally significant. There are, however, notable differences between the *in vivo* and *in vitro* findings for each isotope and between ^{3}H and $^{35}SO_4$ at each age (cf. Table 38).

Because the thymidine and inorganic sulphate pool sizes are not known, including whether there is a change with age, quantitative comparisons of differences between the isotopes in the cartilage zones cannot be made. Nevertheless, conclusions may be validly drawn from changes in costo-sternal ratios for each isotope.

In vitro there is a trend for the ^{3}HTdr ratio to increase with age to a maximum at 37 days PC and then to decrease. *In vivo* there is no such increase, the ratio remains near 2,0 until 37 days PC and then decreases (Tables 37 and 38). Why the *in vitro* ratio is greater than the *in vivo* at the same age is not clear, but there are several speculative possibilities. *In vitro* availability of ^{3}HTdr to the costal zone may have been enhanced by the dissection and stripping of the perichondrium; the adjacent bone and its associated blood vessels *in vivo* may have had an influence on the cartilage; or the *in vitro* stimulus may have been greater than that *in vivo*, and the greater proportional response of the costal zone to a given stimulus compared to the sternal zone (see below) may be an explanation for the difference.

The sulphate ratios are similar in both situations, being just in excess of unity with a tendency to decrease with age. Herbai (1970a,c) showed a greater ratio for sulphate, namely 2,55, than those observed here. The reasons for this difference are not understood.

In vitro serum stimulation has a marked effect on the thymidine ratio in cartilage up to 70 days PC. Stimulation causes a proportionately greater uptake in the costal zone compared to the sternal zone, resulting in a rise in the ratio

with stimulation (Table 38). This does not occur with sulphate, suggesting that all cartilage cells are equally sensitive to sulphation stimulation, whereas for thymidine stimulation those in the growth zone are selectively sensitive.

The slope of a dose-response curve is a valid index of the responsiveness of a biological system (Finney 1964). The use of the slope has a major advantage over use of absolute uptake as an indicator of response as it is independent of variations in basal uptake. In this respect its use is similar to the use of the percentage stimulation (Heins et al. 1970), but where more than one dose is used the slope is probably the more useful parameter.

For thymidine the slope increases with age to a maximum at 22 days PC and then declines rapidly to zero at 105 days PC (Table 38). For sulphate there is a marked change between 18 and 20 days PC with the slope increasing 10-fold to 0,21. Thereafter there is a plateau fluctuating around 0,1, between 22 and 47 days PC, followed by a decline to zero at 305 days PC.

The post-natal sulphate results are similar to those obtained by Heins et al. (1970). The foetal differences have already been discussed.

The correlation between the measures of cartilage response, both in vivo and in vitro, with the exception of the in vitro sulphate slope, and both length and weight velocity suggests that the cartilage response and not the serum SM stimulus is the major determinant of growth velocity (Table 39). Why sulphate slope has such a poor correlation with length and weight velocity is not clear. The correlation between the absolute sulphate uptakes and velocity is equally poor. It may be speculated that sulphation is not as an appropriate index of cartilage growth rate as thymidine uptake, and therefore correlations are lower. However, the excellent correlations obtained for sulphate in vivo mitigate against this argument. This contradiction may be

resolved by investigating other indices of matrix formation such as ^{14}C-leucine incorporation.

11.4 Conclusions

These results indicate that the response of the costal cartilage depends on the age, and therefore growth rate, of the donor, rather than on the stimulus to which the cartilage is exposed.

In these experiments the SM stimulus was manipulated in three separate ways: by keeping the stimulus constant *in vivo* (transplants in adults), by matching it to the age of the transplanted cartilage *in vivo* (age-matched transplants) and by keeping the stimulus constant *in vitro* (incubation in human serum). In all three of these situations the uptake of thymidine and sulphate was closely related to the growth velocity of the cartilage, with the exception of the slopes of the *in vitro* sulphate uptake. In other words, increasing the stimulus to slowly growing cartilage, or decreasing the stimulus to rapidly growing cartilage, does not alter the uptake of the isotopic markers of metabolic activity in the cartilage, indicating that cartilage responsiveness is the major determinant of growth velocity.

CHAPTER 12

CHARACTERISTICS OF THE RESPONSE OF ISOLATED COSTAL CHONDROCYTES TO SERUM SOMATOMEDIN ACTIVITY

The stimulation of thymidine incorporation into DNA is one of the established actions of serum SM activity. The original observations were made in rat costal cartilage segments (Daughaday & Reeder 1966). Since then stimulation of DNA synthesis has also been reported in porcine costal cartilage (Van den Brande 1973), Hela cells (Salmon & Hosse 1971), human glia-like cells (Uthne 1973), isolated chick pelvis chondrocytes (Garland et al. 1972) and isolated rabbit articular chondrocytes (Binet et al. 1975).

The isolated chondrocyte studies are of particular interest for two reasons. Firstly, the preparation of a suspension of isolated cells provides a greater homogeneity of cells in the aliquots than occurs in segments of whole cartilage thus reducing the variability of the responses. This, coupled with the greater number of samples which may be assayed, offers potential advantages over the conventional bioassay. Secondly, removal of the cartilage matrix permits the investigation of a possible role for the matrix in determining chondrocyte function and response to SM.

This chapter describes the thymidine incorporation response to SM by chondrocytes isolated from rabbit costal cartilage.

12.1. Methods and Materials

New Zealand White rabbits from the colony were used (Chapter 7).

All animals were growing at normal body weight velocity. The rabbits were sacrificed under ether anaesthesia by cardiac puncture exsanguination.

Chondrocyte isolation. A modified version of the chondrocyte isolation technique of Smith (1965) was used. The 4th to 7th costal cartilages and adjacent sternum were removed in a block and soaked in Geys' solution (1936) at room temperature. Each costal cartilage was then dissected free, stripped of all adherent perichondrium and divided into approximately equal sternal, middle and costal third segments. Segments were blotted dry, weighed to 0,0001g (Mettler H20 balance) and those from different animals pooled by sternal, middle and costal origin in fresh Geys' solution at room temperature. Between four and seven rabbits from the same litter were used in each experiment.

Portions of approximately 150mg of cartilage were placed in 4ml Geys' solution in 25ml stoppered Erlenmeyer flasks through which air with 5% CO_2 was bubbled for several minutes to give a pH of 7,2. To each flask was added 1ml crude papain solution (BDH, Johannesburg) to give a final concentration of papain of 4mg/ml. Finally a 1ml solution of 24mg cysteine (Merck, Darmstadt) was added. Flasks were incubated in a shaking water bath at 30 strokes/min at 37°C for 2 hours. The softened cartilage segments were washed three times in 10ml Geys' solution, blotted dry and transferred to clean flasks containing 4ml collagenase (from C. histolyticum Type 1, Sigma, St. Louis) at a concentration of 2mg/ml. Incubation was continued for 16 hours. To effect separation of the clumps of cells 1ml trypsin (from bovine pancreas, type III, Sigma, St. Louis) solution was added to give a final concentration of 0,5mg/ml for the last hour of incubation. The enzymes and cysteine were dissolved in Geys' solution at pH 7,2.

The resulting suspension of cells was centrifuged at 1000g

for 10 min, the supernatant aspirated and the pellet resuspended in the medium of amino acids, buffer glucose and antibiotics described previously (Chapter 9). The debris was removed by centrifugation at 60g for 10 minutes and the cells resuspended in 3ml of medium.

Cell numbers were counted at 400x magnification in a haemocytometer. The percentage of viable cells was determined by supravital staining with Trypan blue in a final concentration of 0,2%. The mean viability,measured in 10 samples, varied between 88 and 97% of the total cell count in each experiment.

Isolated cell assay. The technique described by Garland and co-workers (1972) was used and the method is outlined here.

The laboratory reference standard of a pool of adult human serum was used as the SM source throughout and diluted to the appropriate concentrations in the incubation medium described.

To dilutions of serum in 120 x 15mm glass centrifuge tubes were added 400 μl of cell suspension (containing approximately 5×10^5 cells) and 25 μl ^{3}HTdr (Amersham, specific activity 17 Ci/mmole) to give a final concentration of 0,15 μCi/ml in 2,5ml. Immediately prior to addition to each tube the cells were suspended by swirling briefly on a vortex mixer. Cell cultures were performed in triplicate for each serum dilution. Incubation was continued for 24 hours at 37°C in the shaking water-bath.

The tubes were then centrifuged at 1000g for 10 min, the supernatants discarded and the pellets washed twice in isotonic sodium sulphate. The pellet was resuspended in a 0,5ml solution of 200 μg of soluble RNA (from Torula yeast grade VI, Sigma, St. Louis) as a macromolecular carrier (Garland 1975, personal communication). Three ml of 5% trichloroacetic acid (TCA, Riedel De Haën Ag Seelze, Hanover) at 4°C was added and the mixture left

overnight at 4°C. Each tube was then shaken on a vortex mixer and the contents filtered under suction through 25mm cellulose 0,22 µm pore size discs (Millipore Corp, USA). Each disc was rinsed three times with 3ml 5% TCA and three times with 5ml 95% ethanol.

The discs were placed in glass scintillation vials, air dried, dissolved in 0,5ml Soluene-350 (Packard, Johannesburg) and bleached by addition of 0,5ml 30% hydrogen peroxide (Merck, Darmstadt). Finally 0,5ml 0,5N hydrochloric acid and 10ml Instagel (Packard, Johannesburg) were added and the vials counted.

To establish the degree of non-specific uptake potassium cyanide was added to cell cultures at each serum dilution to give a 0,01M solution.

Rib segment assay. To measure the uptake of thymidine by intact segments of rib cartilage the cartilages were stripped of perichondrium as already described and randomly allocated in groups of 8 to 25ml Erlenmeyer flasks containing medium only and dilutions of serum from 2,5% to 40%. Flasks were incubated for 20 hours at 37°C in a shaking water bath. The cartilages were then transferred to flasks containing 2 µCi/ml ^{3}HTdr in incubation medium for a further 4 hours. In all experiments ribs were immersed in solutions providing 1ml/rib. The flasks were then placed in boiling water for 15 minutes, the cartilages removed and washed five times in isotonic sodium sulphate and left overnight in this solution. Cartilages were blotted dry, divided into thirds and weighed to 0,0001g. Each third was dissolved in 0,5ml 23N formic acid at 80°C in a glass scintillation vial. The solution was cooled, 10ml Instagel added and counted.

