GLYCOSAMINOGLYCANS AND INTRAMEMBRANOUS BONE FORMATION

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A histochemical and biochemical investigation of the rat calvarium

PROMOTOR

Prof. Dr. Ch. M A. KUYPER

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG
VAN DE RECTOR MAGNIFICUS MR W C L VAN DER GRINTEN,
HOOGLERAAR IN DE FACULTEIT DER RECHTSGELEERDHEID,
VOLGENS BESLUIT VAN DE SENAAT IN HET OPENBAAR TE
VERDEDIGEN OP DONDERDAG 1 APRIL 1971
DES NAMIDDAGS TE 2.00 UUR PRECIES

DOOR

HENRICUS WILHELM BERNHARD JANSEN

GEBOREN TE NIJMEGEN

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Bij het verschijnen van dit proefschrift wil ik gaarne al diegenen bedanken die mij op enigerlei wijze hebben geholpen bij de totstandkoming ervan.

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De biochemische analyses zijn voor een groot deel uitgevoerd door Piet Hoppenbrouwers, zonder wiens toewijding deze resultaten hier niet gepresenteerd hadden kunnen worden.

De heer van de Kamp ben ik veel dank verschuldigd voor het geduld dat hij met mij vooral in de donkere kamer heeft gehad bij het afwerken van de microfoto's.

Het omslag en de illustraties zijn van de hand van de heer Konings, die mijn ideeën hierover voortreffelijk heeft uitgewerkt.

Voor het leesbaar maken van mijn soms wel zeer onduidelijke handschrift gaat mijn grote waardering uit naar Marianne Gevers en voor de correctie van het Engelse typescript ben ik John Jefferys en Margret Weiss zeer dankbaar.

This investigation was carried out in the research laboratories of the School of Dentistry, University of Nymegen, The Netherlands.

Dedicated to the memory of my father
and my father-in-law

CONTENTS

INTRODUCTION

1.1. BONE FORMATION

The onset of calcification, and the beginning of bone formation in particular, have interested and intrigued many people working in different fields. Embryologists, anatomists, histologists, biochemists and workers in other fields of medicine and science are still investigating this very complicated process, although a great number of facts are already known.

Morphologically two major processes can be distinguished in bone formation: intramembranous ossification and endochondral ossification. The first process is characterized by the fact that bone is formed within a membrane of fibroblasts and collagenous fibers.

The process of endochondral ossification is typified by the replacement of a cartilaginous model of the future structure by bone.

Most of the skull bones are representatives of intramembranous ossification, while the long bones belong to the group of bones formed by endochondral ossification.

A membranous bone grows only by apposition; new bone is laid down on its surface. Long bones grow by two mechanisms: apposition of new bone on the surface of the shaft and interstitial growth within the cartilage of the epiphyseal disk (Ham, 1965).

McLean and Urist (1968) give the different constituents of compact bone. Inorganic matter, calcium and phosphate, comprises almost 65 % of the dry weight; the remaining 35 % is organic bone matrix. This matrix has two chief components: collagen and proteinpolysaccharide, degradable to chondroitin sulphate.

1.2. TERMINOLOGY

The terminology of the constituents of the proteinpolysaccharide mentioned above has fequently been the cause of misunderstanding and confusion. Meyer (1938) introduced the word 'mucopolysaccharide' to describe 'hexosamine-containing polysaccharides of animal origin occurring either in a pure state or conjugated with protein through a salt linkage'. Different substances were subsequently indicated by this term.

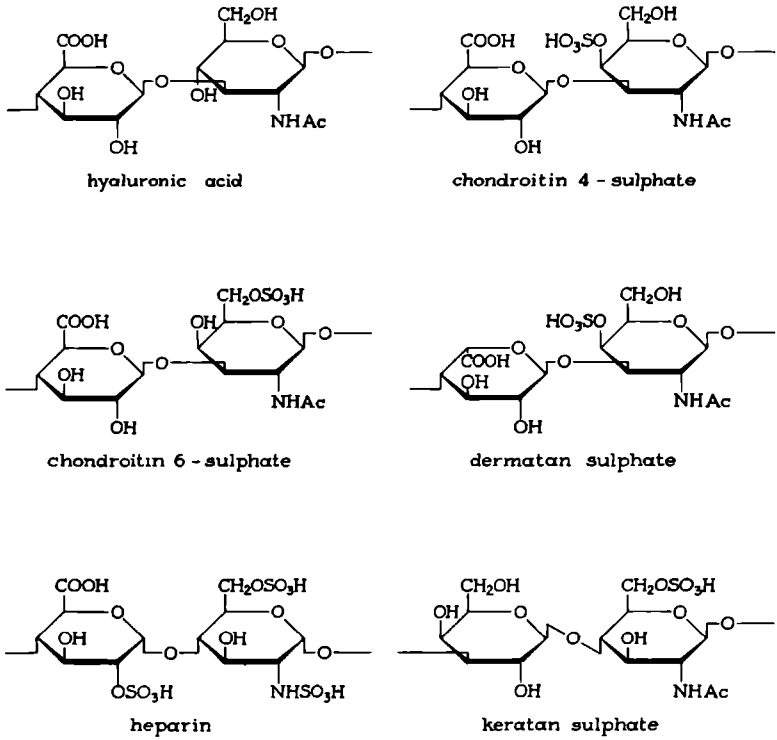


FIGURE I,1. Structural formulas of different glycosaminoglycans.

To solve this problem Jeanloz (1960) suggested a series of new terms which indicate the composition of the substances more precisely. Probably the best term to indicate most of the older 'acidic mucopolysaccharides' is the term glycosaminoglycuronans, mentioning both the amino sugar and the uronic acid moiety. This term, however, does not cover all the substances belonging to this group; therefore the term glycosaminoglycans is more fequently encountered in recent literature, indicating only the common presence of an amino sugar component.

In this thesis this term will be used, as well as the nomenclature suggested further by Jeanloz (1960) and that used by Balazs and Jeanloz (1965).

1.3. AIM OF INVESTIGATION

In studies on bone formation the epiphyseal disk is the principal item of interest.

As mentioned earlier, the matrix contains two chief components: collagen and

glycosaminoglycans. A number of papers exist dealing with the possible changes in content and composition of the glycosaminoglycans in the epiphyseal disk (Hjertquist, 1964a,b; Guri and Bernstein, 1965; Campo and Tourtelotte, 1967; Lindenbaum and Kuettner, 1967; Greer et al., 1968; Hjertquist and Vejlens, 1968; Campo et al., 1969).

An attempt has been made to relate these findings to the mechanism of calcification and bone formation.

A great problem is that in endochondral ossification the cartilage is not replaced by bone, but bone is deposited upon calcified cartilage spicules by osteoblasts.

So the described changes in content and composition are related to calcification of cartilage rather than to bone formation.

The glycosaminoglycans usually found to be present in connective tissue are shown in figure I, 1 on page 10.

Hyaluronic acid and chondroitin sulphates are the most important ones in relation to cartilage and bone formation (a.o. Campo and Tourtelotte, 1967; Hjertquist and Vejlens, 1968) as well as during fracture callus formation (Antonopoulos et al., 1965; Solheim, 1966).

To elucidate the process of bone formation and to investigate the eventual role of glycosaminoglycans present at the time of onset of bone formation it would be better to investigate the intramembranous ossification. The purpose of the present investigation is the demonstration of the presence of glycosaminoglycans and their possible role in intramembranous ossification.

1.4. PLAN OF INVESTIGATION

Having the intention to investigate the presence and composition of glycosaminoglycans during bone formation, it was felt necessary to use both histochemical and biochemical methods. The materials and methods used in this investigation are given in Chapter II.

Histological sections and numerical data from biochemical extractions give in fact only a momentary, static, representation of the continuous, dynamic processes. Therefore experiments were also carried out with radioactive precursors to collect data about the metabolism of these glycosaminoglycans in relation to bone formation.

There is not only confusion about terminology, as mentioned earlier, but even more confusion about the value of many of the current methods used in the histochemistry of glycosaminoglycans. Therefore, it was essential to evaluate the methods used in this investigation. This evaluation is given in Chapter III.

The same has been done for the biochemical methods. Their characteristics and limitations are reported and discussed in Chapter IV.

The findings of the histochemical investigation are presented in Chapter V.

To clarify a special aspect of the metabolism of chondroitin sulphate, an autoradiographic study was made. This is described in Chapter VI. Chapter VII deals with the results of the biochemical investigations and Chapter VIII with the results of some precursor incorporation experiments.

Finally, the conclusions from the histochemical and biochemical approach are combined and discussed in Chapter IX.

MATERIALS AND METHODS

2.1. GENERAL INFORMATION

For the investigations underlying this thesis Wistar Albino rat fetuses were used. The parents originated from Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands. They were kept in stainless steel cages on Hope Farms' Standard Laboratory Diet R.M.H.-B. and water *ad libitum* in the Central Animal Laboratory of the Faculty of Medicine of this University.

A reliable determination of fetal age was necessary. Male and female rats were allowed to mate from 17.00 hours until 09.00 hours next day. At that time vaginal smears were made. Inseminated rats were put in cages, five in one cage, and kept under the conditions mentioned above.

As there is no agreement on the time of onset of pregnancy in rats (Kalter, 1968). midnight was assumed as such. The day on which the positive vaginal smear was made was noted as the first day of pregnancy.

From the sixteenth day of pregnancy to the nineteenth included rats were killed by decapitation at 17.00 hours and the fetuses were taken out.

Some of them were fixed for histological and histochemical examination and the rest frozen in acetone at $-15^{\circ}C$ for biochemical procedures.

2.2. HISTOCHEMICAL METHODS

2.2.1. *Chemicals*

Chemicals used for histochemistry were analytical grade reagents and microscopical stains from E. Merck AG, Darmstadt, W. Germany, and:

5-Aminoacridine hydrochloride	BDH, Poole, Gr. Britain.
Cetylpyridinium chloride	Th. Schuchardt, Munich, W. Germany.
Eukitt mounting medium	O. Kindler, Freiburg/Br., W. Germany.
Staphylococcal Hyaluronidase 10,300E/mg	Organon, Oss, The Netherlands.
Testicular Hyaluronidase 750 NF units/mg Type IV	Sigma, St. Louis, Mo, U.S.A.
Tissuemat 56, 5°C	Fisher Scientific Co., New York, N.Y., U.S.A.

2.2.2. *Fixation of tissues*

For histological investigation tissues were fixed in Heidenhain's SUSA mixture (Romeis, 1968).

For histochemical investigation tissues were fixed at 4°C in a 0.4 % solution of 5-aminoacridine hydrochloride in 70 % ethanol, containing 4% formaldehyde.

After fixation for 24—48 hours tissues were placed in 70 % ethanol for storage until further processing.

2.2.3. *Processing of tissues*

The fetuses were dehydrated in a graded ethanol series and embedded in Tissuemat.

Sections were cut serially 7 μm thick on a Leitz Minot microtome, affixed to Mayer's albumen coated slides and dried in an incubator at 37°C.

2.2.4. *Staining techniques*

For studying skeletal development sections were stained with:
Delafield's hematoxylin and eosin (H.E.),
Van Gieson's connective tissue stain,
Masson's trichrome stain modified after Goldner,
Von Kossa's calcium method,
all as described by Adam and Czihak (1964) and Romeis (1968).

For studying glycosaminoglycans sections from different fetuses of different age groups were stained simultaneously in the same staining solution to enable accurate comparison of staining properties of the tissues. They were stained in the following manner:

Periodic acid – Schiff (PAS): Sections were deparaffinized and brought to distilled water, immersed in 1% aqueous periodic acid for 5 minutes, washed thoroughly in distilled water, incubated with Schiff's reagent for 20 minutes, washed twice in a sulphite bath for 10 minutes, washed in water, dehydrated in a graded ethanol series, cleared and mounted in Eukitt.

Astrablau – PAS: Sections were deparaffinized and brought to distilled water, stained for 10 minutes in a 0.1% solution of Astrablau in 1% acetic acid, washed in distilled water and treated further as described for the PAS staining method.

Hale's colloidal iron, modified after G. Müller, as cited by Adam and Czihak (1964):

Stock solution: 750 ml of distilled water, heated to boiling, mixed with 12 ml of a 32 % solution of ferric chloride.

Staining solution: 100 ml of stock solution diluted with 10 ml of glacial acetic acid.

Sections were deparaffinized and brought to distilled water, incubated for 10 minutes in colloidal iron solution, washed five times for 2 minutes in distilled water, stained for 10 minutes in a freshly prepared solution of 1 gram of potassium ferrous cyanide in 100 ml of 1 % w/v hydrochloric acid, washed in distilled water and counterstained with nuclear fast red, washed, dehydrated in a graded ethanol series, cleared in xylene and mounted in Eukitt.

Hale's colloidal iron after staphylococcal hyaluronidase digestion: Sections were deparaffinized and brought to distilled water, incubated with staphylococcal hyaluronidase at a concentration of 2 mg per ml of saline for 3 hours, washed in distilled water and stained with colloidal iron as indicated above.

Hale's colloidal iron after testicular hyaluronidase digestion: Sections were treated as with staphylococcal hyaluronidase except that the concentration was 1 mg per ml of saline and the incubation period was 2 hours.

Astrablau 0.05 M $MgCl_2$: For this and the next two staining methods the following stock solutions were used:

0.1 M acetate buffer pH 4.0

5 M magnesium chloride ($MgCl_2$) in distilled water

1 % Astrablau in distilled water with a small crystal of thymol added against mould growth.

Sections were deparaffinized and brought to distilled water, stained for 30 minutes in 0.1 % Astrablau in 0.025 M acetate buffer pH 4.0, containing 0.05 M $MgCl_2$, washed in distilled water, dehydrated in a graded ethanol series, cleared in xylene and mounted in Eukitt.

Astrablau 0.3 M $MgCl_2$: Sections were treated in the same way as in 0.05 M $MgCl_2$, but the staining solution was made up to 0.3 M $MgCl_2$ in 0.025 M acetate buffer pH 4.0.

Astrablau 0.5 M $MgCl_2$: Sections were treated as above, but the solution was now made up to 0.5 M $MgCl_2$ in 0.025 M acetate buffer pH 4.0.

Toluidine blue: Sections were deparaffinized and brought to distilled water, stained for 5 minutes in a 0.05 % solution of toluidine blue in distilled water, washed in distilled water, dehydrated in 3 rinses of acetone for 5 seconds each, cleared in xylene and mounted in Eukitt.

2.2.5 *Autoradiography*

Pregnant rats were injected intraperitoneally with a single injection of a solution of $Na_2{}^{35}SO_4$ in saline at a dose of 250 μCi per 100 grams of body weight. The rats were sacrificed 24 or 48 hours after administration of the radioactive substance. The fetuses were taken out and fixed in Bouin's solution. After processing as described above, the sections were coated with strips of Kodak AR 10 film or with Ilford K5 emulsion. The slides were developed after 2–3 weeks and stained with toluidine blue as described by Bergeron (1958).

2.2.6. *Microphotography*

Microphotographs were made with a Zeiss Contarex camera fitted on a Zeiss Universal microscope. (Carl Zeiss, Oberkochen/Württ., W. Germany). Gevaert-Agfa Scientia 45C62 and 50B65 35 mm film were used in combination with filters as indicated in the legends to the figures.