Removal of inhibitors. Heat-treated and acidified serum from the reference pool was prepared. The serum pH was adjusted to 5,5 with 0,1N hydrochloric acid and the solution heated at 80°C for 15

minutes in a water bath. The extract was lyophilized and reconstituted to the original volume with deionized doubledistilled water. The SM activity of the extract was assessed in the porcine cartilage assay and found to have retained 64% of its activity (potency ratio 0,64, index of precision 0,21), when the untreated serum from the same pool was used as the reference standard.

Calculation of thymidine uptake. Serum contains free thymidine in solution at a reported concentration of slightly greater than 10^{-6}M (Hughes et al. 1973). Thymidine uptake in these experiments was calculated assuming a serum concentration of 10^{-6}M. Precipitation of the serum proteins does not alter the free thymidine concentration (Hughes et al. 1973).

12.2 Results

Thymidine uptake, expressed as moles/10^5 viable cells, varied considerably from one experiment to another when chondrocytes were prepared from rabbits of the same age and growth rate. Figure 59 shows a typical example of the nature of the response of the isolated cells of 60 day PC rabbits to increasing concentrations of normal human serum. Costal uptake was significantly greater than sternal uptake ($p < 0,05$) at 1,25, 2,5 and 5,0% serum concentrations. The maximum costo-sternal difference occurred at 2,5% with the costal response being 1,3-fold greater than the sternal.

In this and all other experiments the middle zone uptake was not significantly different from the sternal zone uptake. Therefore, in reporting the rest of the observations only costal and sternal zone responses are described.

In spite of the considerable range of uptakes observed in four separate experiments on 60 day PC rabbits the pattern of the dose-response relationship did not vary. Grouped results from these four experiments are expressed as the percentage stimulation

or inhibition that serum concentrations induce with respect to incubation medium only (Figure 60). A progressive increase in thymidine uptake occurs from incubation medium only up to 2,5% serum, after which the curve declines, falling below zero between 10% and 40%.

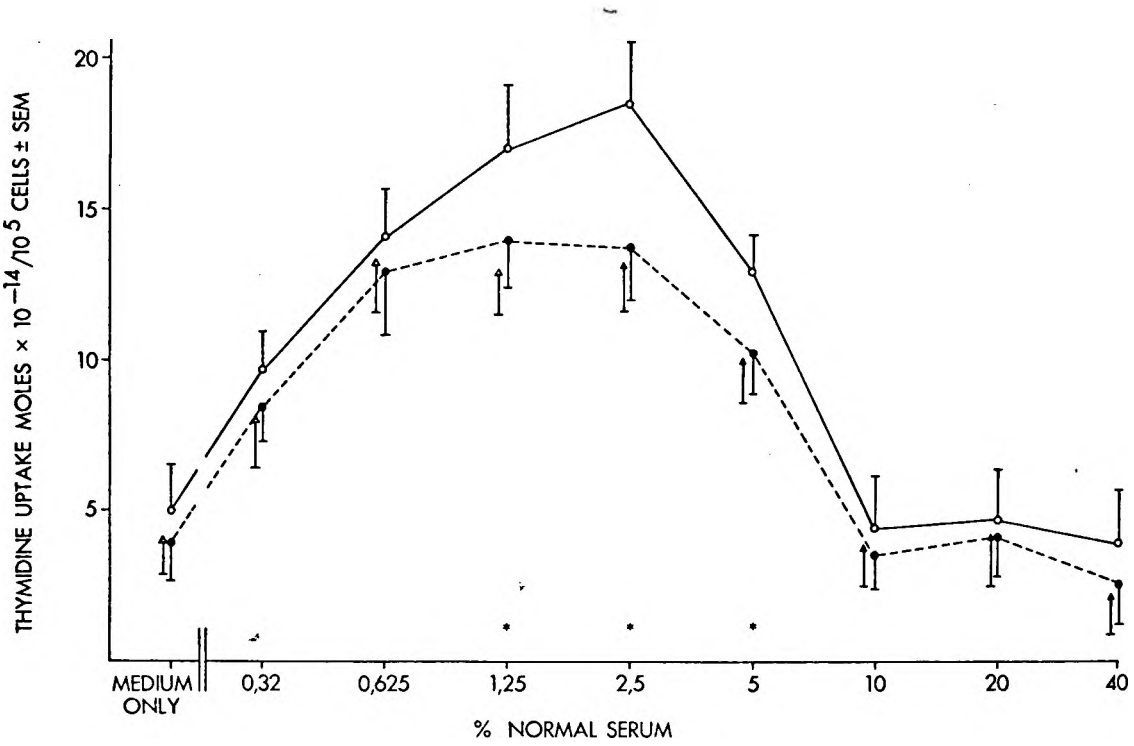


Figure 59. A typical example of the response of isolated rib cartilage chondrocytes to normal human serum for sternal, middle and costal zones of the rib cartilage. The asterisk* indicates that the costo-sternal difference is significant ($p < 0,05$). O: costal zone, ● : sternal zone, △ : middle zone. The serum concentration in this and all subsequent figures in this chapter is shown on a logarithmic scale.

Comparison of 60 day and 35 day PC responses shows that the range of the former is lower than and does not overlap to any greater extent with that of the latter (Table 40). The pattern of a rise to peak stimulation with 2,5% serum and then a decline to inhibition of uptake at concentrations greater than 10% is also found in 35 day PC rabbits.

TABLE 40. The range of thymidine uptake by isolated costal cartilage chondrocytes from 35 and 60 day PC rabbits in response to increasing concentrations of normal human serum. The effect of 0,01M potassium cyanide on uptake is also shown. The number of experiments is indicated by n.

Serum Concentration %	Thymidine Uptake moles x 10^{-14} / 10^{5} cells				
	35 days PC (n = 2)		60 days PC (n= 4)		with 0,01M KCN (n = 2)
	RANGE		RANGE		MEAN (± SEM)
	Costal zone	Sternal zone	Costal zone	Sternal zone	Costal zone
Medium only	9,3 - 14,1	6,0 - 9,9	3,3 - 9,7	2,8 - 8,8	1,1 (0,3)
0,32	8,1 - 11,4	6,1 - 10,3	4,1 - 11,6	3,7 - 6,9	0,8 (0,4)
0,625	13,7 - 17,9	11,4 - 15,8	7,4 - 13,3	8,3 - 10,7	0,9 (0,3)
1,25	21,4 - 27,0	15,1 - 19,3	11,6 - 20,1	10,6 - 14,0	1,2 (0,5)
2,5	26,1 - 29,5	16,3 - 20,0	14,3 - 25,4	11,2 - 16,3	1,0 (0,3)
5,0	19,3 - 24,6	16,0 - 21,1	8,3 - 17,9	9,9 - 15,6	1,4 (0,4)
10,0	11,4 - 14,9	4,8 - 10,1	3,6 - 7,7	2,1 - 7,9	0,7 (0,6)
20,0	5,3 - 9,8	3,2 - 7,6	2,8 - 7,9	3,6 - 7,1	1,3 (0,5)
40,0	6,7 - 9,7	3,1 - 7,9	3,1 - 6,3	2,9 - 6,3	1,4 (0,6)

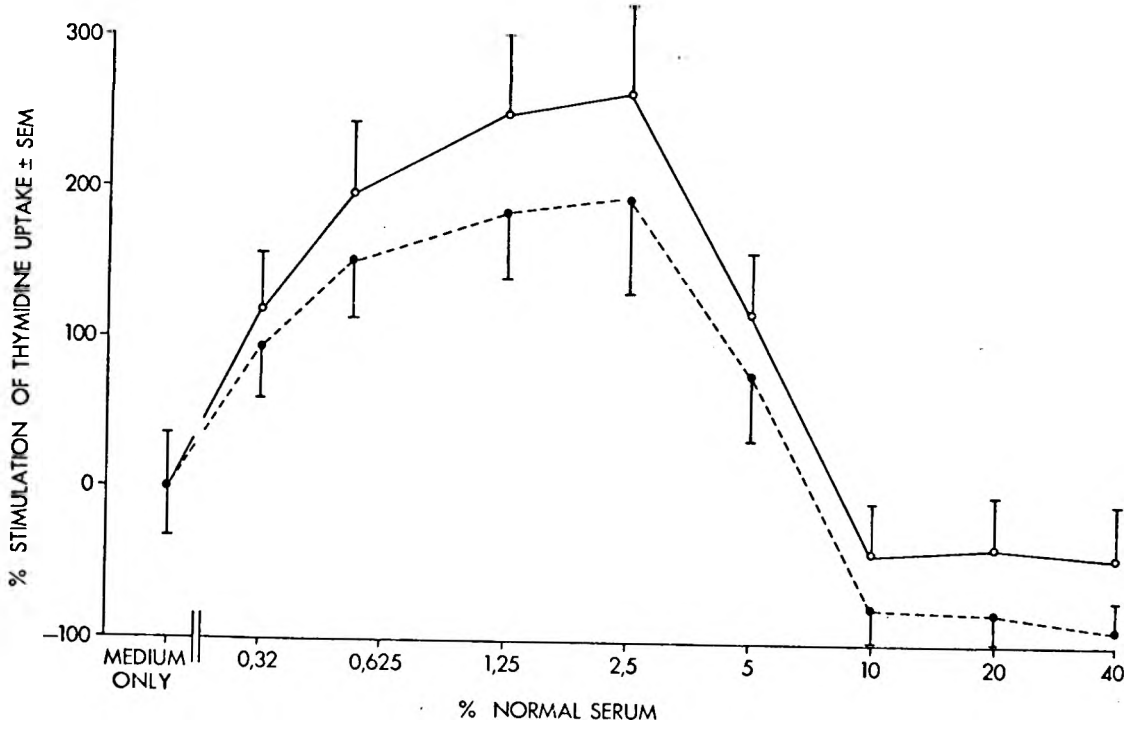


Figure 60 The response of isolated rib cartilage chondrocytes to normal human serum expressed as mean percentage stimulation, or inhibition, with respect to incubation medium only, for four separate experiments. O : costal zone, ● : sternal zone.

Potassium cyanide, 0,01M, causes a marked reduction in uptake (Table 40). The effect was studied in the costal zone of 60 day PC rabbits in two experiments. There was no stimulation or inhibition of uptake with increasing serum concentrations; the binding being constant around $1,0 \times 10^{-14}$ moles/10^5 cells.

The heated and acidified serum extract causes only progressive stimulation with increasing concentration. Figure 61 shows the response to the serum extract of chondrocytes from the same harvest as those shown in Figure 59. There is a rise in uptake between 1,25 and 80% concentrations, with the response becoming significantly greater than that induced by medium only at concentrations of 10% and greater ($p < 0,05$). It should be noted

that the absolute uptake at the point of maximum stimulation by normal serum, viz. 2,5%, was 18,5 x 10^{-14} moles/10^5 cells, compared with 13,5 x 10^{-14} moles/10^5 cells for heated-acidified serum at 2,5%. This is consistent with the reduced potency of the extract in the porcine cartilage assay of 0,64. Costal zone uptake is greater than sternal zone uptake throughout the concentration range, but not significantly so ($p > 0,05$).

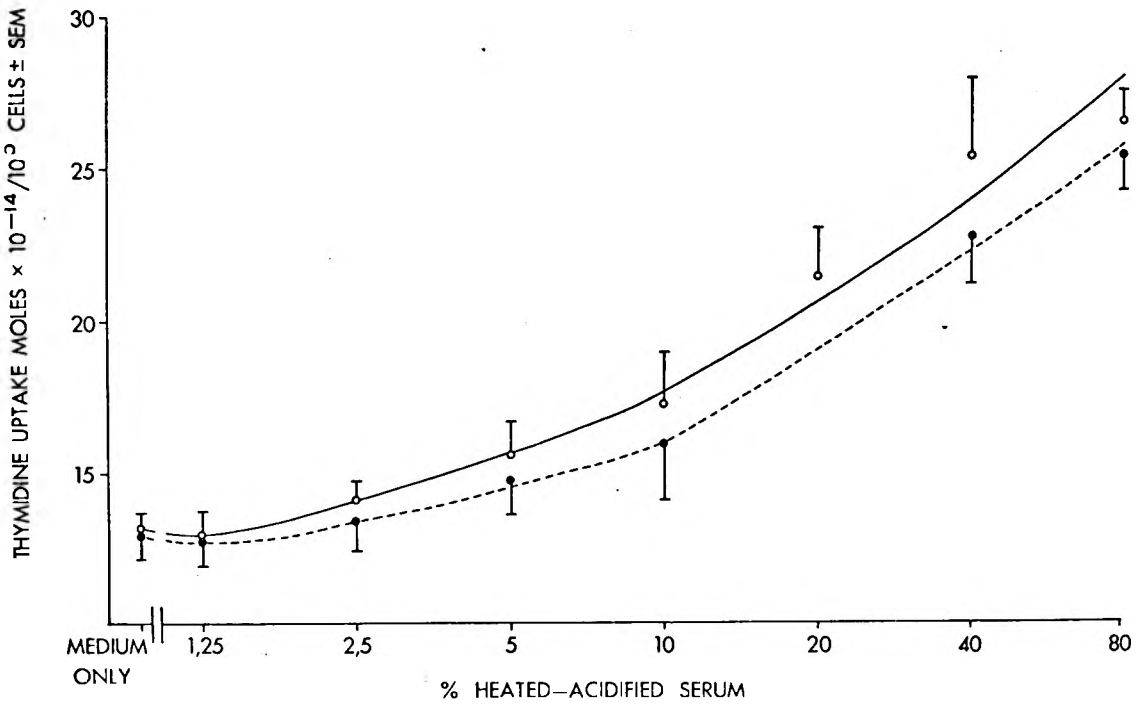


Figure 61. A typical example of the response of isolated rib cartilage chondrocytes to heated-acidified human serum (see text) for sternal and costal zones of the rib cartilage. The cells used in this experiment are from the same harvest as those shown in Figure 59. O : costal zone, ● : sternal zone.

In these experiments on 60 day PC rabbits absolute uptake also varied as greatly from one cell harvest to another as it did with normal serum. Grouped results are therefore expressed for the three complete experiments, and shown in Figure 62 as percentage stimulation with respect to incubation medium. These three experiments were on cell harvests used for three of the four experiments with normal serum.

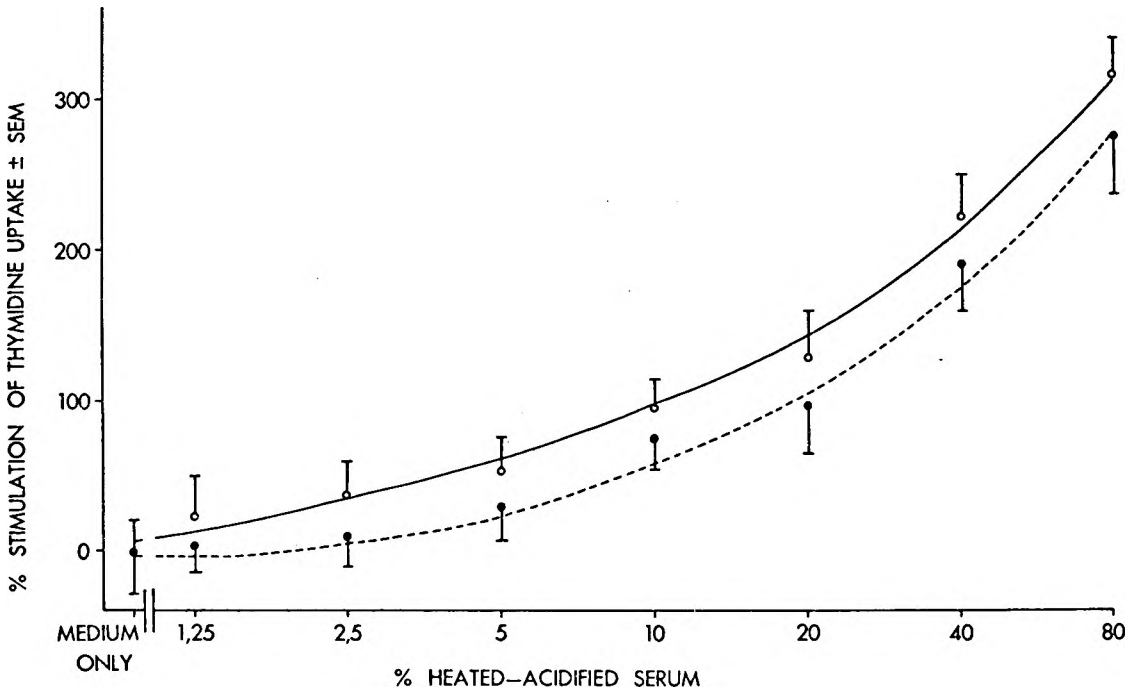


Figure 62. The response of isolated rib cartilage chondrocytes to heated-acidified human serum expressed as mean percentage stimulation, with respect to incubation medium only, for three separate experiments. O : costal zone, ● : sternal zone.

The intact cartilage segments from 57 day PC rabbits do not differ in their responses to normal and heated-acidified sera, except that the latter produces a quantitatively smaller response (Figure 63). The costo-sternal difference in uptake is much greater in the intact cartilage than in the isolated cells, being 3 to 4-fold over the concentration range studied, compared to the 1,3-fold maximum difference for the isolated cells.

Chondrocyte densities were higher in the costal zone than in the sternal, but not significantly so for quadruplicate counts on cell suspensions in each of the zones within the same harvest ($p > 0,1$). Cell harvests ranged from $2,05 \times 10^4$ to $6,03 \times 10^4$ cells/mg in the isolations reported here.

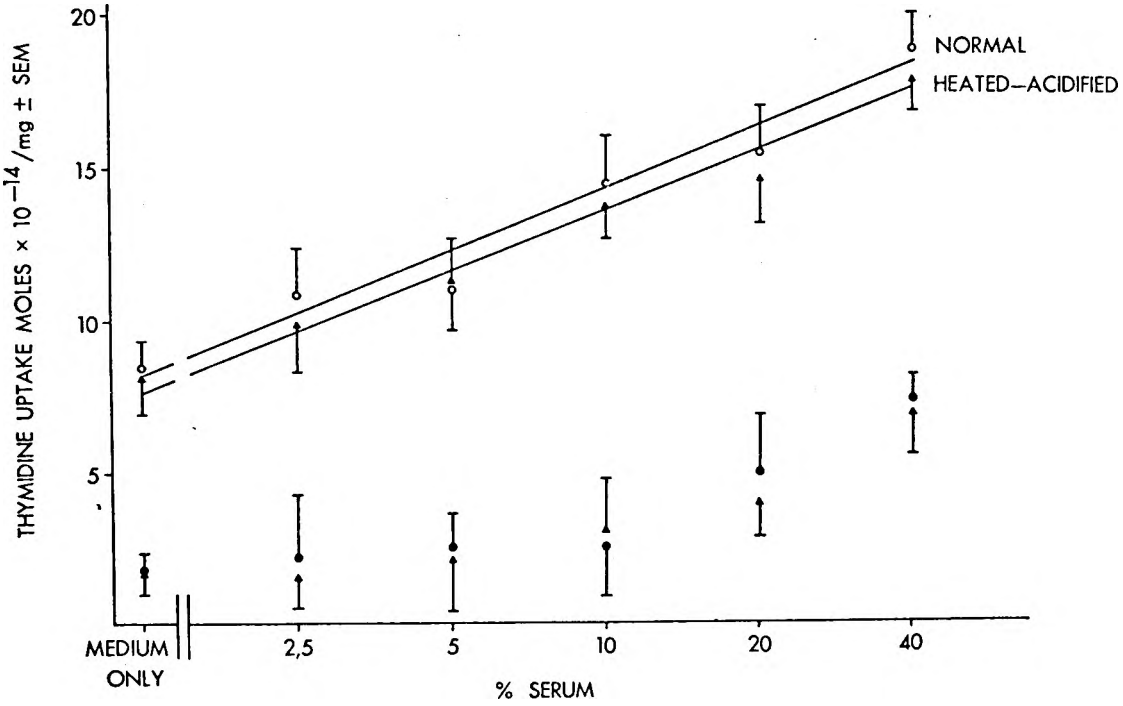


Figure 63. The response of intact rib cartilage segments to both normal (costal zone 0, sternal zone ●) and heated-acidified (costal zone , sternal zone ▲) human serum.