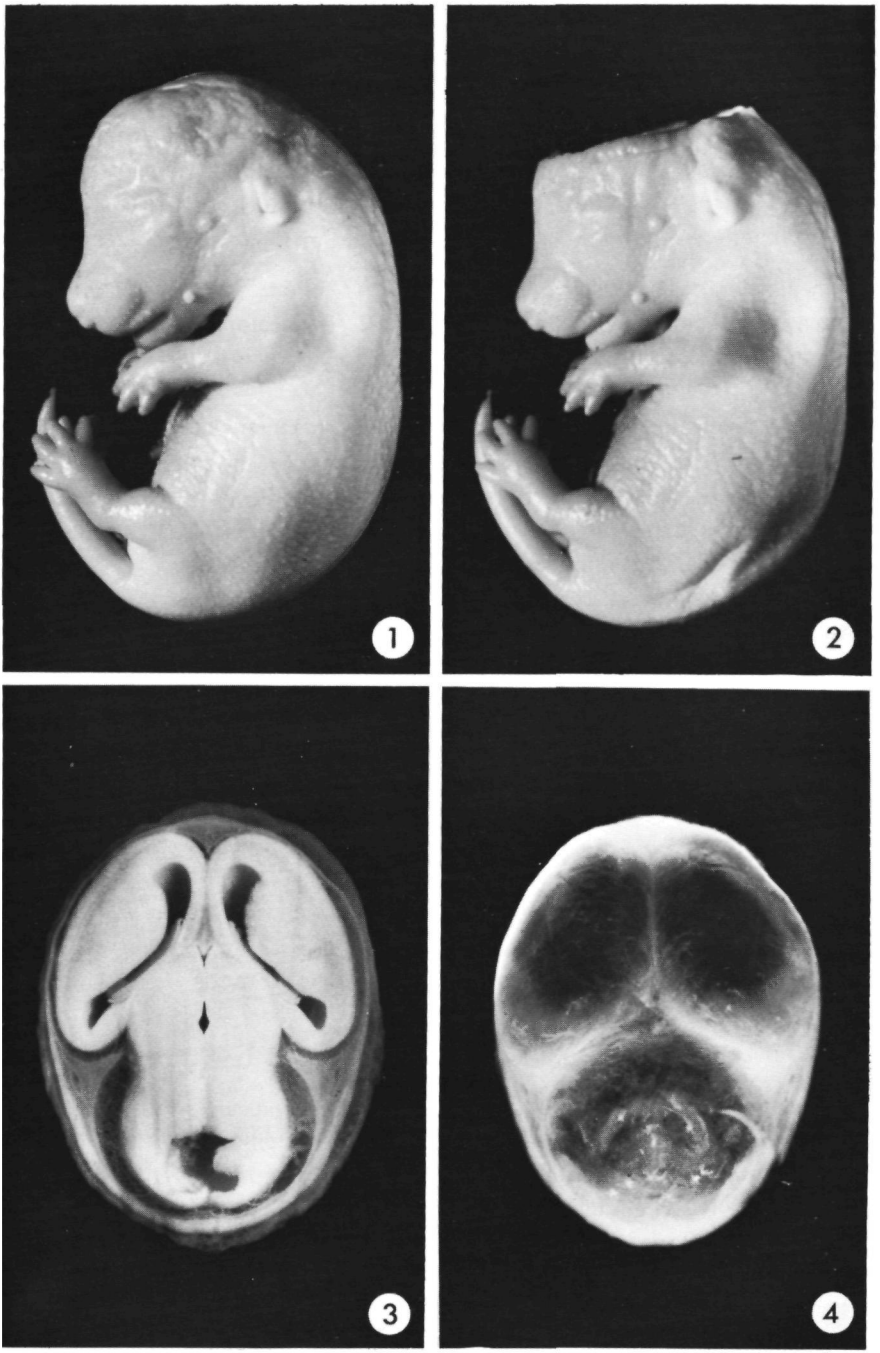
2.3. BIOCHEMICAL METHODS

2.3.1. *Chemicals and reference compounds*

Chemicals used were analytical grade reagents from E. Merck AG, Darmstadt, W. Germany, and:

N-Acetylglucosamine	BDH, Poole, Gr. Britain.
Carbazole	BDH
Chondroitinsulphate, mixed isomers Grade III	Sigma, St. Louis, Mo, U.S.A.
DEAE-Sephadex A 25	Pharmacia AB, Uppsala, Sweden.
Dimethyl-POPOP 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-Benzol	Packard Instruments S.A., Brussels, Belgium.
D-Galactosamine hydrochloride	Sigma
D-Galactosamine-1-^{14}C hydrochloride CFA. 345	The Radiochemical Centre, Amersham, Gr. Britain.
D-Glucosamine hydrochloride	BDH
D-Glucosamine-1-^{14}C hydrochloride CFA. 346	The Radiochemical Centre
D-Glucose-^{14}C(U) CFB. 96	The Radiochemical Centre
D-Glucuronic acid	BDH
Heparin Grade I	Sigma

FIGURE II,1 Different phases of preparation of tissue samples.
1. Rat fetus on the 18th day of gestation. X 4 2. Same fetus after removal of the top of the cranium. 3. Inside view of the top of the cranium. Skin, dura and brain-tissue are visible from outside inwards. X 8 4. Dura alone after removal of skin and brain-tissue. Samples like this one are used for biochemical extractions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2
3
4

Hyaluronic acid Grade I	Sigma
PPO 2,5-Diphenyloxazol	Packard Instruments S.A.
Sodium borohydride	May and Baker, Dagenham, Gr. Britain.
Sodium sulphate-^{35}S DRN 1601	N.V. Philips-Duphar,Petten, The Netherlands.
Triton X-100	Serva Entwicklungslabor, Heidelberg, W. Germany.

2.3.2. *Preparation of tissues*

Immediately after being taken out of the mother complete fetuses or fetal heads were placed and stored in cold acetone at −15°C. The acetone was changed twice within the first 24 hours. The top of the neurocranium was cut off with a scalpel (Fig. II,1).

Care was taken to exclude frontal or occipital cartilaginous portions. With pairs of watchmakers tweezers the calvaria were then freed from the adherent brain- and skin-tissue under a stereomicroscope at low magnification. However, this proved to be impossible for all of the investigated age groups. At 16 and 17 days of prenatal age only the brain-tissue could be removed.

The separated skin-tissue was treated further in the same way as the calvaria to serve as a control and as a reference.

Preceding the extraction procedures specimens were pooled according to age, dried *in vacuo* and weighed.

Subsequently, the tissues were minced with a pair of scissors and homogenized twice for 10 seconds with an Ultra Turrax model TP 18/2 N at top speed (20,000 RPM) in cold acetone in a centrifuge tube.

The homogenate was centrifuged at 3,500 RPM for 10 minutes and the precipitate taken up in 10 ml of ethanol-diethylether 1 : 1. After 16 hours at 4°C the tube was centrifuged again at 3,500 RPM for 10 minutes and the precipitate dried *in vacuo* and stored over silica gel.

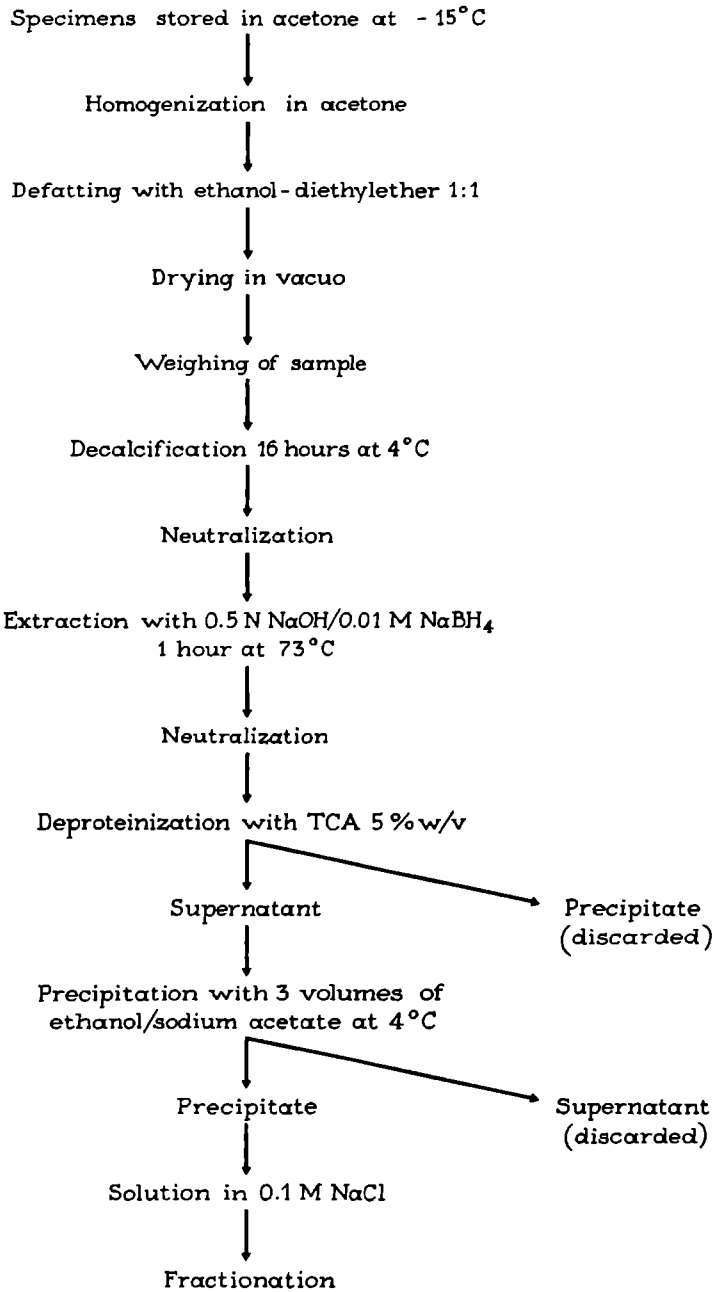
This precipitate will be referred to further as acetone powder.

2.3.3. *Extraction of tissues*

Weighed samples of ± 30 mg of acetone powder were taken up in 1.0 ml of 0.01 N hydrochloric acid in stoppered centrifuge tubes for decalcification. After 16 hours at 4°C the contents were neutralized with 0.1 ml of 0.1 N sodium hydroxide.

1.1 ml of 1 N sodium hydroxide, containing 0.02 M sodium borohydride was added, and the mixture hydrolyzed for 1 hour at 73°C in a thermostat-controlled waterbath. After cooling to room temperature the mixture was neutralized with 0.11 ml of 10 N hydrochloric acid, cooled down further to 4°C and mixed with 0.77 ml of cold 20% w/v aqueous trichloroacetic acid.

ISOLATION OF GLYCOSAMINOGLYCANS



After 10 minutes at 4°C the tubes were centrifuged for 20 minutes at 3,500 RPM and decanted. The residue was washed with 1.0 ml of cold 5% w/v aqueous trichloroacetic acid and centrifuged at 3,500 RPM for 10 minutes. The precipitate was discarded.

The supernatants were combined and made up to 75% ethanol with 12.25 ml of cold ethanol saturated with sodium acetate and kept at 4°C for 24 hours. The tubes were centrifuged for 20 minutes at 3,500 RPM, decanted and the precipitate dissolved in 1.0 ml of distilled water. Glycosaminoglycans were precipitated again with 3 ml of ethanol saturated with sodium acetate. After 24 hours at 4°C the precipitate was centrifuged and dissolved in 1.1 ml of 0.1 M sodium chloride pH 7.0 for fractionation. This procedure is schematically presented on page 19.

2.3.4. *Fractionation of glycosaminoglycans*

2.3.4.1. Ion exchange chromatography

A column of 0.9 x 5 cm of DEAE-Sephadex A 25 was prepared as indicated in the instructions of the manufacturers. The column was equilibrated with 0.1 M sodium chloride pH 7.0. The sample was allowed to drain in by gravity and eluted stepwise with the following solutions:

15 ml of 0.1 M sodium chloride pH 7.0
30 ml of 0.5 M sodium chloride pH 7.0
15 ml of 0.75 M sodium chloride pH 2.0
40 ml of 1.5 M sodium chloride pH 2.0
10 ml of 3 M sodium chloride pH 7.0

The flow rate was kept constant at 0.5 ml per minute by means of a peristaltic pump. Fractions of 2.5 ml were collected in a LKB fraction collector regulated by the time-controller. Four columns could be run simultaneously.

The contents of each tube were tested for the presence of glycosaminoglycans by the carbazole method as described in paragraph 2.3.5. of this chapter. The contents of positive tubes were then pooled peak by peak, dialyzed against distilled water in Visking dialysis tubing for 24 hours and lyophilized.

2.3.4.2. Electrophoresis

Electrophoresis was performed on Sepraphore III cellulose polyacetate strips 2.5 x 17 cm (Gelman Instr. Cy., Ann Arbor, Mich., U.S.A.), according to the method described by Stefanovich and Gore (1967), in a Shandon Universal Electrophoresis Apparatus after Kohn, at a constant current of 2 mA per strip for 1 hour.

2.3.5. *Analysis*

Tubes that might contain glycosaminoglycans were tested for uronic acid contents with the carbazole method as described by Bitter and Muir (1962) using 0.5 ml of sample, 3.0 ml of sulphuric acid reagent and 0.1 ml of carbazole solution.

The standard curve was prepared from D-glucuronic acid.

Glycosamine and galactosamine were determined in the pooled and lyophilized material following the procedure of Good and Bessman (1964).

2.3.6. *Calcium determination*

For the determination of calcium both the method described by Ray Sarkar and Chauhan (1967) and flame photometry (MacIntyre, 1957) were used for samples prepared in the following manner:

1 mg of acetone powder was extracted with 10 ml of 0.3 N hydrochloric acid for 24 hours at room temperature and centrifuged.

Samples were allowed to have a maximum calcium concentration of 6 micrograms per ml. In order to obtain this, extracts from calvaria of 16 to 19 days of prenatal age were not diluted, those from 19 to 22 days of prenatal age were diluted 1 : 1 with 0.3 N hydrochloric acid and extracts from calvaria older than 21 days of prenatal age were diluted 1 : 2. All glass equipment used in the calcium determination was treated beforehand for 24 hours with 0.3 N hydrochloric acid and air dried.

2.3.7. *Liquid-scintillation counting*

For some precursor incorporation experiments pregnant rats were injected intraperitoneally with a single dose of a solution of a radioactive substance in saline as listed below:

^{14}C-galactosamine, spec.act. 3.5mCi/mM	3.3μCi/100 grams of body weight
^{14}C-glucosamine, spec.act. 3.7mCi/mM	2,5μCi/100 grams of body weight
^{14}C-glucose, spec. act. 309mCi/mM	12.5μCi/100 grams of body weight
Sodium ^{35}S-sulphate, carrier-free	250μCi/100 grams of body weight

The rats were sacrificed 24 or 48 hours after injection and the fetuses taken out.

They were treated in the same way as described above in paragraph 2.3.2., 2.3.3. and 2.3.4.1.

From the carbazole positive tubes 1.0 ml was brought into a liquid-scintillation vial containing 10 ml of a mixture of 2 parts toluene and 1 part Triton X-100 in which 5.5 grams of PPO and 0.1 gram of dimethyl-POPOP were dissolved per liter.

The vials were counted in a Packard Tricarb liquid-scintillation spectrometer system model 3375 at 10°C.

EVALUATION OF HISTOCHEMICAL METHODS

3 1 FIXATION OF TISSUES

There is no general agreement on the best fixative for glycosaminoglycans The final conclusion of the Deutsche Gesellschaft fur Histochemie at the VIII Symposium at Vienna in 1962 (Lindner, 1965) was that formalin still came out as the most satisfactory one

This conclusion is correct in so far as we are dealing with glycosaminoglycans bound to tissue proteins In this case formalin fixes the proteins and keeps the glycosaminoglycans within the tissues, even when they come into contact with water On the contrary, the glycosaminoglycans not linked to tissue proteins are not really fixed, but more or less occluded within the tissue proteins and will dissolve and diffuse if they come into contact with water

As we wish to deal with the early stages of synthesis of glycosaminoglycans we can expect glycosaminoglycans not linked to tissue proteins Therefore we need a fixative that also fixes these substances Curran (1964) reviewed the effects of a number of different fixatives He came to the conclusion that fixatives that form a water insoluble complex with glycosaminoglycans are the best Fixatives that form such a complex with glycosaminoglycans are described by Williams and Jackson (1956) They recommended 0 5% cetylpyridinium chloride in 4% aqueous formaldehyde or 0.4% 5-aminoacridine hydrochloride in 50% aqueous ethanol

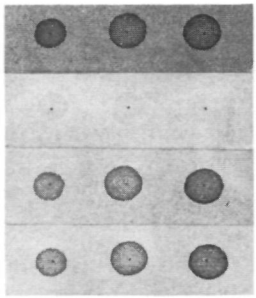
We have tested both solutions 2 µl Samples of glycosaminoglycans were spotted on filter paper They were placed in different fixatives for 5 minutes and washed in tap water. The spots were stained with a 0.1% Astrablau solution for 5 minutes and compared with unfixed spots directly stained.

The test-substances are specified in 2 3.1 (page 16)

The results are shown in figure III,1.

After cetylpyridinium chloride fixation staining is completely absent, while after 5-aminoacridine hydrochloride fixation, even after prolonged washing in tap water, the same staining intensity as in unfixed spots is seen This means that a 0 1% aqueous solution of Astrablau is not able to dissociate the cetylpyridinium-glycosaminoglycan complex and to form a new one with the glycosaminoglycan moiety, at least not within the staining time of 5 minutes applied here

Theoretically, the possibility exists that the glycosaminoglycans are dissolved in



unfixed control

0.5 % cetylpyridinium chloride

5-aminoacridine in 50 % ethanol

5-aminoacridine in 70 % ethanol
with 4 % formaldehyde

FIGURE III,1. Test of fixatives.

2 μg samples of hyaluronic acid (left), chondroitin sulphate (middle) and heparin (right) are spotted on filterpaper and stained after the indicated fixation with 0.1 % Astrablau pH 4.0 for 5 minutes.

the cetylpyridinium solution. This is the case with substances of low molecular weight in relation to the molecular weight of the cetylpyridinium micelle, but the presence of salt (Scott, 1968) can prevent this. It was shown with the ion association technique (Zugibe and Fink, 1966) that cetylpyridinium-glycosaminoglycan complexes were still present on the filter paper.

We can state that cetylpyridinium chloride gives too strong a complex; it blocks the substances of interest.

With 5-aminoacridine hydrochloride this is not the case; it also gives complexes, but these can be dissociated by the staining solution and new ones are formed.

Generally, fixation is optimal if it takes place at a low temperature; this is impossible, however, with cetylpyridinium chloride because it crystallizes below 25°C.

Because of the indicated disadvantages of the cetylpyridinium solution we chose 5-aminoacridine as the fixative to be used for the histochemistry of glycosaminoglycans.

In order to prevent shrinkage and to improve the preservation of the morphology of the tissues we changed the alcohol concentration of the 5-aminoacridine hydrochloride solution to 70 % and added 4 % formaldehyde.

3.2. STAINING TECHNIQUES

Up to now there have been no conclusive staining procedures that adequately differentiate the different glycosaminoglycans. Many prescriptions are given in the literature, and we will discuss here the methods we used and give the arguments as to why we chose them.

3.2.1. *Periodic acid – Schiff (PAS)*

This is a staining method which is chemically well understood and most widely used in carbohydrate research.

When paraffin embedded tissues are treated, a red stain may be regarded as nearly specific for neutral carbohydrates. Pearse (1968) gives an excellent review about the use and mechanism of the reaction.

Periodic acid is an oxidant which breaks the C-C bond in various structures where these are combined with vicinal hydroxylgroups (CHOH–CHOH) or with vicinal hydroxyl- and aminogroups ($CHOH–CHNH_2$), thereby converting them into aldehyde groups. These can be detected by combination with Schiff's reagent. A large number of carbohydrates contain these groups and give a positive result, but compounds in which the hydroxyl- or aminogroups are substituted do not. Hyaluronic acid and chondroitin sulphate have substituted groups in the hexosamine moiety and are PAS negative, as demonstrated among others by Hooghwinkel and Smits (1957).

In the uronic acid moieties the hydroxyl groups react very slowly with periodate, but do not form any demonstrable aldehyde groups (Hooghwinkel and Smits, 1957). Complete reaction takes 5 days (Hoffman, 1968a).

Glycans, sialoglycans, glycopeptides are, among others, PAS positive tissue compounds. The glycan which is stored within the cytoplasm of connective tissue cells is glycogen.

Several carbohydrates are found to be involved in binding glycosaminoglycuronoglycans to proteins (Helting and Rodén, 1968). They are also found to be bound firmly to collagen (Fullmer, 1965).

So if we see a positive reaction in our material, we are demonstrating carbohydrate, but it is impossible to conclude with which one of the mentioned substances we are dealing with, except that we can be sure they are neither hyaluronic acid nor chondroitin sulphate.

3.2.2. *Astrablau-PAS*

This staining method is a combination of the above mentioned periodic acid - Schiff reaction with the reaction of glycosaminoglycans with Astrablau which will be discussed below.

The combination of both reactions offers a good differentiation between neutral carbohydrates and glycosaminoglycans in the same tissue section.

3.2.3. *Hale's colloidal iron method*

This method depends on the affinity of acidic groups for ferric ions at pH 2.0.

The bound ions are demonstrated by conversion to prussian blue.

Hale (1946) stated that this method would stain non-sulphated and sulphated glycosaminoglycans. Although the method was not highly specific, it was a useful one.

After its introduction many attempts have been made to improve this method, and to investigate its specificity.

Müller (1955) modified the staining solution into colloidal ferric hydroxide. This increased the specificity and reduced staining of cytoplasm and nuclei. It is accepted that this solution is able to stain both unsulphated and sulphated glycosaminoglycans.

In order to make a distinction between these two groups, we treated the sections with different types of hyaluronidase and stained them afterwards with colloidal iron.

Staphylococcal and testicular hyaluronidase digestion: Different types of hyaluronidase are often used to distinguish between unsulphated and sulphated glycosaminoglycans, i.e. hyaluronic acid and chondroitin sulphates.

Staphylococcal hyaluronidase specifically breaks down hyaluronic acid, while testicular hyaluronidase digests both hyaluronic acid and chondroitin sulphates as mentioned by Brimacombe and Webber (1964). We checked our enzymes on substrate-impregnated gelatin plates and according to the spectrophotometric method of Nakada et al. (1960).

As a result we can state that the staphylococcal hyaluronidase used is highly specific for hyaluronic acid, but the testicular hyaluronidase used shows great proteolytic activity besides its ability to break down both hyaluronic acid and chondroitin sulphates.

3.2.4. *Astrablau*

Copperphthalocyanin dyes, and Alcian blue 8 GS in particular, were introduced by Steedman (1950) as stains for mucins.

Since that time many papers have been published about the use of Alcain blue and similar dyes.

A number of publications by Scott, Quintarelli and Dellovo (Scott et al., 1964; Quintarelli et al., 1964a,b; Quintarelli and Dellovo, 1965) describe the chemical and histochemical properties of Alcian blue.

Scott and Dorling (1965) give the prescriptions for a differential staining method for glycosaminoglycans with Alcian blue:

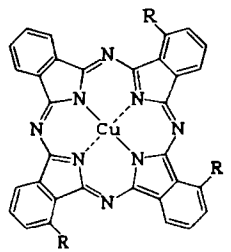
Saunders (1964) used a similar differential technique with acridine orange for the histochemical identification of these substances.

All the methods described go back to the finding of Scott (1955) that glycosaminoglycans are precipitated by ammonium compounds, which precipitates dissolve on the addition of inorganic salts.

It appeared that solution of a particular precipitate depends upon the salt concentration, and that each glycosaminoglycan precipitate had its own so-called critical electrolyte concentration.

Pioch (1957) introduced another phthalocyanin dye for the detection of glycosaminoglycans: Astrablau, trademark of Bayer Werke, Leverkusen, W. Germany. This basic dye has the net formula $C_{53}H_{64}CuN_{14}O_{12}S_3$ and a molecular weight of 1248.94.

The molecular structure is shown in figure III,2.



$$R = -SO_2-\underset{H}{N}-C-C-C-N(CH_3)_2 \cdot CH_3COOH$$

FIGURE III,2. Structural formula of Astrablau.

Bloom and Kelly (1960) also investigated the staining properties of Astrablau. They came to the conclusion that under standardized conditions Astrablau shows a greater specificity for glycosaminoglycans than does Alcian blue. Moreover, it appeared to be highly suitable for microspectrophotometric procedures. Therefore, we chose Astrablau for our investigations instead of Alcian blue.

Astrablau also belongs to the group of ammonium compounds mentioned above, and so we tried to determine the critical electrolyte concentration for that dye.

Model experiments on strips of filter paper: 2 µl samples of glycosaminoglycans, specified in Chapter II, at a concentration of 1 mg per ml distilled water were spotted on strips of Whatman no. 1 filter paper and dried in air.

The strips were placed in a 0.1% solution of Astrablau in 0.025 M sodium acetate buffer pH 4.0 containing different amounts of $MgCl_2$. After 10 minutes the strips were taken out of the solution, washed in tap water for 5 minutes and dried.

The experiments were repeated at pH 2.0 without, and at pH 4.0 with, 5-amino-acridine fixation before staining.

The results are shown in figure III,3.

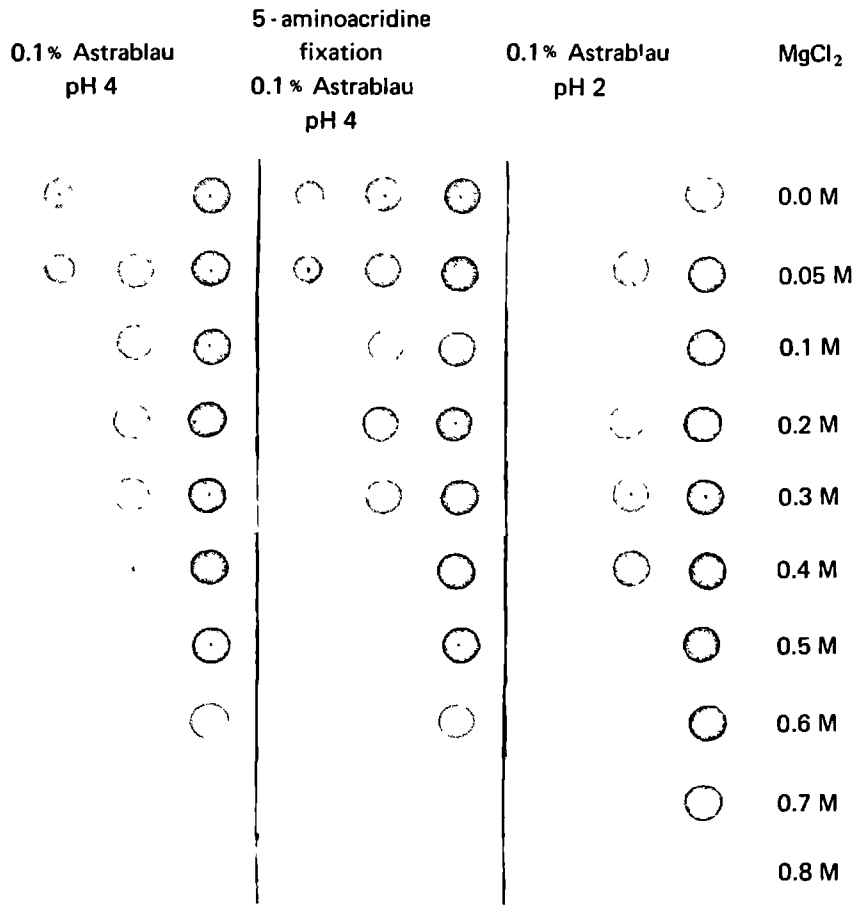


FIGURE III,3. Test of different electrolyte concentrations in Astrablau staining.

2 μg samples of hyaluronic acid (left), chondroitin sulphate (middle), and heparin (right) are spotted on filterpaper and stained after the indicated fixation with 0.1% Astrablau at the pH and concentrations of $MgCl_2$ indicated.

The first thing seen was a background suppression in the presence of a small amount of electrolyte: 0.05 M $MgCl_2$. This can be explained by the fact that a low electrolyte concentration neutralizes the negative charges in the filter paper. The

same will be seen in tissue sections: negative charges of proteins are shielded.

As the concentration of $MgCl_2$ increased first hyaluronic acid failed to stain (> 0.2 M), then chondroitin sulphate (> 0.4 M), and finally heparin (> 0.7 M).

At pH 2.0 we see that the critical electrolyte concentrations are shifted to higher values, just as indicated by Scott and Dorling (1965) but the staining of hyaluronic acid is less than at pH 4.0.

After 5-aminoacridine fixation the critical electrolyte concentrations were the same as without fixation.

Our conclusion is that by means of this staining pattern of Astrablau at different concentrations of $MgCl_2$ we can distinguish unsulphated from sulphated glycosaminoglycans qualitatively. In practice, however, it will be rather difficult to distinguish them quantitatively.

Scott and Dorling (1965) found that the critical electrolyte concentration in filter paper tests is generally somewhat lower than in tissue, so we chose 0.05 M, 0.3 M and 0.5 M $MgCl_2$ as concentrations for the differential staining of tissue sections.

We checked these concentrations on section of rat epiphyseal cartilage.

3.2.5. *Toluidine blue*

This dye can show metachromasia under specific conditions. Primarily, it is a basic stain which combines with negatively charged molecules. Water molecules can be intercalated between the dye molecules when these are at a certain distance from each other. The distance between charged groups on some glycosaminoglycans is such that they have the potential to form these toluidine blue-water aggregates. Hyaluronic acid has obviously not the minimum distance between the negative charges to create metachromasia and can therefore not be detected by toluidine blue staining.

Sulphated glycosaminoglycans are stained metachromatically with toluidine blue because they have a smaller distance between the negatively charged groups than hyaluronic acid (Pearse, 1968).

As mentioned above, water molecules are important for metachromasia of this kind. Therefore it is impossible to dehydrate the sections completely before mounting.

Dehydration with three short rinses of acetone, 5 seconds each, gave almost the same results for permanent slides, as for water-mounted sections.

Summarizing we can state that the staining techniques discussed above were chosen for the following reasons:

PAS: to detect neutral carbohydrates,

Astrablau - PAS: to distinguish neutral carbohydrates from glycosaminoglycans

in the same section,

Hale's colloidal iron with hyaluronidase treatment: to distinguish between different glycosaminoglycans,

Astrablau with different concentrations of magnesium chloride: to distinguish between unsulphated and sulphated glycosaminoglycans,

Toluidine blue: to detect sulphated glycosaminoglycans.

EVALUATION OF BIOCHEMICAL METHODS

4.1. EXTRACTION

4.1.1. *Literature*

The main problem in the analysis of pure glycosaminoglycans is the dissolution of these substances out of the tissues in which they are more or less firmly bound to other structural substances such as collagen.

Because the aim of the investigation is the comparison of the amounts of different glycosaminoglycans present at several stages of development of the same tissue, a reproducible percentage of extraction of each glycosaminoglycan is necessary.

It may be possible, however, that these percentages change from stage to stage. In that case, comparison of the amounts of different glycosaminoglycans becomes rather difficult.