In three separate harvests the number of cells exhibiting amoeboid movements was counted immediately after isolation and again 24 hours later after incubation in 2,5% serum. For each harvest counts on five groups of 500 cells each from both costal and sternal zones were made blind by two observers. The means of these 10 observations are expressed as the percentage of amoeboid cells. After isolation 11% (range 5 - 17%) of costal cells exhibited amoeboid movement and following 24 hours in culture 23% (range 15 - 37%) of these cells were amoeboid. This difference is significant ($p < 0,01$). The corresponding results for the sternal zone were 4% (range 1 - 9%) and 19% (range 12 - 29%). This difference is significant ($p < 0,01$). The difference between costal and sternal zones is significant after isolation ($p < 0,05$), but not after 24 hours in culture ($p > 0,05$).

12.3 Discussion

The chondrocyte culture technique used in this study maintains cells in suspension for 24 hours after isolation. Abbott and Holtzer (1966) have demonstrated that when chondrocytes in culture are in close contact with each other, either in intact matrix or as isolated cells, 98% of cells continue to synthesize metachromatic matrix and show no signs of mitotic activity. However, when cells are dispersed in monolayer culture they rapidly de-differentiate, cease specialized metabolic activity and begin DNA synthesis. This change was morphologically correlated with the onset of amoeboid cell movement, and adoption of a stellate rather than round configuration. In the present study the cell suspension used lies between the extremes of the centrifuged pellet and the monodispersion studied by Abbott and Holtzer. Our results are consistent with those of these workers in that both studies showed evidence of increasing DNA synthesis and/or mitotic activity within 24 hours of freeing chondrocytes from their matrix (Table II and Figure 12 of Abbott & Holtzer 1966 and the significant increase in the percentage of amoeboid cells in the present study).

The very small costo-sternal difference in thymidine uptake for the isolated cells compared to the 3 to 4-fold difference in the whole segments is consistent with the observation that there is a greater increase in the percentage of amoeboid cells in the sternal zone than in the costal, and that after 24 hours there is no significant difference between the zones in this respect. These findings may be explained by dedifferentiation having a proportionately greater effect in the sternal zone, where mitotic activity is confined to the sub-periosteal region, than in the costal zone, where the large interstitial growth centre lies. An alternative or additional factor contributing to the reduced costo-sternal difference may have been enzymatic damage to the

cells with the more active costal zone cells being more affected than the quiescent sternal zone cells. Furthermore the finding that cell densities in the costal and sternal zones were not significantly different also requires some comment in this respect. The autoradiographs of Chapter 10 show much higher cell densities in the region of the columns of the growth centre than in the rest of the cartilage. The active growth region occupies about a quarter of the costal zone at 60 days PC of age and the fact that cell counts in the costal zone were higher, but not significantly so, than in the sternal suggests that some costal cells were lost during isolation. It is possible that the actively dividing cells were more susceptible to damage and were lost, thus reducing the costal zone density, and therefore also reducing the costo-sternal difference in thymidine uptake.

It is not known why uptake varied so considerably from one cell harvest to another. Cell viability after isolation was high and fairly consistent as assessed by the supravital staining method. Although uptake was expressed in terms of the number of viable cells two sources of error may have contributed to the variability. The first is the possibility that cells judged to have been viable by the trypan blue technique may not have been capable of DNA synthesis, and the converse may also have been true. Secondly viability was only assessed after isolation and not during or after the subsequent 24 hour incubation. It is possible that the variability in percentage viability at the end of this period was greater than after isolation. The consistent qualitative reproduction of the results when expressed as the percentage changes from the base-line (Figures 60 and 62) is, however, reassuring and indicative of a significant biological phenomenon.

Putative SM stimulation of isolated chondrocytes at low serum concentrations reaching a peak at 6% and declining at 10% (Garland

et al. 1972) and dose-related up to 10% (Binet et al. 1975) have been reported. Neither of these groups of authors report events at higher concentrations. The present study shows a peak stimulation at 2,5% and inhibition from 10% normal serum concentration upwards. This inhibition is interpreted to indicate further evidence for the presence of somatomedin inhibitors in normal serum. Such inhibitors have been postulated in normal rat serum (Salmon 1972, p.180) and in serum of malnourished children (Van den Brande & Du Caju 1973). The inhibitory material in malnutrition is a neutral, thermolabile molecule with a molecular weight in excess of 100 000 daltons (Van den Brande 1974, personal communication). Our results with heated-acidified serum support the thermolability and acid-sensitivity of the inhibitor, but its further characteristics have not been determined in these experiments. We only used a single reference pool of three adult sera as a source for SM. It is not known whether all human sera contain inhibitory factors. The relationship of this inhibitor in normal human serum to that in rat serum and that in the serum of malnourished children is not known.

The vigorous handling of the serum to remove inhibitory material resulted in some loss of SM activity, and a potency of 0,64 is consistent with previous observations (Van den Brande et al. 1971).

The stimulatory activity of serum at low concentrations suggests that the activity of the inhibitor is concentration dependent, for once effective concentrations are reached, at 10%, little or no further inhibition occurs (Figure 60). This suggests that inhibition is not competitive as SM activity is dose-responsive up to the maximum concentration studied, namely 80%, once inhibitory material is removed (Figure 62).

Somatomedin inhibition in cartilage segment assays with normal human serum has not been reported (Van den Brande 1973)

and has not been observed in our laboratory using the porcine cartilage assay. The dose-response characteristics of the rabbit costal cartilage were the same to both normal and heat-treated serum; there was no evidence of inhibition (Figure 63). This difference between the intact segments and the isolated chondrocytes suggests that the matrix plays an important role in mediating the net SM effect *in vivo*. The greater molecular weight of the inhibitor, ca. 100 000, compared to that of SM, ca. 4000, may be a determining factor, although the details of the diffusion characteristics of large molecules through cartilage are very poorly understood (Nachemson *et al*. 1970; Campo & Dziewiatowski 1961; Kantor & Schubert 1957). Again the possibility of enzymatic damage to or alteration of the chondrocyte membranes cannot be excluded as a factor causing or contributing to the nature of the response of the isolated cells.

The age-related differences in response of intact costal cartilage reported in Chapter 10 also occur in the isolated cells (Table 40). Chondrocytes from 35 days PC costal cartilage with a growth velocity of 0,57mm/day (Chapter 10, Table 26) have a 2 to 3-fold greater uptake of thymidine than those from 60 days PC cartilage with a velocity of 0,35mm/day. Ehatever the as yet unrecognized effects of enzymatic isolation and de-differentiation in culture, the inherent responsiveness of the cartilage cell appears to remain a function of its age and growth velocity.

CHAPTER 13

SOMATOMEDIN IN PROTEIN - ENERGY MALNUTRITION

The study of disease processes in man often yields early insight into normal function and provides the basis for later experimental design either in man or other animals. Protein-energy malnutrition (PEM) is one such situation which provided opportunities for the study of growth hormone (GH) kinetics in man leading to experimental liver perfusion and the investigation of hepatic metabolism of GH (Pimstone et al. 1972). The finding of an elevated GH secretion in PEM led to the study of serum SM in this condition by Pimstone and co-workers. They showed that SM was reduced in five children admitted to hospital with low body weight, hypoalbuminaemia and oedema, rising in all patients after nine days of treatment (Grant et al. 1973). Prior to this study Daughaday and Kipnis (1966) had demonstrated that SM levels were reduced in the sera of some starved rats, which suggested the possible presence of serum factors inhibiting SM activity. Recently Van den Brande and Du Caju (1973) showed that there are thermolabile, macromolecular proteins in serum of some patients with PEM that inhibit the stimulatory activity of SM.

In view of the limited number and extent of the studies on SM in PEM we have investigated a larger number of children and related our findings to an age-matched control series. Further, in view of the somewhat paradoxical demonstration of low SM and high GH in PEM patients on admission to hospital, we have questioned whether the endogenous GH in these patients is biologically inactive with respect to SM generation or whether there is end-organ insensitivity to a normally active GH. In an

attempt to answer the first part of this question, namely whether endogenous GH is inactive, we have infused GH into children with PEM and measured their serum SM response.

13.1. Subjects and Methods

13.1.1. Patients and control subjects. Two Caucasian children (a boy aged 4,5 years and a girl aged 3,0 years) with proven isolated hGH deficiency were studied in the Growth and Endocrine Clinic of the Transvaal Memorial Hospital for Children, Johannesburg. Serum samples were obtained prior to and two weeks after commencement of therapy with hGH (Crescormon, Kabi) 0,5 i.u./ kg body weight in two divided doses per week.

A series of clinically normal subjects were studied to establish the range of normal serum SM in this laboratory. Forty one fasting members of the Medical School staff aged 18 to 61 years (South African Bantu-speaking Negroes : 7 male and 9 female; Caucasians : 14 male and 11 female) donated serum between 07h00 and 10h00. Thirteen Negro children, admitted to Baragwanath Hospital for minor surgical procedures, who were all clinically normal in terms of nutritional and growth status had fasting serum collected at the time of routine pre-operative haematological assessment. The five girls and eight boys ranged in age from two to fortyeight months.

Serum from five patients with hypopituitarism, two with isolated GH deficiency and three with panhypopituitarism (two due to tuberculous meningitis and one after hypophysectomy),were assayed prior to institution of therapy.

Twelve Negro children admitted to Baragwanath Hospital, Johannesburg with a primary clinical diagnosis of PEM, and in some cases associated complications, were studied. The seven boys and five girls were aged from two to twenty nine months. Based on the Wellcome criteria (Editorial 1970) two were diagnosed as having marasmus, three marasmic-kwashiorkor and seven kwashiorkor.

Treatment in hospital consisted of protein supplements, maternal instruction in feeding, and, where necessary, intravenous fluids, antibiotics, potassium chloride and vitamin supplements.

Five Cape Coloured children, aged 13 to 27 months, were admitted to the Red Cross Children's Hospital, Cape Town with a primary clinical diagnosis of PEM, all of the kwashiorkor type (Wellcome criteria). Treatment was similar to that of the children admitted to Baragwanath Hospital.

Informed consent was obtained from all subjects, or a parent, and this work was approved by the ethical committees for human experimentation of the University of the Witwatersrand, Johannesburg and, in the case of the Cape Coloured children, the University of Cape Town.