Therefore it is necessary to get the glycosaminoglycans completely into solution.

Many methods have been used in the past for the extraction of particular glycosaminoglycans, which can broadly be divided into three groups of extraction procedures:

water or saline extraction
alkaline extraction
proteolytic enzyme digestion

A short review of these methods is presented here.

4.1.1.1. Water or saline extraction

The single blending of tissues with water or saline offers mild conditions for glycosaminoglycan extraction, although complexed with protein.

From tissues such as synovial fluid or nasal cartilage it is possible to extract hyaluronic acid or chondroitin sulphate as glycosaminoglycan - protein complexes.

Under these mild conditions, however, only the soluble glycosaminoglycan - protein complexes are extracted.

It has been proven that it is impossible to extract all the glycosaminoglycans from

some tissues without first causing almost complete protein breakdown.

Besides, fractionation will be beter if the amount of protein in the extract is minimal.

Therefore, other methods such as alkaline extraction and proteolytic enzymes are more suitable for general purposes. (Scott, 1960; Brimacombe and Webber, 1964).

4.1.1.2. Alkaline extraction

In cases where it is necessary to obtain glycosaminoglycans practically protein-free this method offers good prospects, but loss of material may occur. The use of sodium hydroxide for the extraction of glycosaminoglycans was abandoned more or less because it degraded the glycosaminoglycan-protein complex too far. This occurred especially in those cases where the protein linkages were of interest.

Hoffman (1968b) compared different methods in order to obtain glycosaminoglycans with different amounts of protein to study the nature of the linkages. With a solution of sodium hydroxide mixed with sodium borohydride as an anti-oxidant he extracted calf nasal septum material for a short time at high temperature. The yield expressed as the percentage of dry weight was the same as for the other methods, but the protein content was almost negligible. The addition of the anti-oxidant and a short time of extraction apparently minimize the loss of material previously encountered.

4.1.1.3. Proteolytic enzyme digestion

Proteolytic enzymes have been widely used in the extraction of glycosaminoglycans, and the results have been quite satisfactory. It is assumed that there is no action on the glycosaminoglycans themselves (Scott, 1960). The possibility exists that not all the protein is removed from the glycosaminoglycans, and that by subsequent deproteinization the glycosaminoglycans bound to this protein are lost.

A variety of proteolytic enzymes have been used by different investigators.

Pepsin has the possible disadvantage of its low optimum pH (pH 1.0-2.0), and native collagen is not attacked by trypsin without pre-treatment in order to break the hydrogen bonds. This pre-treatment, mostly done by boiling with water, also seems necessary when using proteolytic enzymes from plants or bacteria. Papain, bromelin and ficin are such enzymes prepared from plants. Especially papain is used by many investigators (Scott, 1960; Schiller et al., 1961; Greiling et al., 1964; Searls, 1965; Solheim, 1965; Lovell et al., 1966; Stefanovich and Gore, 1967).

One of the disadvantages of papain is the fact that polyanions liberated inhibit

the proteolytic activity and form complexes with the enzyme which even can precipitate. This may be overcome by adding strong salt solutions, for hyaluronic acid up to 0.3 M and for chondroitin sulphate even up to 1.0 M. Most of the authors mentioned above do not pay any attention to this because their material is in excess compared to the amount of enzyme. In the case of very little material however, this must be kept in mind.

Bacterial proteases are also used for extraction (Svejcar and Robertson, 1967) with the same results and disadvantages as mentioned for papain.

After proteolytic digestion the glycosaminoglycans are recovered mostly by deproteinization and ethanol precipitation. Extracted substances are then described as 'pure glycosaminoglycans'. In fact they contain still a number of amino acid residues as shown by Hoffman (1968b).

4.1.2. *Choice of method*

The papain method as described by Scott (1960) was applied to acetone powder of pig nasal septum cartilage, as a trial for the calvarial tissues to be studied later on.

Even after prolonged incubation at 60 °C there was a high amount of residue. The total neutralized digestion mixture was applied to an anion exchange column to fractionate the glycosaminoglycans by column chromatography without further treatment after the enzymatic digestion, but papain appeared to interfere with the carbazole reaction in the eluates. So it was necessary to deproteinize, which was done with 5 % trichloro-acetic acid (TCA). Even after this treatment there was still an interference with the carbazole reaction. Control experiments demonstrated that the interfering substances were derived from the papain, probably as oligopeptides. To overcome this the glycosaminoglycans were isolated after TCA treatment by precipitation with three volumes of ethanol saturated with sodium acetate.

After these preliminary experiments the same method was applied to acetone powders of calvaria. Because this material contains bony structures a decalcification step was necessary.

In order to incur no loss of glycosaminoglycans during this step, decalcification was done in the extraction tube with 1 ml of a 0.1 M EDTA solution buffered at pH 6.35. After standing for 24 hours at 4 °C 5 mg of papain and 0.6 mg of cystein dissolved in 1 ml of a 0.1 N sodium acetate buffer of pH 6.35 were added, and the tube placed at 60 °C for 24 hours.

Undissolved material was spun down, and the precipitate extracted for another 24 hours at 60 °C with the same buffer solution and papain concentration. After deproteinization and ethanol precipitation the glycosaminoglycans were recovered, dissolved in water and fractionated on an ion exchange column.

By stepwise checking of the procedure it appeared that:

extraction was not complete, because extraction of the sediment with sodium hydroxide yielded additional glycosaminoglycans,

papain precipitated previously dissolved material because of the excess of this enzyme,

admixtures of the crude papain were not precipitated by TCA, but were precipitated by ethanol.,

EDTA was occluded within, or precipitated with, the ethanol precipitated material and interfered with the carbazole reaction afterwards.

The interference of the EDTA was overcome by using 0.01 N HCl as decalcifying solution with neutralization afterwards. To overcome the other shortcomings of this method the alkaline extraction method as given by Hoffman (1968b) was investigated.

This resulted in the procedure as given in 2.3.3. (page 18).

4.1.3. *Control experiments*

Hoffman (1968b) added $NaBH_4$ to prevent oxydative degradation, but he did not mention the concentration used. Different concentrations were tried, and 0.01 M was found to be optimal.

The result of a typical control experiment on the influence of $NaBH_4$ is given below.

Pig nasal septum cartilage 20 mg sample 1 hour 73°C

SOLVENT	COLOR	YIELD*
0.5 N NaOH	Brown	12%
0.5 N NaOH/0.01 M $NaBH_4$	Yellowish	30%

*Determined with the carbazole reaction, expressed as % of dry weight of the sample.

Another control experiment was made with the reference substances specified in 2.3.1.

Hyaluronic acid, chondroitin sulphate and a combination of both were treated in exactly the same way as in the extraction procedure given in 2.3.3.

The results are given below.

Recovery percentages of reference substances after alkaline extraction with $NaBH_4$

SAMPLE	WEIGHT	RECOVERY *
Hyaluronic acid	2 65 mg	85±1%
Chondroitin sulphate	2 20 mg	92±1%
Hyaluronic acid + Chondroitin sulphate	1 50 mg + 1 75 mg	87±2%

*Determined with the carbazole reaction

4 2 FRACTIONATION BY ION EXCHANGE CHROMATOGRAPHY

4 2.1. *Literature*

To fractionate glycosaminoglycans a number of different techniques have been used in column chromatography Scott (1955, 1960) based the fractionation on the selective dissociation of cetylpyridinium - glycosaminoglycan complexes with increasing ion concentration. This method was further developed by Schiller et al. (1961), and is now frequently used in the modification of Antonopoulos et al. (1964). In several other methods anion exchangers, such as Dowex 1 (Schiller et al., 1961), ECTEOLA-cellulose (Ringertz and Reichard, 1960) and DEAE-Sephadex (Schmidt, 1962) are used.

An investigation on the optimal conditions for these anion exchangers was published by Pearce et al. (1968) However, exact figures for the recovery percentages were only given for resin columns, and most experiments were made with Dowex 1.

4.2 2. *Choice of method*

Since Schmidt (1962) in his communication on the fractionation of glycosaminoglycans showed a clear-cut separation of the mixture applied to a DEAE-Sephadex column, this method was chosen.

It seemed possible to adapt this method to a microscale, in order to fractionate the very small amounts of material available in our study

4.2.3. *Control experiments*

As shown in the leaflet of the manufacturer, DEAE-Sephadex A-25 has more than one type of charged groups. This could explain why at pH 2 0 a separation of sulphated glycosaminoglycans is obtained, and why no separation occurs at pH 7.0 To obtain reproducible results the exact equilibration of DEAE-Sephadex

A 25 with respect to pH is a prerequisite In order to get reproducible fractionations is was tried to elute with a continuous gradient of NaCl, but a change in pH from 7 0 to 2 0 after the gradient had reached a molarity of 0 5 was hardly obtainable. As a consequence no complete separation resulted.

Therefore elution was carried out by stepwise changes in molarity as described in 2 3 4 1

The result of a typical fractionation is shown in figure IV, 1

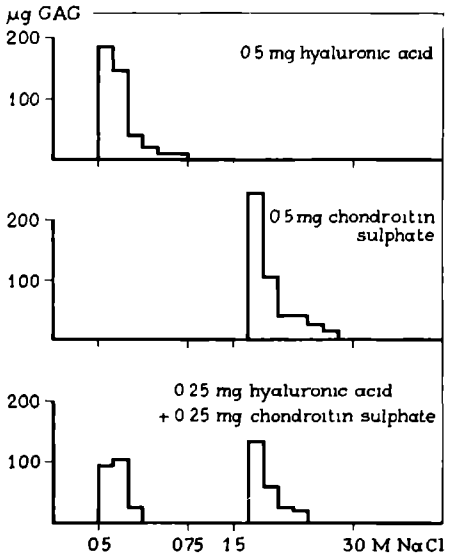


FIGURE IV,1 Elution patterns of commercial glycosaminoglycans on DEAE Sephadex columns

At a later stage of the investigation a 0 75 M fraction was introduced in the elution system between the 0 5 M and the 1 5 M fraction In the control experiments no glycosaminoglycans were found in the 0 75 M fraction, the fractionation of extracts, however, showed that the 0 75 fraction did contain glycosaminoglycans This fact will be discussed later on

The column was washed with 3 0 M NaCl for regeneration

Recovery experiments were made with the commercial products specified in 2 3 1 and given below

Recovery percentages of reference substances after column chromatography

SAMPLE	WEIGHT	RECOVERY*
Hyaluronic acid	0.5 mg	82±2%
Chondroitin sulphate	0 5 mg	93±1%
Hyaluronic acid + Chondroitin sulphate	0 25 mg + 0 25 mg	85±4%

*Determined with the carbazole reaction.

TABLE V, 1. Staining intensity of intercellular substances

	PAS	AB/PAS	KOSSA	COLL.I.	COLL.I. BACT.H.	COLL.I. TEST.H.	AB 0.05M	AB 0.3M	AB 0.5M	TOL. BLEU
17th day										
Skin	+	- / +	-	+ + +	+	-	+	-	-	-
Fibrous layer (dura)	+	+ / +	-	+ + +	+ +	-	+ +	+	+	-
Orb.parietal commissure	-	+ + / -	-	+ + +	+ + +	+	+ + +	+ +	+	+ + +
Parietal bone										
Fibrous periosteum	+	+ / +	-	+ +	+	-	+	+	-	-
Matrix	-	+ / -	-	+ +	+ +	-	+	+	-	-
Squamosal bone										
Periosteal tissue	+	+ / +	-	+ +	+	-	+	+	-	-
Matrix	+	+ / +	-	+ +	+ +	-	+	+	-	-
18th day										
Parietal bone										
Fibrous periosteum	+	+ / +	-	+ + +	+ +	-	+	+	+	-
Matrix	+	+ / +	-	+ + +	+ +	+	+	+	-	-
Bone surface	-	+ + / -		+ + +	+ +	+	+ + +	+ +	+ +	+
Bone matrix	+ + +	- / + +	+ + +	-	-	-	+ +	+	-	+ +
Squamosal bone										
Periosteal tissue	+	+ / +	-	+ +	+	-	+	-	-	-
Matrix	+	+ / +	-	+ + +	+ +	+	+	+	-	-
9th day										
uamosal bone										
riosteal tissue	+	+ / +	-	+ +	+ +	-	+	+	-	-
trix	+	- / +	-	-	-	-	-	-	-	-
e surface	-	+ + / -		+ + +	+ + +	+	+ + +	+ +	+ +	+
e matrix	+ + +	- / + + +	+ + +	-	-	-	+ +	+	-	+ +

TABLE V, 1. Staining intensity of intercellular substances FOLD OUT

HISTOCHEMICAL INVESTIGATION OF INTRAMEMBRANOUS BONE FORMATION

5.1. INTRODUCTION

Several attempts have been made to investigate the cytological and histochemical aspects of intramembranous ossification (Bevelander and Johnson, 1950; Pritchard, 1952; Curran and Collins, 1957).

However, none of these studies was purposely designed to investigate the presence, localization and function of glycosaminoglycans in the intramembranous ossification process.

The aim of this chapter is to describe and interprete the responses of an area of intramembranous bone formation at succeeding stages of development to various histochemical procedures for the identification of glycosaminoglycans.

Most of the bones of the skull are formed by intramembranous ossification. A good object for this histochemical investigation would be the mandible, the first bone to form, originating along Meckel's cartilage (Duterloo and Jansen, 1969). It is easy to locate, creates enough matrix for reliable histochemical reactions, but has the disadvantage that it cannot be removed easily free from surroundig tissue – especially Meckel's cartilage – for biochemical investigations.

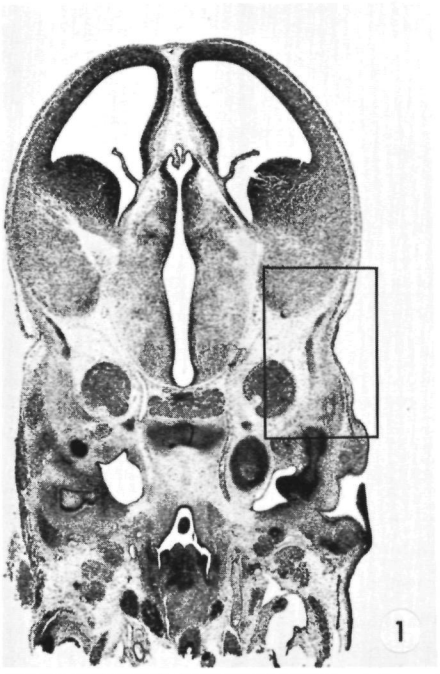
This difficulty does not pertain to the parietal bones. These structures, however, are thin and show little matrix. Therefore, the squamosal bone was also investigated in the same frontal sections as the parietal bone.

At three ages, the 17th, 18th and 19th day of gestation respectively, serial sections were made and stained by the methods described in Chapter II; adjacent sections were stained by a different stain. In this manner it became possible to combine histological and histochemical details and differences.

To compare the possible differences in reaction, it is necessary to make sure that the bone has been investigated at the same place at different ages.

Therefore, the frontal section in which that part of Meckel's cartilage was seen which forms the future malleus was selected as a reference plane. In this section the formation of the parietal bone is seen lateral to a cartilaginous plate, known as the orbitoparietal commissure (Youssef, 1966, 1969), and the formation of the squamosal bone is seen slightly cranial and lateral to the malleus (figure V, 1).

In this investigation for the morphology the terminology as used by Pritchard (1952) will be followed. He identified 4 zones at the site of intramembranous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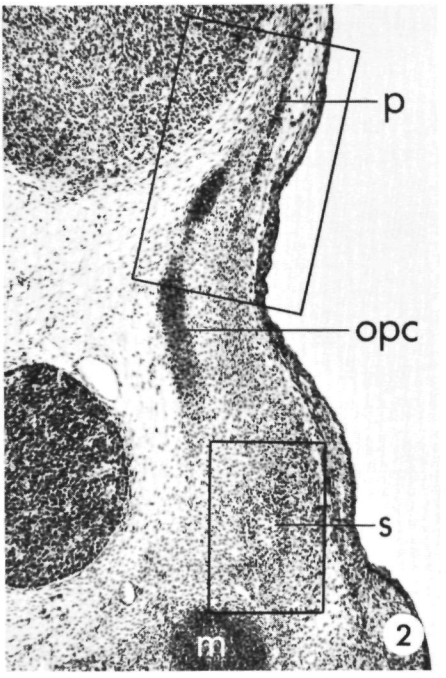
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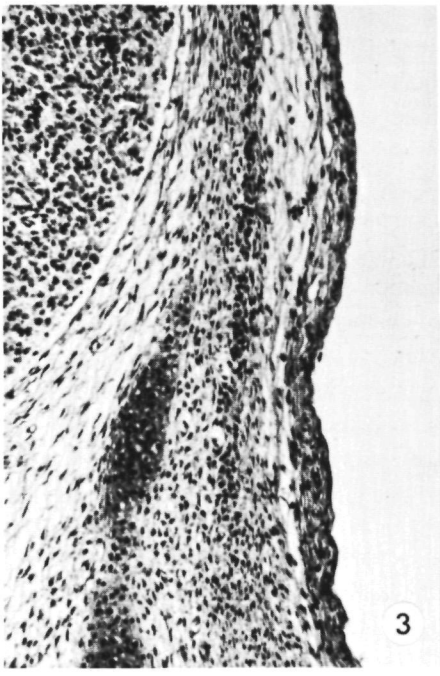
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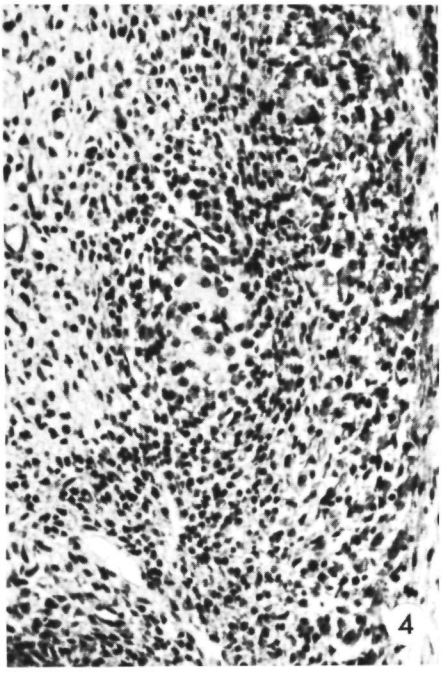
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ossification:

1. a zone of periosteal (fusiform) fibroblasts and tangentially oriented coarse collagen fibres,

2. a zone of proliferating and differentiating (round) pre-osteoblasts in a network of fine collagen fibres,

3. a zone of definitive periosteal osteoblasts arranged along radially directed bundles of coarse collagen fibres which merge into trabeculae of uncalcified bone matrix (osteoid) and then into calcified bone,

4. a network of primary cancellous bone whose cavities are lined with medullary osteoblasts.

In histochemistry it is very difficult, and perhaps disputable, to describe changes in staining intensities with words. It is also dangerous to indicate staining intensities in terms of +, ++, +++ and -, because sometimes this will indicate a greater difference than present in the section.

Despite this disadvantage the last method was given the preference.

5.2. FINDINGS

5.2.1 *17th day of gestation*

Morphology:

Parietal bone: Fig. V, 1.

Between the skin of the cranium and the brain-tissue a fibrous layer can be seen. Within this layer - origin of the future periosteum and dura - the shape of cells changes from fusiform to round towards the orbitoparietal commissure.

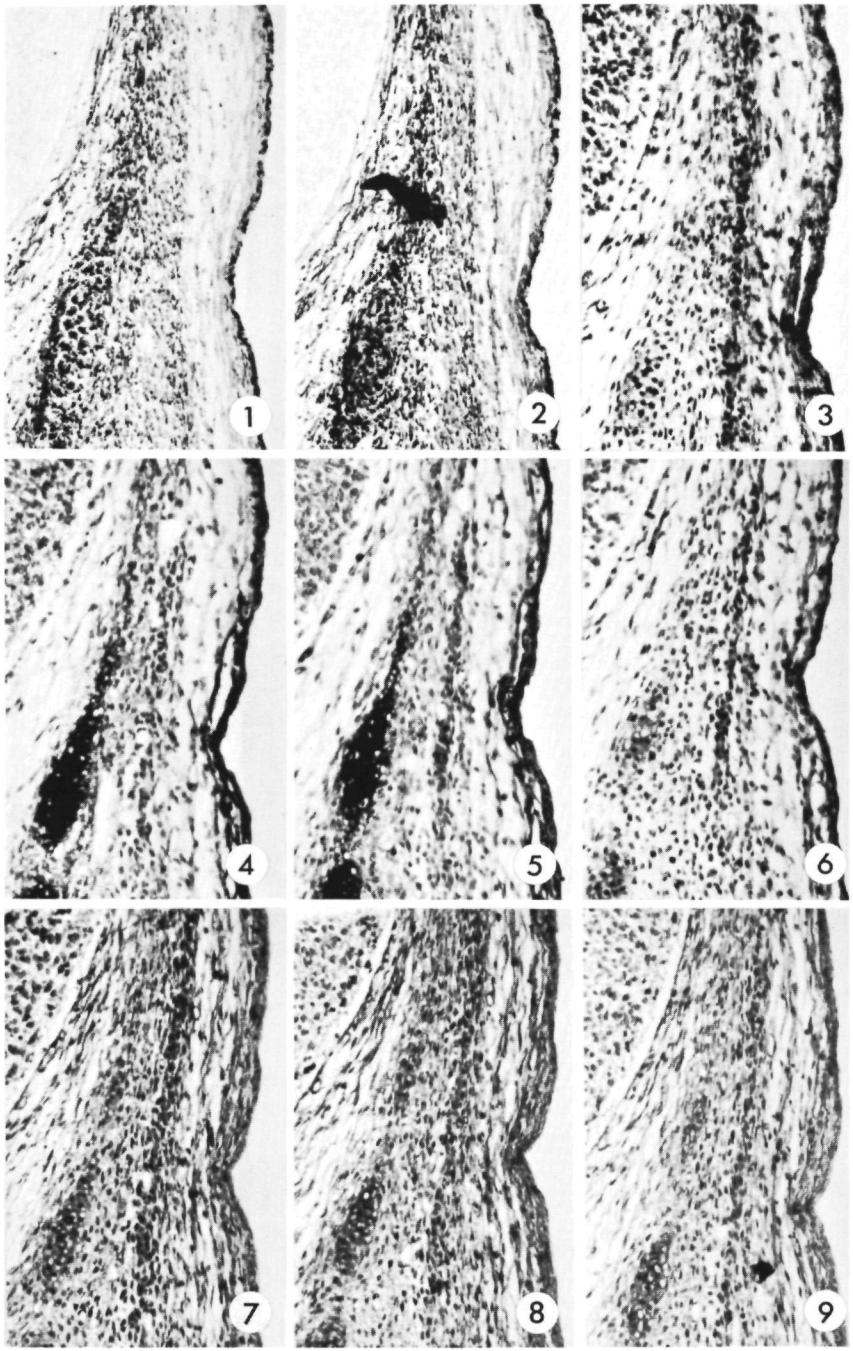
The first 3 zones as defined by Pritchard (1952) can be identified at this stage.

Squamosal bone: Fig. V, 1.

In the anlage of this bone only two of the zones described above can be seen. Pre-osteoblasts and definitive osteoblasts are differentiated, but the zone of periosteal fibroblasts is not yet formed. At this stage matrix is already present.

FIGURE V,1. Frontal section of a rat fetus on the 17th day of gestation. Hematoxylin-eosin, green filter VG 9

1 Low magnification showing the total section. X 12. 2. Higher magnification of area outlined in figure 1, representing the spatial relation between malleus (m), orbitoparietal commissure (opc), parietal bone (p), and squamosal bone (s). X 50 3. Higher magnification of area outlined in figure 2 X 125 4. Higher magnification of area outlined in figure 2, showing the matrix of the squamosal bone. X 200.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Histochemistry.

Parietal bone: Fig. V, 2.

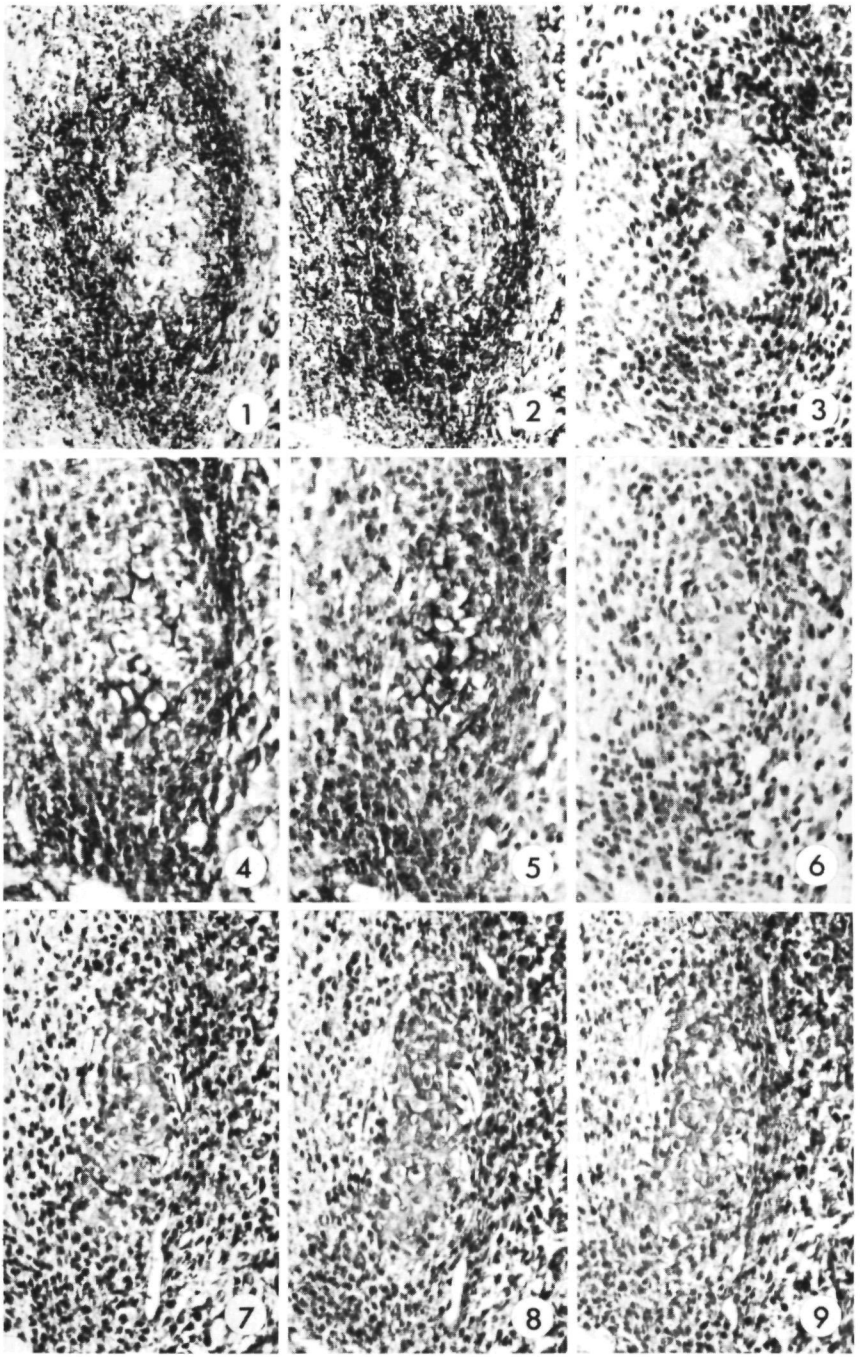
The details about the results of the different staining techniques are given in table V, 1., at least as far as the intercellular substances are concerned.

At the cellular level a strong PAS positive reaction is seen in cells of the skin, of the orbitoparietal commissure and of the fibrous periosteum.

Because of the fact that not much matrix is formed yet, it is rather difficult to evaluate the staining intensities of this matrix, and therefore conclusions about the composition of the matrix at this stage can hardly be drawn.

FIGURE V,2. Histochemical reactions of serial sections of the parietal bone area on the 17th day of gestation. X 125.

1. PAS reaction (green filter VG 9), 2. Astrablau-PAS (green filter VG 9), 3. Von Kossa, 4. Colloidal iron, 5. Colloidal iron after bacterial hyaluronidase, 6. Colloidal iron after testicular hyaluronidase, 7. Astrablau 0.05 M $MgCl_2$, 8. Astrablau 0.3 M $MgCl_2$, 9. Astrablau 0.5 M $MgCl_2$.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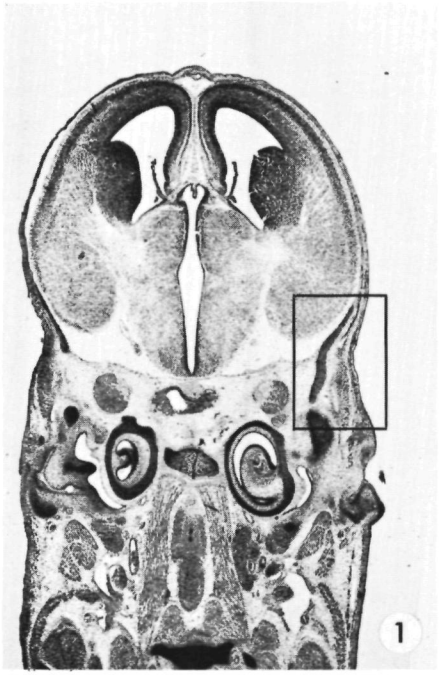
Squamosal bone: Fig. V, 3.

The details of the histochemical results are given in table V, 1. The surrounding mesenchymal cells, forming the future periosteum, contain much PAS positive material. This can be identified as glycogen, and it is a general finding that glycogen is present before or at the moment that cell differentiation takes place (Milaire, 1968).

Matrix has already been formed to some extent, and the results of the histochemical methods indicate that glycosaminoglycans are present in this matrix.