13.1.2. Methods. In the hospital patients serum was collected with the children at bedrest by venepuncture between 07h00 and 10h00 on the day after admission after an overnight fast, immediately before starting re-feeding, and stored at -20°C until assayed. Further fasting specimens were drawn at approximately weekly intervals, the last being obtained before discharge, at a stage of clinical and biochemical recovery.

In the Cape Coloured children 4mg of hGH (Kabi) in 5ml saline was infused intravenously over 5 minutes on the day after admission. Blood was collected 3 and 5 hours after the infusion. After recovery, prior to discharge from hospital, the hGH infusion and blood collection were repeated.

Serum SM was estimated by the porcine cartilage method using 6- or 8- point designs, depending on the availability of serum (described in Chapter 9). Growth hormone was measured by a modification of the double antibody radioimmunoassay of Morgan and Lazarow (1963). Insulin was measured by the radioimmunoassay method of Morgan and Lazarow (1963). Serum albumin was estimated

by the biuret method (Wolfson et al. 1948), blood glucose by Somogyi - Nelson technique (1952) and free fatty acids (FFA) by the colorimetric method (Duncombe 1964).

Attempts were made to remove possible SM inhibitors in some sera by the heating and acidification procedure of Cohen et al. (1975), described in Chapter 9.

13.2. Results

13.2.1. GH-dependence of serum SM. Table 41 shows the marked rise that occurred in serum SM in both GH-deficient children during GH therapy. Only sulphation results are shown, those for thymidine being similar.

TABLE 41. Serum SM (sulphation) during GH therapy in GH-deficient children

Child	Serum Somatomedin ($\pm$ 95% limits)	
	Prior to therapy	After two weeks therapy
A	0,14 (0,004 - 0,29)	0,56 (0,34 - 0,76)
B	0,19 (0,09 - 0,31)	0,74 (0,43 - 0,96)

13.2.2. Normal range of Serum SM. Figure 64 shows the range of serum SM (measured by sulphation) of the control series of adults and children. There is no difference between male and female, nor between Negroes and Caucasians. The range of the small series of children falls within the larger series of adults and no trend with age is apparent. The indices of precision for the three standards of the assays for these samples were 0,12, 0,16 and 0,18. The indices of precision for the samples ranged from 0,10 to 0,29. Simultaneously determined potency ratios for thymidine uptake yielded similar ranges, but the range of indices of precision was greater, namely 0,13 to 0,42.

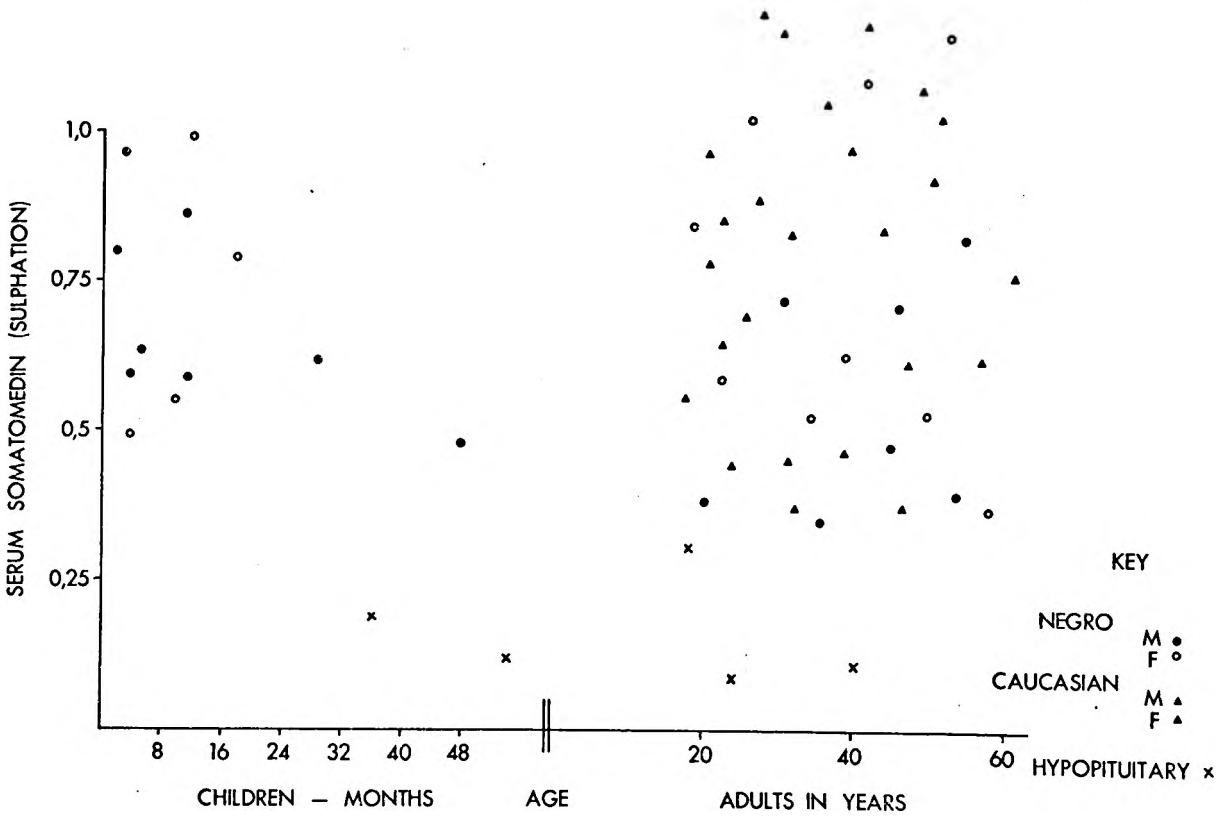


Figure 64. Human serum SM (sulphation) in 54 normal subjects, aged 2 months to 61 years and in 5 hypopituitary subjects, aged 3 to 40 years.

13.2.3. Serum SM in children with PEM. The two or more serial samples from each child with PEM were assayed together so that possible inter-assay variation does not influence the comparison of results obtained on each child.

In two children (numbers 1 and 8) none of the sera gave dose-response curves parallel to the standard at the 95% level of significance, and potency ratios could not therefore be computed. In four other children (numbers 4,6,7 and 17) admission and first week samples did not give parallel responses, but later samples did do so. For all other serum samples Table 42 shows the diagnostic category, serum hGH, serum albumin, and serum SM for both sulphation and thymidine uptake.

TABLE 42. Changes in serum SM in children with PEM from the time of admission to hospital to the stage of clinical recovery

Case No.	Age (months)	Diagnosis	Days after admission	Serum hGH (ng/ml)	Serum albumin (g/100ml)	Somatomedin Potency Ratio Sulphate	Thymidine
1	2	M*	1	5,5	2,3		
			8	1,5	-	none achieve parellelism	
			15	1,1	3,1		
			23	0,8	2,9		
			35	0,6	4,1		
			49	0,6	3,5		
3	4	K	1	3,1	1,7	0,22	0,31
			8	0,4	3,0	0,76	0,78
4	10	MK	1	16,1	2,7	neither achieve parallelism	
			8	12,4	3,0	0,13	0,10
			13	2,3	-	0,23	0,26
			38	4,6	3,4	0,10	0,11
			58	0,3	4,5	0,50	0,54
6	29	M	1	4,6	2,9	neither achieve parallelism	
			8	2.1	-	" " "	
			15	0,1	3,7	0,31	0,24
7	18	K	1	5,2	1,8	neither achieve parallelism	
			7	0,8	2,7	0,51	0,42
			14	0,1	3,6	0,84	0,74
8	9	MK	1	6,0	2,2	none achieve parallelism	
			7	3,1	2,9		
			14	0,5	3,9		
9	14	K	1	0,3	1,9	0,31	0,48
			6	0,1	2,7	0,42	0,63
14	13	K	1	1,6	2,1	0,73	0,64
			7	1,1	1,7	0,58	0,69
			14	0,2	1,9	0,84	0,79
			26	1,0	3,0	0,81	0,83
15	3	K	1	6,8	2,9	0,22	0,31
			7	2,4	2,7	0,72	0,49
			16	1,3	3,7	0,75	0,61
			25	1,2	3,0	0,74	0,60
16	24	K	1	12,5	1,6	0,10	0,09
			8	3,3	1,7	0,17	0,21
			17	1,0	1,8	0,49	0,53
			23	1,6	3,0	0,73	0,69
			29	0,8	4,0	0,84	0,88
17	17	K	1	8,6	1,7	neither achieve parallelism	
			9	2,1	3,1	0,19	0,11
			19	0,6	2,6	0,46	0,33
			25	0,7	3,4	0,48	0,31
18	20	MK	1	8,7	2,0	0,29	not assayed
			6	3,4	2,7	0,34	
			14	0,2	3,7	0,38	

Note: * M indicates marasmus, MK marasmic-kwashiorkor and K kwashiorkor

The hGH concentrations exhibit the characteristic elevation in severe, untreated PEM and rapid fall on institution of therapy. The number of cases is too small to decide whether there is any difference in hGH response between kwashiorkor and marasmus. Serum albumin was low in all cases on admission and rose on therapy. Transient decreases during recovery (case 17, for example) may have been related to intravenous fluid therapy.

In those cases in which multiple samples achieved statistically acceptable parallelism in the assay SM exhibited a consistent trend, namely a progressive rise from very low levels, some in the hypopituitarism range, towards normal. Some patients (cases 6,9, 17 and 18), however, although discharged as 'normal', had not achieved normal serum SM as judged by the values for the control children (cf. Figure 64).

Further details of the nature of the dose-response curves are seen in the reproductions of the calculator plots in Figures 65 and 66. These show the uptake of $^{35}SO_4$ by porcine costal cartilage in response to three concentrations of serum. The non-parallelism of the first serum sample with parallelism of later samples is shown in Figure 65, while Figure 66 demonstrates the progressive rise of serum SM that accompanies recovery.

There is a small negative correlation between hGH and SM in the serum of patients. For sulphate uptake the coefficient is -0,34 ($p > 0,05$) and for thymidine uptake it is -0,37 ($0,01 < p > 0,01$). There is no correlation between serum albumin and SM (sulphate 0,06; thymidine 0,02). There is a significant correlation between the sulphate and thymidine potency ratios ($r = 0,84$ $p < 0,001$).