FIGURE V,3 Histochemical reactions of serial sections of the squamosal bone area on the 17th day of gestation X 200

1 PAS reaction (green filter VG 9), 2 Astrablau-PAS (green filter VG 9), 3. Von Kossa, 4. Colloidal iron, 5 Colloidal iron after bacterial hyaluronidase, 6 Colloidal iron after testicular hyaluronidase, 7 Astrablau 0 05 M $MgCl_2$, 8 Astrablau 0 3 M $MgCl_2$, 9. Astrablau 0 5 M $MgCl_2$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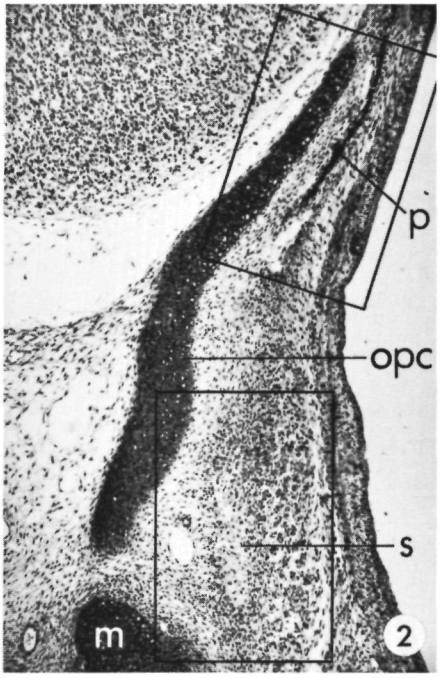
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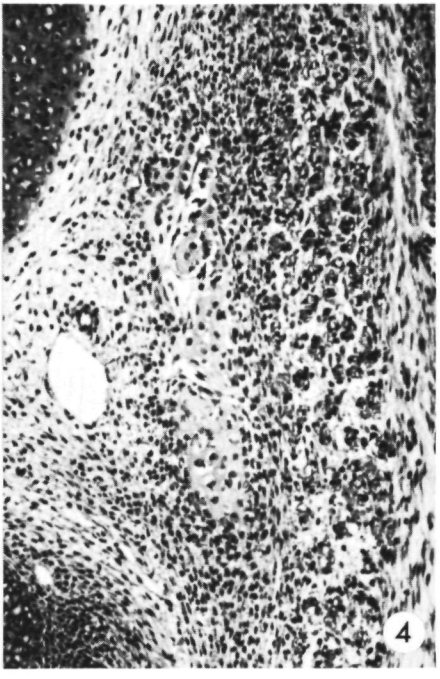

4

5.2.2. *18th day of gestation*

Morphology:

Parietal bone: Fig. V, 4.

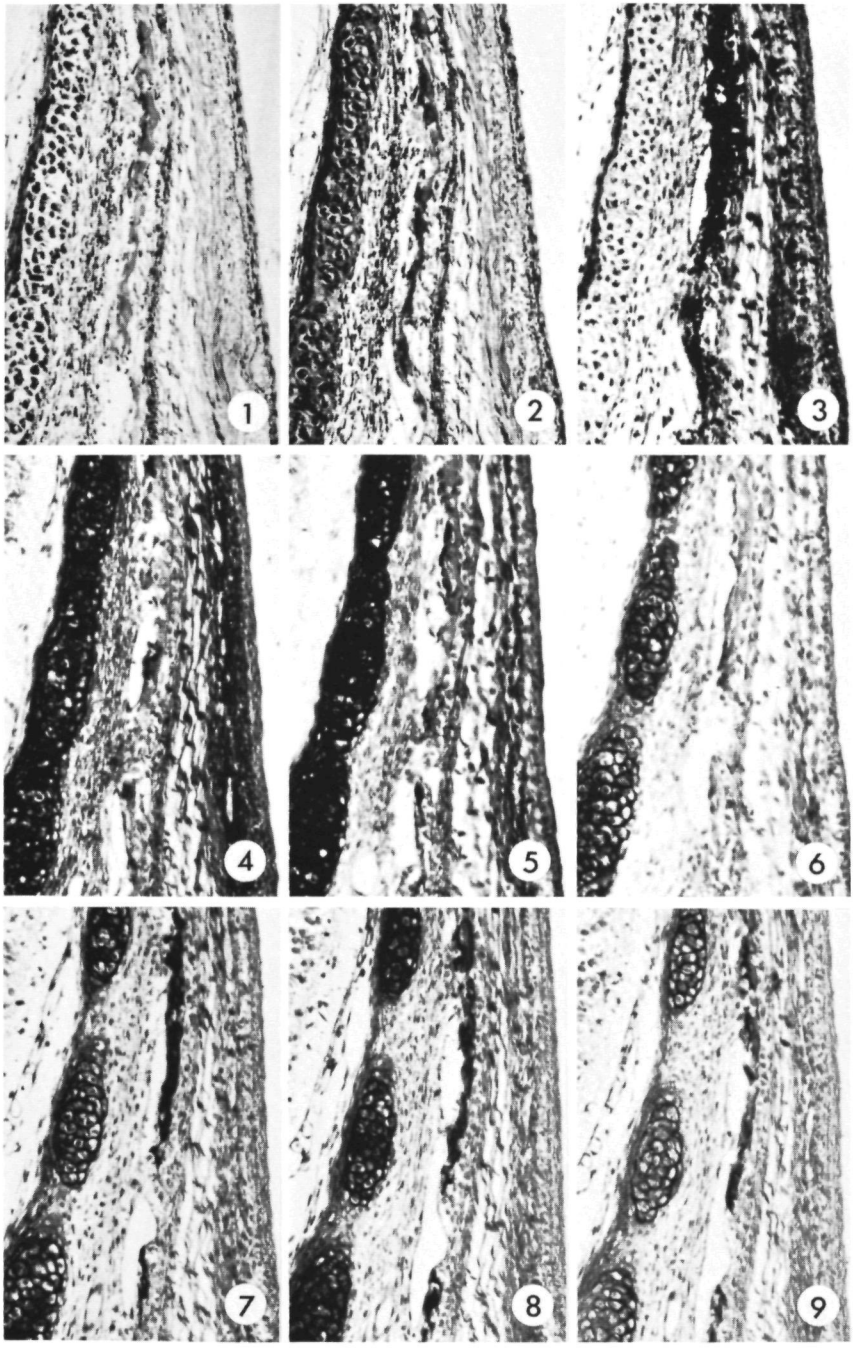
In comparison to the 17th day stage large amounts of matrix are present and partly calcified. The parietal bone has grown in a cranial direction. The bone material has the appearance of primary cancellous bone.

Squamosal bone: Fig. V, 4.

The amount of matrix has increased, but calcification has not yet started. Osteoblasts are embedded in this uncalcified matrix.

FIGURE V,4 Frontal section of a rat fetus on the 18th day of gestation Hematoxylin-eosin, green filter VG 9

1. Low magnification showing the total section X 10 2. Higher magnification of area outlined in figure 1, representing the spatial relation between malleus (m), orbitoparietal commissure (opc), parietal bone (p), and squamosal bone (s) X 50. 3 Higher magnification of area outlined in figure 2 X 125 4 Higher magnification of area outlined in figure 2, showing the matrix of the squamosal bone X 125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Histochemistry:

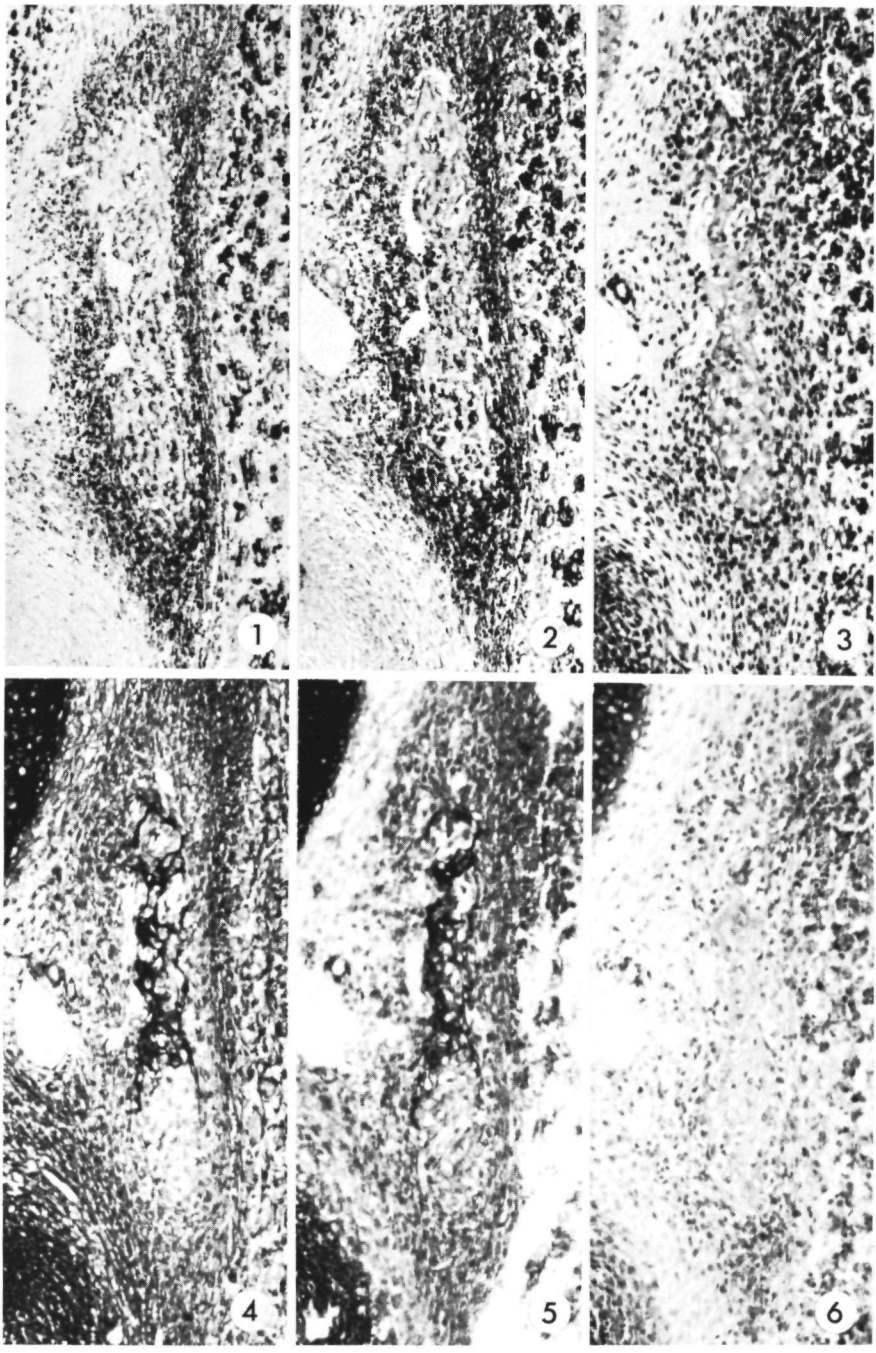
Parietal bone: Fig. V, 5.

The details of the results of the different staining techniques are given in table V, 1. In this table the staining intensities of different structures are evaluated. Fibrous layer indicates the layer between skin and brain-tissue at places where no sign of bone formation is seen. This layer will be referred to also by the term dura. Matrix stands for uncalcified intercellular substance. Bone surface indicates the osteoid tissue and the surface of the calcified matrix seen as a line in the sections, and bone matrix the Von Kossa positive material seen between the bone surfaces.

From the table and the figures it can be seen that the bone matrix does not take up colloidal iron, but stains intensely with Astrablau. The bone surface, however, is comparable to the uncalcified matrix when stained with colloidal iron, but not when stained with Astrablau. The change from uncalcified to calcified matrix goes hand in hand with increased PAS staining intensity.

FIGURE V,5. Histochemical reactions of serial sections of the parietal bone area on the 18th day of gestation X 125.

1. PAS reaction (green filter VG 9), 2. Astrablau-PAS (green filter VG 9), 3. Von Kossa; 4. Colloidal iron, 5 Colloidal iron after bacterial hyaluronidase; 6. Colloidal iron after testicular hyaluronidase; 7 Astrablau 0.05 M $MgCl_2$, 8. Astrablau 0.3 M $MgCl_2$, 9. Astrablau 0.5 M $MgCl_2$.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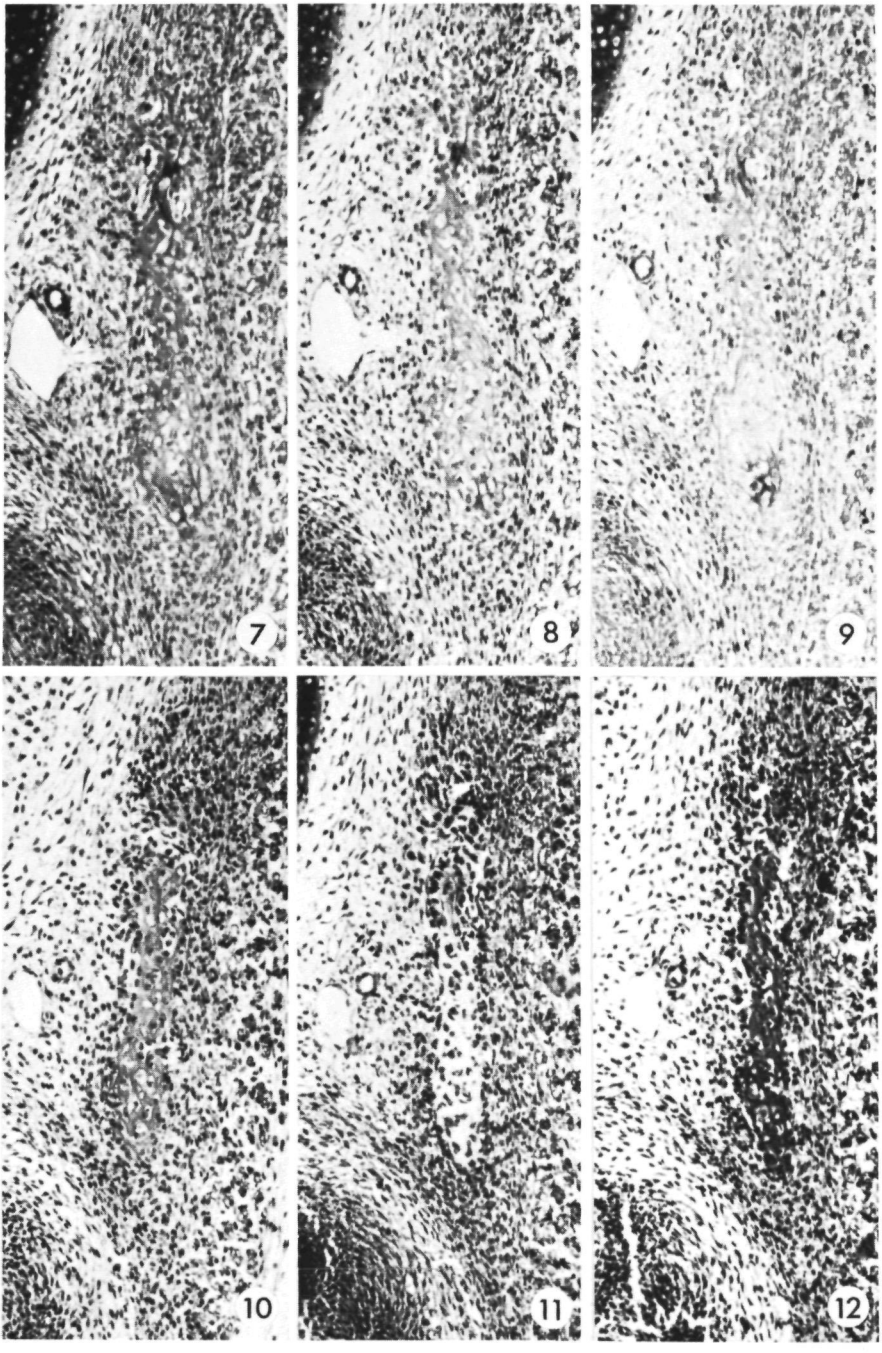
Squamosal bone: Fig. V, 6.

Only the intensity of the colloidal iron staining of the matrix has increased in comparison to that on the 17th day.

FIGURE V,6. Histochemical reactions of serial sections of the squamosal bone area on the 18th day of gestation X 125.

1. PAS reaction (green filter VG 9); 2. Astrablau-PAS (green filter VG 9); 3. Von Kossa; 4. Colloidal iron; 5. Colloidal iron after bacterial hyaluronidase; 6. Colloidal iron after testicular hyaluronidase, 7. Astrablau 0.05 M $MgCl_2$, 8. Astrablau 0.3 M $MgCl_2$, 9. Astrablau 0.5 M $MgCl_2$, 10. van Gieson, 11. Toluidine blue, 12. Masson's trichrome (mod. Goldner).


7
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5.2.3. *19th day of gestation*

Morphology:

Parietal bone

The parietal bone is larger as compared to 18th day stage, but maintains the same organization as described for that stage. The bone is now covering the brain over two-thirds of its surface.

Squamosal bone: Fig. V, 7.

The matrix area has increased again and is partially calcified. This provides a good model to follow the changes related to the calcification process.

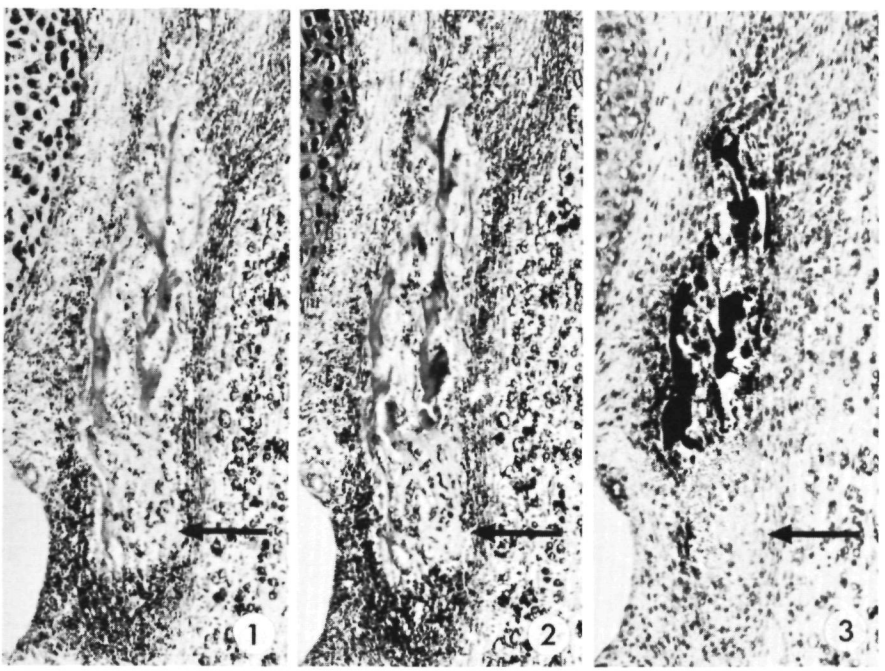
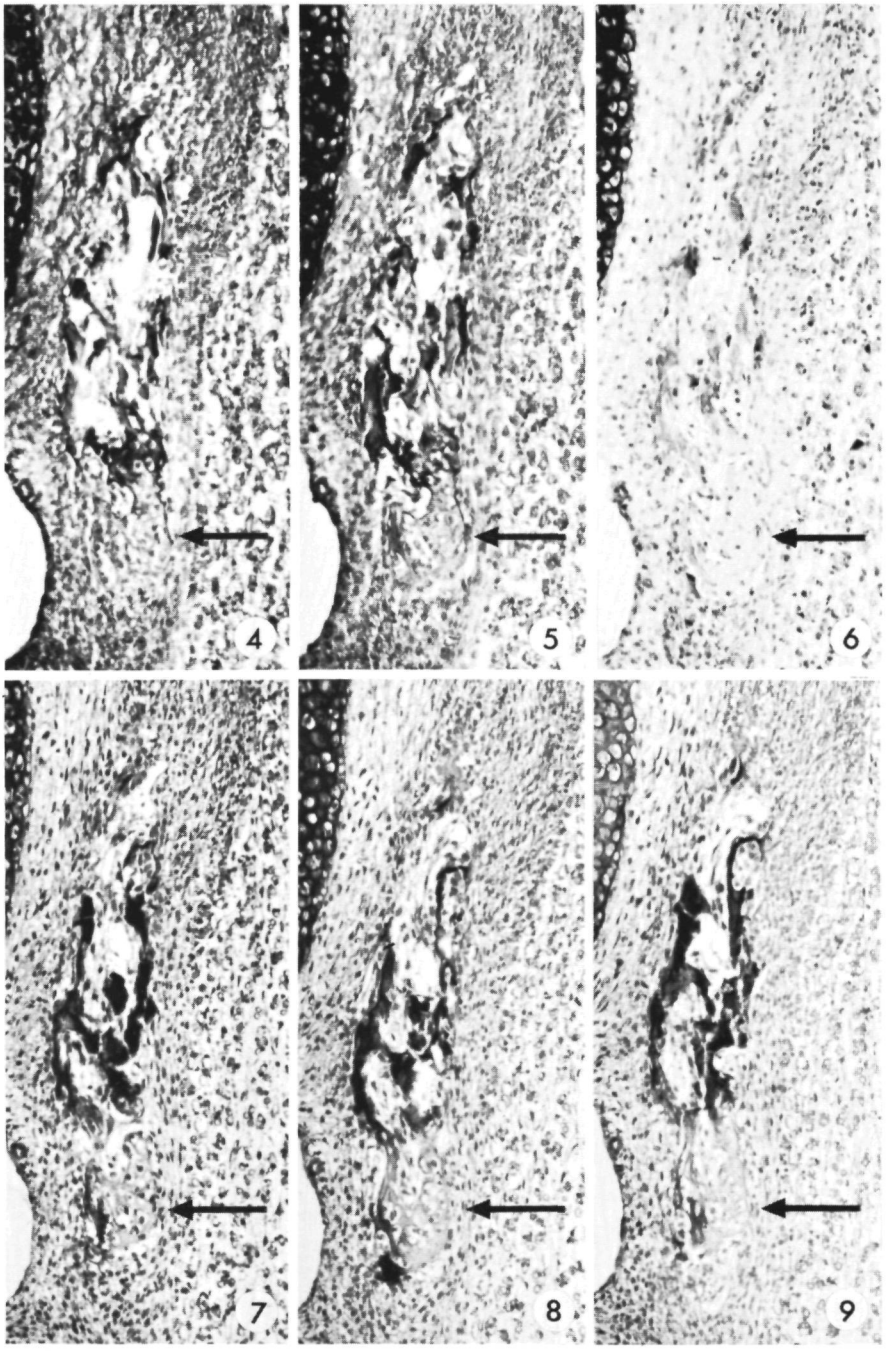


FIGURE V,7. Histochemical reactions of serial sections of the squamosal bone on the 19th day of gestation. X 125. Arrow points to uncalcified part.

1. PAS reaction (green filter VG 9); 2. Astrablau-PAS (green filter VG 9); 3. Von Kossa; 4. Colloidal iron; 5. Colloidal iron after bacterial hyaluronidase; 6. Colloidal iron after testicular hyaluronidase; 7. Astrablau 0.05 M $MgCl_2$; 8. Astrablau 0.3 M $MgCl_2$; 9. Astrablau 0.5 M $MgCl_2$.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Histochemistry:

Parietal bone:

The findings were essentially the same as on the 18th day of gestation. Therefore they are neither shown in the table, nor in the figures.

Squamosal bone: Fig. V, 7.

As already mentioned above this bone has at this age almost as much uncalcified as calcified matrix which makes a reliable evaluation of different staining methods possible.

The uncalcified part is indicated by an arrow. With the hematoxylin-eosin stain the calcified part is basophilic, the uncalcified eosinophilic. The calcified part is strongly PAS positive, even in the combined Astrablau-PAS reaction; the uncalcified part shows a faint, but definitely positive, PAS reaction in both cases.

A remarkable finding is that the uncalcified part reacts negatively in the colloidal iron reaction and is negative to Astrablau staining. It must be mentioned that the reaction of the uncalcified part of the squamosal bone at this stage is also different from that of the uncalcified matrix at the 18th day of gestation. It seems that here we encounter the stage just prior to calcification.

5.3. DISCUSSION

Weighing all the findings with respect to the histochemistry of the intramembranous ossification, the following conclusions can be drawn:

Uncalcified matrix is barely PAS positive, but the reaction becomes more intense after calcification.

Uncalcified matrix stains well with colloidal iron, but fails to stain just prior to and after calcification.

Uncalcified matrix stains slightly with Astrablau, but after calcification the bone surface stains very intensely, and the bone matrix somewhat less.

Toluidine blue metachromasia is present only in calcified material.

At this point it seems necessary to check the correctness of our staining methods.

Skin and dura react strongly with colloidal iron. Both structures are of mesenchymal origin and are known to contain hyaluronic acid. After treatment with bacterial hyaluronidase the staining intensity is much less, indicating the presence of hyaluronic acid in both.

The orbitoparietal commissure shows a strong positive colloidal iron reaction which diminishes in intensity after treatment with different hyaluronidases, a dif-

ferential staining with Astrablau at different molarities of $MgCl_2$ and metachromasia with toluidine blue. All of these indicate the presence of a sulphated glycosaminoglycan, such as chondroitin sulphuric acid, known to be the main glycosaminoglycan of cartilage matrix.

The findings given in table V, 1. concerning skin, dura – being the fibrous layer between skin and brain tissue – and the cartilage matrix of the orbitoparietal commissure, indicate that the principles of the staining methods described in Chapter III are correct.

Out of these findings and the results of the different techniques with the uncalcified matrix at different stages of development we conclude that glycosaminoglycans are present before calcification. There is substantial evidence that hyaluronic acid and chondroitin sulphate are the main components of these glycosaminoglycans.

The more or less opposite findings about the matrix after calcification suggest that

1. glycosaminoglycans present before calcification have been catabolized or have been changed into other substances prior to calcification or

2. glycosaminoglycans stain differently before and after calcification because of binding to other substances, such as collagen or calcium phosphate.

The change in reactivity with the PAS method argues in favour of the first possibility. Glycosaminoglycans present before calcification react negatively, but after calcification the reaction is positive.

This could indicate a replacement of the glycosaminoglycans by another type of glycans. The same change in reactivity of the matrix relative to the PAS method is described by Van den Hooff (1964). His observations, however, were made during the investigation of the endochondral ossification of the tibia of the rat.

The differences in the outcome of the colloidal iron technique, the Astrablau reactions and the toluidine blue reaction before and after calcification give support to the second possibility.

Calcification of the matrix could make the anionic groups of the glycosaminoglycans part of the crystal structure. In that case they can no longer react with the different stains. On the other hand, the presence of calcium phosphate could be the cause of the positive reaction with Astrablau and of metachromasia of toluidine blue.

Pearse (1955, 1961) used copperphthalocyanin dyes as stains for calcium deposits. This holds true also for Astrablau in our experience. This reaction of Astrablau explains the high increase in staining intensity of the bone surface. The calcified matrix, however, is stained less intensely because of the slow diffusion into bony structures.

The metachromatic reaction with toluidine blue could possibly be explained by

the presence of a number of phosphate groups.

Although different explanations may be called upon for the differences in staining of the matrix before and after calcification, it is not possible to prove that glycosaminoglycans are still present in the matrix.

Therefore both possibilities mentioned above are still valid, and have to be investigated by other means.

One of them is the investigation with radioactive sulphate as precursor which will be presented in the next chapter.

AUTORADIOGRAPHIC STUDY OF ^{35}S-SULPHATE INCORPORATION IN INTRAMEMBRANOUS BONE

6.1. INTRODUCTION

The results of the previous chapter appear to indicate that there is a great change in glycosaminoglycan content just before calcification. Especially the negative staining reactions of the matrix and the change in toluidine blue metachromasia could give rise to the statement that glycosaminoglycans are no longer present.

One could conclude that the disintegration or the disappearance of chondroitin sulphate is necessary before calcification can start. This theory was already stated by Sylvén (1947), and was based upon the change in toluidine blue metachromasia during endochondral ossification. This change in glycosaminoglycan stainability has been detected by many investigators, and Hirschman and Dziewiatkowski (1966) have shown that the protein moiety of the glycosaminoglycan - protein complex also changes just before calcification.

On the other hand, glycosaminoglycans are considered prerequisites for the formation of the initial calcification loci (a.o. Weidmann, 1963; Bernard and Pease, 1969).

These two opposite opinions, however, are not mutually exclusive. The amount or configuration of glycosaminoglycans could be of vital importance.

Radioactive sulphate has been used by many investigators to study glycosaminoglycan metabolism.

Dziewiatkowski (1949, 1956) et al. (1949) made extensive studies of the uptake of inorganic radioactive sulphate by cartilage and bone. Chemical analysis showed that most of the radioactive material was present in the form of chondroitin sulphate. Later investigations brought about that the incorporation was an enzymatic process (D'Abramo and Lipmann, 1957). Amprino (1955) made an extensive study on the sulphate metabolism in cartilage and bone differentiation and growth. He reports that it is rather difficult to follow the incorporation of radioactive sulphate in bone because of the coexistence in bone of two components, the organic and the inorganic material. These components seem to fix radioactive sulphate by a different mechanism. According to him radioactive sulphate is incorporated in the organic matrix in the form of chondroitin sulphate and in the inorganic material through ionic exchange.

Pregnant rats were injected intraperitoneally with sodium ^{35}S-sulphate as described in 2.5.

Two groups were injected on the 17th day of gestation. The fetuses were recovered respectively 24 and 48 hours after injection. One group was injected on the 18th day, and 24 hours later the fetuses were recovered. In this way three groups were arranged with different incorporation periods: a 17–18th day group, a 17–19th day group and a 18–19th day group. Each group consisted of 3 fetuses recovered from different mothers. The sections to be studied were taken in the same plane as mentioned before in the histochemical study. Both the parietal bone and the squamosal bone were investigated.

To rule out differences in exposure time, development etc., relevant sections of the three different groups were combined on the same slide.

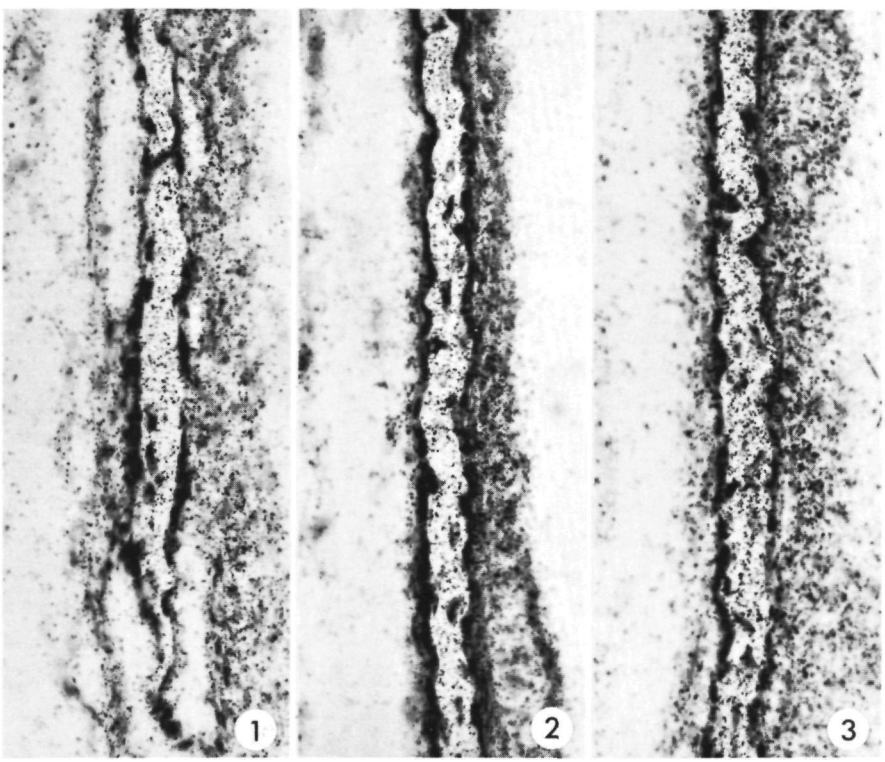


FIGURE VI,1. Autoradiographs of sections of the parietal bone. X 300.