The indices of precision of the standards in these assays ranged from 0,11 to 0,21 (sulphate) and 0,17 to 0,28 (thymidine) and for the samples from 0,08 to 0,30 (sulphate) and 0,12 to 0,39 (thymidine).

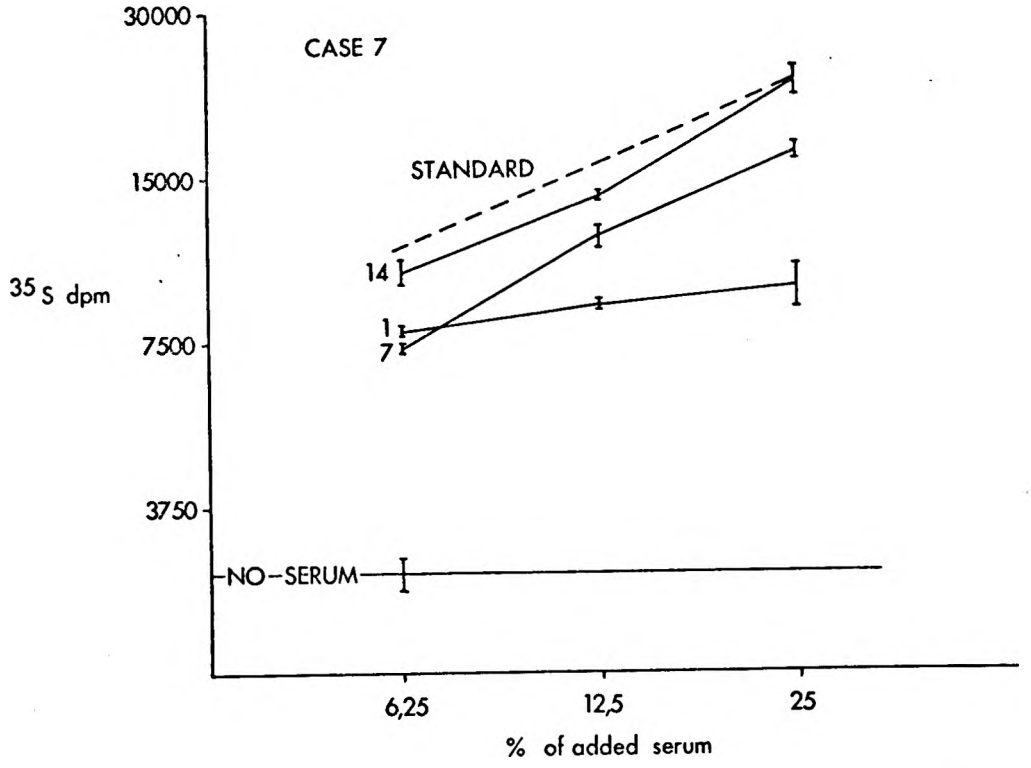


Figure 65. Calculator plot of dose-response curves for serum samples from a child with PEM during recovery (case 7). The numerals opposite each curve represent the days after admission to hospital and "Standard" represents the dose-response curve of the serum from the reference standard pool. Each point represents the mean ± sem.

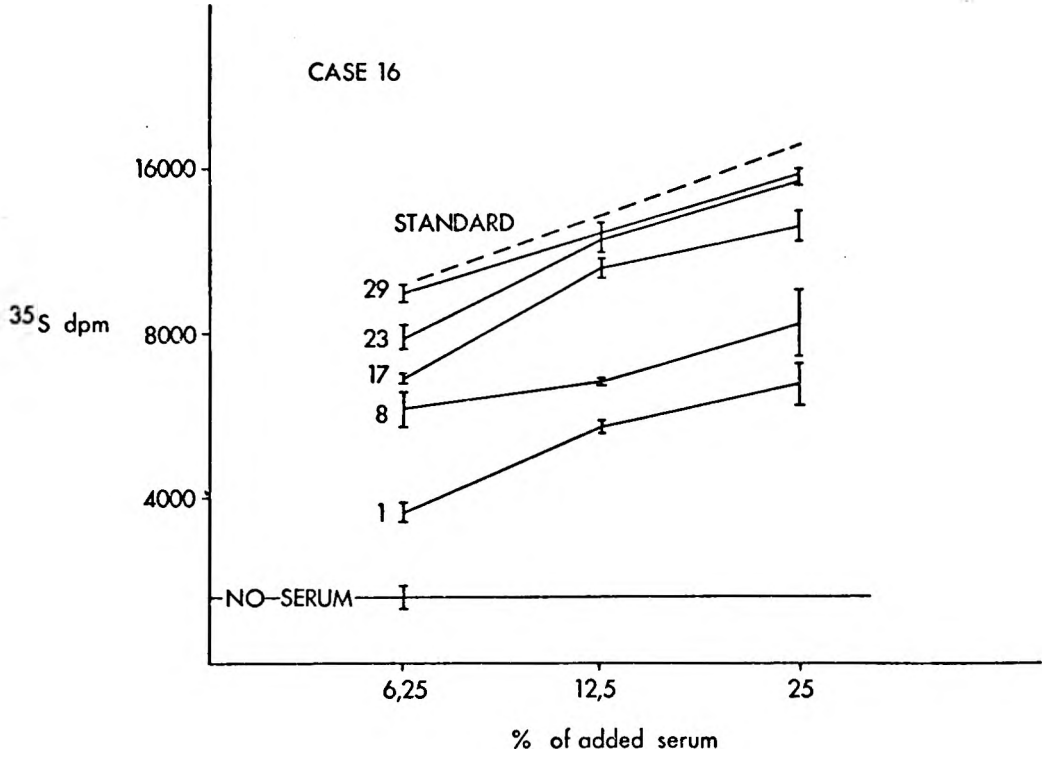


Figure 66. Calculator plot of dose-response curves for serum samples from a child with PEM during recovery (case 16). For further details see Figure65.

13.2.4. Effect of infusion of hGH on SM in children with PEM. (Table 43). Fasting levels of hGH are significantly higher on admission than after recovery in each child ($p < 0,001$). Five hours after the infusion the hGH concentration had returned to near baseline level in all but one child (case 4) on admission. There are no consistent changes in glucose or insulin concentrations. Basal FFA concentrations are lower after recovery than on admission in three of the five patients, but there is no statistically significant difference ($p > 0,05$). Growth hormone provokes a small rise in FFA on admission (range 4 - 55%), but after recovery there is a much more dramatic rise after infusion (range 76 - 421%).

Potency ratios could not be calculated for SM in three patients on admission because of non-parallelism. To assess whether any change had occurred in SM at 3 and 5 hours after infusion the sulphate uptakes at these times were compared with uptake at time zero, i.e. fasting. In this way parallel dose-response curves were obtained and potency ratios calculated. The potency ratio of 3 and 5 hour samples showing parallelism to the standard was calculated in the conventional manner (with reference to the standard, results shown in Table 43) and also with reference to the fasting sample.

Table 43 shows that SM was low on admission and rose on recovery. No change in SM occurred after hGH, either on admission or on recovery. The small fluctuations in serum SM that do occur at 3 and 5 hours are within the 95% confidence limits of the fasting sample and are not considered significant.

13.2.5. Heated - acidified serum. Serum from 7 of the 13 normal young children and from 6 of the children with untreated PEM was acidified and heated (HA), and then assayed at the same time as the untreated serum. All HA sera showed less SM activity than the paired native sera (Table 44). In the three PEM cases where the serum response was not parallel to that of the standard,

TABLE 43. Effect of infusion of hGH in children with PEM on serum hGH (ng/ml), blood glucose (mg/100ml) serum insulin (μU/ml), free fatty acids (μ/Eq/l) and somatomedin (sulphation) potency ratio.

Case No	Age (months)	Hour	on admission					after recovery				
			hGH	glucose	insulin	FFA	SM	hGH	glucose	insulin	FFA	SM
1	13	0	32	60	4,8	745	0,54	9,2	75	7,2	282	0,85
		3	55	63	3,3	778	0,45	20	71	6,2	1027	0,63
		5	30	-	-	-	0,38	6,7	-	-	-	0,75
2	18	0	47	78	4,1	704	0,22	13,5	61	6,1	700	0,61
		3	80	67	4,5	797	0,31	49	56	6,2	1373	0,81
		5	43	-	-	-	0,24	21	-	-	-	0,79
3	27	0	35	82	3,7	1245	n.p.	19	74	6,7	582	0,81
		3	70	89	5,1	1593	n.p.	22	69	6,6	1027	0,68
		5	41	-	-	-	n.p.	8	-	-	-	0,98
4	21	0	29	70	6,6	573	n.p.	13	72	6,4	864	0,57
		3	68	47	4,6	890	n.p.	33	69	6,8	1922	0,62
		5	52	-	-	-	n.p.	14	-	-	-	0,84
5	19	0	50	62	8,2	631	n.p.	4,2	109	2,0	291	1,48
		3	66	68	6,7	932	n.p.	15,7	65	7,0	1515	1,41
		5	56	-	-	-	n.p.	8,5	-	-	-	1,10

heating and acidification did not alter the lack of parallelism and in all three cases the sulphate uptake induced by the HA serum was lower than that induced by native serum response was not

TABLE 44. Effect on SM (sulphation) of heating and acidifying the serum in normal and malnourished children. The case numbers of PEM patients correspond with those in Table 42. λ indicates the index of precision.

CASE No.	Serum SM Potency Ratio			
	native		heated-acidified	
Normal		λ		λ
101	0,55	0,22	0,21	0,17
103	0,99	0,16	0,15	0,28
108	0,96	0,17	0,48	0,29
110	0,59	0,15	0,57	0,10
111	0,62	0,29	0,08	0,28
112	0,79	0,13	0,31	0,16
113	0,96	0,09	0,58	0,15
PEM				
4	not parallel		not parallel	
6	not parallel		not parallel	
9	0,31	0,29	0,09	0,26
15	0,22	0,16	0,17	0,24
16	0,10	0,12	0,17	0,23
17	not parallel		not parallel	

parallel to that of the standard, heating and acidification did not alter the lack of parallelism and in all three cases the sulphate uptake induced by the HA serum was lower than that induced by native serum.

13.3. Discussion

The bioassay used in this study has been shown to measure a GH-dependent factor(s) in human serum. In spite of this, and an

identical assay design to that used by Van den Brande and Du Caju (1974), a markedly different pattern of serum SM values in childhood has been found compared to that described by Van den Brande (Van den Brande and Du Caju 1973). These authors described a series of 45 normal subjects, of whom 12 were aged four years or less (see Figure 2 of their 1973 publication). In these 12 children there was a clear rise in SM with age from about 0,4 to 0,8. In the present study no such trend occurred (Table 64). The only other published work on children in this age range are the papers of Kogut et al. (1963) who studied 10 children and found no trend and Almquist and Rune (1961) who studied 10 children and found a very modest increase between the 6 month (0,51) and 4 year (0,59) means. Before any conclusions can be reached in this field longitudinal studies of large numbers of children need to be undertaken.

The findings in PEM confirm the work of Grant et al. (1973). Somatomedin is low in severe untreated PEM and recovers rapidly with re-feeding. Whether this rise in SM is causally related or only associated with the catch-up growth of these children is not known, and whether the serum SM returns to normal in all patients who are refed for prolonged periods of time also remains to be investigated.

Non-parallelism was a problem also encountered by Grant et al. (1973). This phenomenon has been speculatively ascribed to serum SM inhibitors, although Grant's mixing experiments and the heating-acidification experiments of this study (Table 44) failed to indicate the presence of inhibitors. However, these negative findings in the small number of patients studied and the limited nature of the experimental approaches to removing the putative inhibitors does not exclude their presence.

It is attractive to speculate on the possibility of a negative feed-back loop between SM and GH. Although a negative correlation

between SM and GH was found in this study it cannot be construed as evidence for such a feed-back. Such evidence will have to be based on short- to medium-term dynamic experiments using purified SM preparations. The significant correlation (r = 0,84) between the SM potency ratios, based on sulphate and thymidine uptake, is reassuring evidence that the assay system has internal consistency.

The only previous study of the effect of intravenous GH on SM is that of Hall (1971). The 7 patients with hypopituitarism were infused with 2mg of GH and showed a considerable and consistent rise in SM 3 hours after infusion. In the present study exogenous GH did not cause any discernable rise in SM. The change in the FFA response with recovery may suggest an end-organ resistance to GH in PEM, but there are alternative possibilities that may explain this change that must be considered. The simple fact that the fat mass of the children increases during recovery may influence the response to GH or the explanation may lie in an alteration of the lipolytic activity of adipose tissue. Furthermore the results of these short-term intravenous studies do not preclude the possibility that intramuscular GH over several days may raise serum SM (should the ethical and clinical problems of such a study be overcome).

In summary, the basis of a low serum SM in the face of an elevated GH in patients with PEM has not been elucidated.

Finally, the difference between fasting levels of GH in patients with PEM in Cape Town (Table 43) and Johannesburg (Table 42) bears comment. The same assay technique was used but in different laboratories in each city, introducing the possibility of the difference being due to technical discrepancies, although the range of normal values adopted by the two laboratories is similar. The alternative explanation lies in the possible pleomorphic nature of the disease and the variable response of different populations (Gardner, L.I. and Amacher, P. 1973).

This question warrants further investigation in South Africa.

Acknowledgment. I am grateful to Professor Bernard Pimstone for the measurement of GH, FFA, glucose and albumin in the Cape Town children and Ms. Marie van As for the measurement of GH in the Johannesburg children.

CHAPTER 14

SUMMARY OF THE MAIN CONCLUSIONS

"To be conscious that you are
ignorant of the facts is a
great step to knowledge"

Benjamin Disraeli

The research described in this thesis explores some of the factors that regulate the velocity of growth in mammals. On theoretical grounds such regulatory relationships would be most fruitfully investigated by simultaneous longitudinal measurement of the regulating factors and the growth velocity of the animal. This theoretical ideal can rarely, if ever, be achieved for technical, cost-benefit and, in the case of man, ethical reasons. The results of the present research have added new knowledge to this field, but the experimental investigation of the regulatory inter-relationships of mammalian growth control is far from complete.

The measurement of changes in tissue responsiveness with age requires controlled in vitro experimentation. This implies, except possibly in the case of blood and skin, that tissue can only be acquired post-mortem on a cross-sectional basis, or by serial operative procedures that would seriously interfere with normal growth, on a longitudinal basis. Lack of tissue homogeneity is a further problem that would beset the sampling of tissue on a serial longitudinal basis. These difficulties also hamper attempts to collect longitudinal series of serum samples until well after

birth, usually at an age when growth has slowed considerably.

The main obstacle that the work in this thesis did not overcome was the technical difficulty of sampling serum and cartilage from animals growing at their normal velocity. Therefore, while the growth study was of a longitudinal design, the physiological study of serum SM and cartilage responsiveness was of a cross-sectional nature. This shortcoming is common to all previous work of this nature. The limitations imposed on the interpretation of the results by this situation have been partly offset by using a highly inbred strain of rabbits and rearing them under strictly controlled uniform conditions. A further strength of the present studies, lacking in previous published work, is the attempt to relate cartilage responsiveness to the actual velocity of growth of the cartilage under study at the time. This novel approach yielded one of the most significant results of the thesis, namely the demonstration that the degree of in vitro responsiveness of costal cartilage does not depend on the stimulus to which it is exposed. That is to say, cartilage responsiveness appears to be determined by some "intrinsic growth capacity" (Needham 1964) that is a function of the age of the animal and the growth velocity of the cartilage. The demonstration of a close correlation between the metabolic responsiveness and growth velocity of costal cartilage under several different experimental conditions suggests that the prime determinant of growth velocity is the growing tissue itself. It may then be asked, what is the role of serum and other micro-environmental stimuli in the regulation of normal growth? It is suggested that in this situation the role of serum stimuli such as somatomedin may be one of superimposing fine controls on the growth process. Such control may be envisaged as modulating the influences of external and internal environmental influences on growth.

Evidence is accumulating that this type of cell-mediated

control of growth does occur. Harris (1970) has elegantly shown that "the same sets of genes in the same cytoplasm may be switched on at very different rates ..... determined ..... by differences in the physiological states of the nuclei which contain them." The nature of the switching signals remains unknown, but the eventual elucidation of their mechanism of action and control will expand much of our understanding of the regulatory processes involved in growth.

It is submitted that although the results presented and discussed in this thesis may have advanced our knowledge of the control processes of mammalian growth, more new questions have undoubtedly been raised than answers provided. In the final paragraphs some areas for future research are suggested.

Single static measurements of a hormone yield very limited information. Techniques that will permit the estimation of secretory and turnover rates of SM are needed and will have to be applied to the longitudinal study of the role of SM in the control of growth in order to provide maximum understanding of this role. Such studies may indicate sex differences and a role for serum SM in the primate pubertal growth spurt.

To facilitate the investigation of the role of the end-organ in growth control other tissues need to be studied. Somatomedin-sensitive cells in the blood, skin or other readily accessible, reasonably homogeneous tissues should be sought and examined in a longitudinal manner. Such experimental situations would provide the opportunities to investigate the possibilities of sex differences in responsiveness and the contribution that other endocrine factors may make to their ontogenesis. These studies would also provide insights into the relationship of SM to other serum and tissue growth factors.

The studies on isolated chondrocyte have raised the

fascinating possibility of the occurrence of SM inhibitors in normal serum. It is conceivable that a dynamic balance exists between stimulatory and inhibitory serum fractions throughout life. More sensitive and selective assay systems, such as the radioreceptor and radioimmunoassay, may separate these fractions and assist in the elucidation of their control systems, functions and inter-relationships. Nevertheless, the bioassay will not lose its usefulness since it measures the true biological, or net, SM activity in serum, and it is this activity that most closely approximates the in vivo situation. The isolation and identification of SM inhibitory fractions from the sera of patients with PEM will provide further insight into the metabolic disorders of this disease.

APPENDIX A

Programme for data handling and spline-set interpolation

FORTRAN IV

```
0001            REAL X(100),Y(100),C(4,100-
0002            REAL XIN(69,20),YIN(69,20),XINTV(67),YINTV(69,67),YDASH(69,67)
0003            REAL HEAD1(10),HEAD2(10)
0004            REAL S1(67,2),S2(67,2),V1(67,2),V2(67,2),D1(67,2),D2(67,2)
0005            INTEGER ISEX(69)
0006            READ(5,140)(XINTV(I),I=1,16)
0007    140     FORMAT(16F4.C)
                XINTV CONTAINS THE POINTS AT WHICH WE WISH TO INTERPOLATE
0008            DO 15 I=17,69
0009            J=I+33
0010    15      XINTV(I)=J
0011            DO 20 I=1,69
0012            DO 20 J=1,20
0013            XIN(I,J)=0
0014    20      YIN(I,J)=0
0015            READ (5,130)NS,HEAD1
```

```
0016     130     FORMAT(13,10A4)
0017             DO 30 I=1,69
0018     30      READ(5,150)(XIN(I,J),J=1,20)
0019     150     FORMAT(7X,20F3.0)
0020             READ(5,130)NS,HEAD2
0021             WRITE(6,300)HEAD2
0022     300     FORMAT('1',10A4)
0023             DO 40 I=1,69
0024             READ(5,159)ISEX(I),(YIN(I,J),J=1,18)
0025     40      READ(5,160)(YIN(I,J),J=19,20)
0026     159     FORMAT(3X,I1,3X,18F4.2)
0027     160     FORMAT(7X,18F4.2)
                 WE HAVE READ THE DATA
                 NOW ELIMINATE ALL ZERO ORDINATES
0028             DO 60 I=1,69
0029             KK=21
                 ASSUME 20 ELEMENTS
                 SAMPLE SIZE=69
0030             DO 51 J=1,20
0031             KK=KK-1
0032             IF(YIN(I,KK).GE.0.0)GO TO 52
```

```
0033      51    CONTINUE
0034            WRITE(6,900-
0035      900   FORMAT('0ERROR 1 - NULL RECORD')
0036      52    M=0
                KK IS NO OF ELEMENTS IN ROW I
0037            DO 55 J=1,KK
0038            IF(YIN(I,J).EQ.0.0)GO TO 55
                SCAN FOR EMPTY SPACES
0039            M=M+1
0040            X(M)=XIN(I,J)
0041            Y(M)=YIN(I,J)
                AND COMPRESS THE VECTOR TO WHAT WE ACTUALLY KNOW
0042      55    CONTINUE
0043            WRITE(6,260)I,M
0044      260   FORMAT('0',I3,2X,I3)
                I=ROW NUMBER, M=NUMBER OF COORDINATES POINTS
0045            WRITE(6,265)(X(JJ),JJ=1,M)
0046            WRITE(6,265)(Y(JJ),JJ=1,M)
0047      265   FORMAT(8(2X,E13.7))
0048            IF(M.LT.4)GO TO 111
                WE CAN NOW INTERPOLATE FROM THIS DATA
```

```
0049                CALL SPLCON(X,Y,M,C)
0050                DO 57 J=1,69
0051                YINTV(I,J)=0
0052                YDASH(I,J)=1.E-7C
0053                XINT=XINTV(J)
0054                CALL SPLINE(X,Y,M,C,XINT,YINT,YD)
0055                YINTV(I,J)=YINT
0056                YDASH(I,J)=YD
                    DISPLACEMENT AND VELOCITY
0057      57        CONTINUE
0058                GO TO 333
0059      111       CONTINUE
0060                DO 222 J=1,69
0061                YINTV(I,J)=-1
0062      222       YDASH(I,J)=-1
0063      333       WRITE(6,270)(YINTV(I,JJ),JJ=1,16)
0064      270       FORMAT(16(1X,F7.1))
0065      60        CONTINUE
0066                WRITE(6,280)HEAD2
0067      280       FORMAT('1ALL INTERPOLATED DATA - ',10A4,'DISPLACEMENT')
0068                WRITE (6,284)(XINTV(J),J=1,16)
0069      284       FORMAT( '0X=',16(F7.1,1X)/'0')
```

```
0070              DO 70 I=1,69
0071      70      WRITE(6,286)I,(YINTV(I,J),J=1,16)
0072      286     FORMAT(' ',I2,16(F7.3,1X))
0073              WRITE(6,287)HEAD2
0074      287     FORMAT('1ALL INTERPOLATED DATA',10A4,' VELOCITY')
0075              WRITE(6,284)(XINTV(J),J=1,16)
0076              DO 72 I=1,69
0077      72      WRITE(6,286)I,(YDASH(I,J),J=1,16)
0078              DO 80 KS1=1,3
0079              KS2=KS1-1
0080              WRITE(6,288)HEAD2
0081      288     FORMAT('1',1CA4/'CNO',3X,'N',8X,'X VALUE',6X,'Y VALUE',
                  16X,'DEVIATION',5X,'Y VEL',7X,'DEVIATION')
0082              DO 80 J=1,69
0083              SM=0
0084              DF=0
0085              SD=0
0086              SMD=0
0087              DFD=0
0088              SDD=0
0089              DO 75 I=1,69
0090              IF(KS2.EQ.2) GO TO 601
```

```
0091              IF(ISEX(I).NE.KS2)GO TO 75
0092      601     CONTINUE
0093              IF(YINTV(I,J).EQ.0.0)GO TO 75
                  ONLY SUM DISPLACEMENTS .GT. ZERO
0094              DF=DF+1
0095              SM=SM+YINTV(I,J)
                  ACCUMULATE THE TOTAL
0096      75      CONTINUE
0097              SM=SM/DF
0098              DO 76 I=1,69
0099              IF(KS2.EQ.2)GO TO 602
0100              IF(ISEX(I).NE.KS2)GO TO 76
0101      602     CONTINUE
0102              IF(YDASH(I,J).LE.0.0)GO TO 76
                  ONLY SUM VELOCITIES .GT. ZERO
0103              DFD=DFD+1
0104              SMD=SMD+YDASH(I,J)
0105      76      CONTINUE
0106              SMD=SMD/DFD
                  SAMPLE MEAN
0107              DO 77 I=1,69
```

```
0108              IF(KS2.EQ.2)GO TO 603
0109              IF(ISEX(I).NE.KS2)GO TO 77
0110      603     CONTINUE
0111              XT=YINTV(I,J)
0112              IF(XT.EQ.0.0)GO TO 77
0113              SD=SD+(XT-SM)*(XT-SM)
                  SUM OF SQUARES
0114      77      CONTINUE
0115              SD=SD/(DF-1.)
0116              DO 78 I=1,69
0117              IF(KS2.EQ.2)GO TO 604
0118              IF(ISEX(I).NE.KS2)GO TO 78
0119      604     CONTINUE
0120              XT=YDASH(I,J)
0121              IF(XT.LE.0.0)GO TO 78
0122              SDD=SDD+(XT-SMD)*(XT-SMD)
0123      78      CONTINUE
0124              SDD=SDD/(DFD-1.)
0125              IF(KS1.EQ.3)GO TO 400
0126              SI(J,KS1)=SM
0127              S2(J,KS1)=SMD
```

```
0128                D1(J,KS1)=DF
0129                D2(J,KS1)=DFD
0130                V1(J,KS1)=SD
0131                V2(J,KS1)=SDD
0132      400       CONTINUE
0133                SD=SQRT(SD)
0134                SDD=SQRT(SDD)
                    STANDARD DEVIATION
0135                WRITE(6,302)J,DF,XINTV(J),SM,SD,SMD,SDD
0136      302       FORMAT(' ',I2,2X,F3.0,5(3X,F10.4))
0137      80        CONTINUE
0138                DO 420 J=1,2
0139                WRITE(6,300)HEAD2
0140                DO 420 I=1,67
0141                WRITE(6,480)S1(I,J),D1(I,J),V1(I,J)
0142      420       WRITE(6,482)S2(I,J),D2(I,J),V2(I,J)
0143      480       FORMAT('0',3(2X,F9.3))
0144      482       FORMAT(' ',3(2X,F9.3))
                    NOW DO THE T-TESTS
0145                WRITE(6,300)HEAD2
0146                DO 450 I=1,67
0147                DBM1=S1(I,1)-S1(I,2)
```

```
0148              DBM2=S2(I,1)-S2(I,2)
0149              WRITE(6,470)DBM1,DBM2
0150              DF1=D1(I,1)+D1(I,2)-2
0151              DF2=D2(I,1)+D2(I,2)-2
0152              WRITE(6,470)DF1,DF2
0153              R1=V1(I,1)/D1(I,1)+V1(I,2)/D1(I,2)
0154              R2=V2(I,1)/D2(I,1)+V2(I,2)/D2(I,2)
0155              WRITE(6,470)R1,R2
0156    470       FORMAT(' ',2(2X,F8.3))
0157              T1=DBM1/SQRT(R1)
0158              T2=DBM2/SQRT(R2)
0159              WRITE(6,460)I,DF1,T1,DF2,T2
0160    460       FORMAT(' ',I2,2(3X,F3.0,3X,F8.3))
0161    450       CONTINUE
0162    1000      STOP
```

SUBROUTINE 1:

```
0001            SUBROUTINE SPLCON(X,Y,M,C)
0002            REAL X(100),Y(100),D(100),P(100),E(100),C(4,100),A(100,3)
0003            REAL B(100),Z(100)
0004            MM=M-1
0005            DO 2 K=1,MM
0006            D(K)=X(K+1)-X(K)
0007            P(K)=D(K)/6.
0008    2       E(K)=(Y(K+1)-Y(K))/D(K)
0009            DO 3 K=2,MM
0010    3       B(K)=E(K)-E(K-1)
0011            A(1,2)=-1.-D(1)/D(2)
0012            A(1,3)=D(1)/D(2)
0013            A(2,3)=P(2)-P(1)*A(1,3)
0014            A(2,2)=2.*(P(1)+P(2))-P(1)*A(1,2)
0015            A(2,3)=A(2,3)/A(2,2)
0016            B(2)=B(2)/A(2,2)
0017            DO 4 K=3,MM
0018            A(K,2)=2.*(P(K-1)+P(K))-P(K-1)*A(K-1,3)
0019            B(K)=B(K)-P(K-1)*B(K-1)
0020            A(K,3)=P(K)/A(K,2)
0021    4       B(K)=B(K)/A(K,2)
```

```
0022            Q=D(M-2)/D(M-1)
0023            A(M,1)=1.+Q+A(M-2,3)
0024            A(M,2)=-Q-A(M,1)*A(M-1,3)
0025            B(M)=B(M-2)-A(M,1)*B(M-1)
0026            Z(M)=B(M)/A(M,2)
0027            MN=M-2
0028            DO 6 I=1,MN
0029            K=M-I
0030    6       Z(K)=B(K)-A(K,3)*Z(K+1)
0031            Z(1)=-A(1,2)*Z(2)-A(1,3)*Z(3)
0032            DO 7 K=1,MM
0033            Q=1./(6.*D(K))
0034            C(1,K)=Z(K)*Q
0035            C(2,K)=Z(K+1)*Q
0036            C(3,K)=Y(K)/D(K)-Z(K)*P(K)
0037    7       C(4,K)=Y(K+1)/D(K)-Z(K+1)*P(K)
0038            RETURN
0039            DEBUG SUBCHK
0040            END
```

SUBROUTINE 2:

```
0001                SUBROUTINE SPLINE(X,Y,M,C,XINT,YINT,YD)
0002                REAL X(100),Y(100),C(4,100)
          C         M=NO OF INITIAL POINTS
0003                IF(XINT-X(1))7,1,2
0004         1      YINT=Y(1)
0005                YD=YINT
0006                RETURN
0007         2      K=1
0008         3      IF(XINT-X(K+1))6,4,5
0009         4      YINT=Y(K+1)
0010                YD=C(4,K)-C(3,K)+3.*(C(2,K)*(XINT-X(K))*(XINT-X(K))
                   1-C(1,K)*(XINT-X(K+1))*(XINT-X(K+1)))
0011                RETURN
0012         5      K=K+1
0013                IF(M-K)7,7,3
0014         6      YINT=(X(K+1)-XINT)*(C(1,K)*(X(K+1)-XINT)**2.+C(3,K))
0015                YINT=YINT+(XINT-X(K))*(C(2,K)*(XINT-X(K))**2.+C(4,K))
0016                YD=C(4,K)-C(3,K)+3.*(C(2,K)*(XINT-X(K))*(XINT-X(K))
```

```
                    1-C(1,K)*(XINT-X(K+1))*(XINT-X(K+1)))
0017                 RETURN
0018      7          WRITE(6,101)
0019      101        FORMAT(' OUT OF RANGE FOR INTERPOLATION')
0020                 YINT=0
0021                 YD=-1.E-70
0022                 RETURN
0023                 DEBUG SUBCHK
0024                 END
```

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