1. 17–18th day incorporation period. 2. 17–19th day incorporation period. 3. 18–19th day incorporation period.

6.3. FINDINGS

On all the slides of the different groups a high grain density is seen above cartilaginous structures indicating the incorporation of sodium ^{35}S-sulphate into chondroitin sulphate. Besides, various other structures show an accumulation of silvergrains, such as intramembranous bone, arterial walls and dura. A low, but significant, labeling in mesenchyme and brain is found. Due to the low dose of radioactive material and a relatively long exposure time the background is not as low as possible, although this does not interfere with the evaluation.

Autoradiographs of the parietal bone of the different age groups are shown in Fig. VI,1.

Due to the opacity of the matrix it is rather difficult to show the grains above these structures correctly in the photographs. Furthermore, over the neighbouring orbitoparietal commissure many silvergrains have also accumulated, thus giving a somewhat complicated picture.

One of the most striking findings of this autoradiographic study is the even distribution of silvergrains seen above the whole matrix of the different bones indicating an even distribution of incorporated ^{35}S-sulphate, both in the parietal and in the squamosal bone. The number of grains, and therefore the amount of incorporated ^{35}S-sulphate, in the 18-19th day group is about twice that in the 17–18th day and 17–19th day group.

The amounts in the 17–18th and 17–19th day group are almost the same.

6.4. DISCUSSION

Most of the injected sodium ^{35}S-sulphate is rapidly eliminated from the body, predominantly in the urine. This gives rise to the assumption that the amount of sodium ^{35}S-sulphate not excreted after 24 hours must be present in a bound form.

Conversion of sulphate sulphur to cystine or methionine occurs only to a negligible extent (Dziewiatkowski, 1958).

It is generally accepted that ^{35}S-labelled substances present 24 hours after injection are sulphated glycosaminoglycans.

From the two mechanisms of fixation of radioactive sulphate as mentioned earlier (Amprino, 1955) the incorporation in the inorganic material can be ruled out, as we are dealing with Bouin fixed, and consequently decalcified sections.

Hence we must conclude that the presence of silvergrains above certain structures can only indicate sulphated glycosaminoglycans. In this way the labelling with sodium ^{35}S-sulphate is almost equivalent to a staining method.

The results of the autoradiographic study show that there is an increase in the amount of sulphated glycosaminoglycans in bone matrix, even after calcification,

although it is not possible to see in these sections to what extent the matrix was calcified.

From the fact that the amounts of labelled material are equal in the 17–18th day group and the 17–19th day group it can be concluded that there is no loss of sulphated glycosaminoglycans from the 18th till the 19th day.

These findings rule out the possibility, mentioned in the previous chapter to explain the differences in histochemical reaction of the matrix before and after calcification, that a loss of glycosaminoglycans has occurred.

On the contrary, from the 18th till the 19th day the amount of sulphated glycosaminoglycans synthesized is about twice that of the previous day.

Up to now, indirect methods have been used in this investigation to show whether or not glycosaminoglycans are involved in intramembranous bone formation. In the next chapter biochemical investigations will be described to reveal directly the presence, amount and composition of these glycosaminoglycans.

BIOCHEMICAL INVESTIGATION OF INTRAMEMBRANOUS BONE FORMATION

7.1. INTRODUCTION

Most of the data available on the presence, composition, and amount of glycosaminoglycans in bone are derived from adult cortical bone (McLean and Urist, 1968). Many similar data are also available for cartilage and calcifying cartilage, present in the epiphyseal plate of long bones (a.o. Hjertquist, 1964a,b; Campo and Tourtelotte, 1967; Greer et al., 1968; Campo et al., 1969), but data on intramembranous bone are much less frequent.

Especially about the time of onset of intramembranous ossification no data are available. This might be explained by the fact that it is very difficult to prepare enough homogeneous material for the investigation of this particular phase.

The rat calvarium was the material of choice for the collection of the aforementioned data because of its homogeneity and ease of access. The term calvarium is given to that part of the skull that comprises the frontal bones, the parietal bones, and the interparietal bone. Before ossification has started it is in fact impossible to write about a calvarium proper. In this investigation, however, this term will also include the earlier stages, although only a fibrous layer, the dura, is present at that time.

The advantage of this material for investigation of the onset of bone formation is that it does not all become calcified homogeneously at the same time, but calcification starts at different sites and progresses with time. This means that the quantitative relationship between uncalcified material, the dura, and calcified material, the differentiated bones, changes with time.

Thus, if glycosaminoglycans are implicated in calcification this must be the case in this calvarial material in particular.

It appeared possible to achieve good reproducibility of the method by which the tissues were collected, although it remains very tedious work to prepare enough homogeneous material for biochemical investigation.

7.2. FINDINGS

7.2.1. *Glycosaminoglycan content at different ages*

Histograms of the quantities of glycosaminoglycans recovered in different frac-

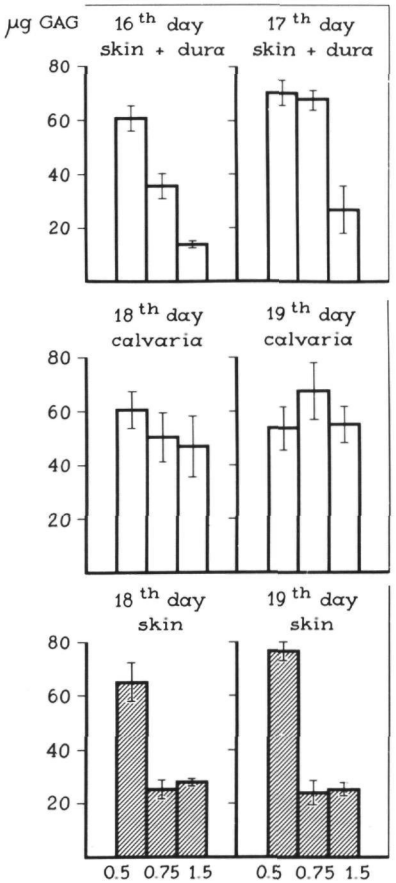


FIGURE VII,1. Histograms of the amounts of different fractions of glycosaminoglycans extracted from 20 mg samples of indicated material. The standard deviation, calculated from at least 4 fractionations, is indicated.

tions after separation on DEAE-Sephadex are presented in Fig. VII,1. They show the mean and standard deviation of at least 4 fractionations. To facilitate comparison each histogram has been recalculated for a sample weight of 20 mg of dry weight, which corresponds to about 80 calvaria on the 16th day and to about 20 calvaria on the 19th day of gestation.

The total amount of glycosaminoglycans in calvaria increases with age from 0.5 % of the dry weight on the 16th day to 0.9 % on the 19th day of gestation.

It appeared impossible to separate dura from skin on the 16th and 17th day. Therefore, the histograms of the fractionation of samples of skin from the 18th day on are represented in this figure.

7.2.2. *Identification of glycosaminoglycans*

The effluents of different peaks were pooled, dialyzed and lyophilized. Glucosamine and galactosamine were determined by the differential method of Good and Bessman (1964). The total amount of hexosamines of the 0.5 M fraction contained 90% glucosamine, that from the 0.75 M fraction 55% glucosamine and 45% galactosamine, and that from the 1.5 M fraction 99% galactosamine.

For all the fractions the molar ratio of hexosamine and uronic acid was nearly 1 to 1. The amount of extracted glycosaminoglycans was insufficient to allow a reliable sulphate determination.

On the basis of these results it seems justified to assume that the 0.5 M fraction contains hyaluronic acid, and that the 1.5 M fraction consists of chondroitin sulphate, although no distinction can be made between chondroitin-4,-6 and dermatan sulphate. The 0.75 M fraction cannot be identified with any of the known glycosaminoglycans. Since keratan sulphate consists of equimolar amounts of N-acetyl-glucosamine, galactose and sulphate (see Fig. I,1 on page 10) it is not detected by the carbazole reaction for uronic acids. The glucosamine moiety, however, is detectable by the hexosamine method. Theoretically the possibility exists that keratan sulphate is present in the 0.75 M fraction, but the molar ratio of 1 : 1, i.e. no excess in hexosamine, contraindicates this possibility. Another possibility is the presence in this fraction of heparan sulphate which contains N-acetyl-glucosamine, glucuronic acid and sulphate in equimolar amounts. The molar ratio of hexosamine to uronic acid favours this last possibility. Besides the possible presence of this substance in the 0.75 M fraction, partially sulphated chondroitin sulphate must also be present on the basis of the positive galactosamine reaction.

The presence of dermatan sulphate, containing iduronic acid instead of glucuronic acid, can be ruled out because of the observed molar ratio 1 : 1, since iduronic acid gives only 40% of the extinction of glucuronic acid in the carbazole reaction. In that case an excess in hexosamines should have been found.

The identification of hyaluronic acid with the 0.5 M fraction and of chondroitin sulphate with the 1.5 M fraction is confirmed by electrophoresis of the fractions together with reference substances on the same strip of cellulose acetate. The results are shown in Fig. VII,2. Due to the fact that only very little material was available, further identification was not possible within the group of the chondroitin sulphates, which thus could not be differentiated further.

7.2.3. *Quantitative relation of different fractions of glycosaminoglycans*

The histograms of Fig. VII,1. show only slight variations in the amount of glycosaminoglycans in the 0.5 M fraction. In calvaria there is a tendency for

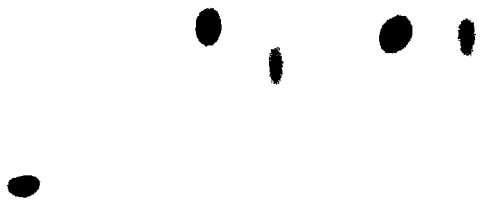


FIGURE VII,2. Electropherograms of the three different fractions of extracted glycosaminoglycans together with reference substances (on the left side of each strip). Start at bottom of the figure.

Left: 0.5 M fraction together with hyaluronic acid. Middle: 0.75 M fraction together with chondroitin sulphate (–4 and –6). Right 1.5 M fraction together with chondroitin sulphate (–4 and –6).

glycosaminoglycans to decrease, while they tend to increase in skin. The amounts of the 0.75 M and 1.5 M fractions, on the other hand, change distinctly. These absolute amounts, however, are not quite comparable because of the change in composition of the sample material, which becomes calcified.

Therefore, a better approach to these changes can be made by comparing the relative amounts in the 0.5 M fraction, containing hyaluronic acid, to those in the combined 0.75 M and 1.5 M fractions containing sulphated glycosaminoglycans.

The percentage of glycosaminoglycans in the 0.5 M fraction with respect to the total amount of glycosaminoglycans was calculated.

The results are presented graphically in Fig. VII,3.

As mentioned before, it is impossible to separate skin and dura on the 16th and 17th day. Therefore, values for skin from the 18th day on are shown in this figure also, to enable extrapolation.

7.2.4. *Calcium content at different ages*

If one studies the relation between glycosaminoglycans and the onset of calcification in intramembranous bone it is necessary to have a parameter for calcification. The calcium content was chosen as such.

The percentages of calcium present in acetone powders of calvaria at different ages are shown in Fig. VII,3.

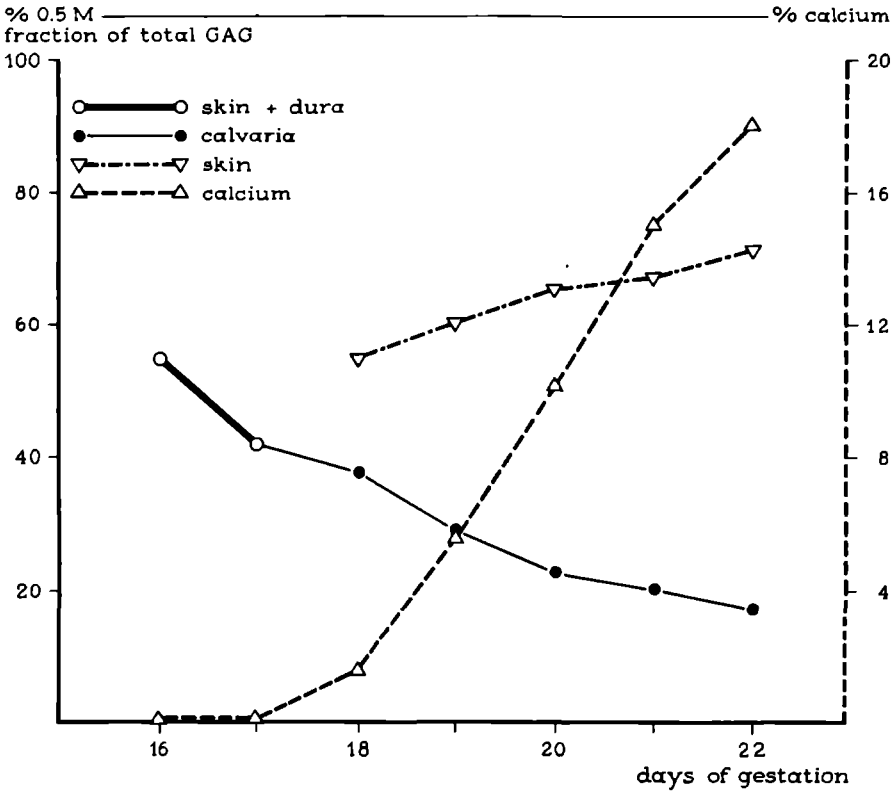


FIGURE VII,3. Graphical representation of the percentage of the 0 5 M fraction of different samples at different ages related to the total glycosaminoglycan content. Also represented is the change in calcium content of different samples of calvaria from different ages.

A significant increase is seen between the 17th and 18th day of gestation. This indicates that calcification has started and confirms the histological findings (table V,1). From the 18th day on there is an almost linear increase levelling off from the 21st day.

The final percentage for bone is 26.7% (McLean and Urist, 1968).

7.3. DISCUSSION

From the findings given above we may conclude that hyaluronic acid, partially sulphated chondroitin sulphate and chondroitin sulphate are components of the glycosaminoglycans extracted from developing calvaria.

As can be seen from Fig. VII,1. the total amount of glycosaminoglycans increases per unit of dry weight. This increase is even greater if calcification is taken into account. If we assume that all calcium is present as hydroxyapatite, it is possible to calculate the percentages of inorganic material present in different samples. These percentages can be calculated from the calcium percentages given in Fig. VII,3. by multiplying the values with a factor of 2.5. This implies that the amount of glycosaminoglycans in calvaria from the 18th day of gestation is 5% more, and in calvaria from the 19th day of gestation 15% more than indicated in Fig. VII,1.

After this correction the amount of hyaluronic acid appears to remain the same from the 18th till the 19th day of gestation, but the increase in sulphated glycosaminoglycans is 10 % more than shown in this figure.

It is also possible to express the increase in glycosaminoglycan content per calvarium. From the 18th till the 19th day of gestation the dry weight of a single calvarium increases from 0.6 mg to 1.2 mg.

Hence, the increase in hyaluronic acid is 100%, at the same time increase in sulphated glycosaminoglycans is 180%.

It is difficult to obtain these data from calvaria proper from the 16th till the 17th day of gestation. If we assume, by extrapolation, that the percentage of the 0.5 M fraction in relation to the total amount of glycosaminoglycans for skin on the 16th day and the 17th day of gestation is the same or even lower than on the 18th day, we may conclude that within this period there has been a high rate of synthesis of sulphated glycosaminoglycans. The change in amount of glycosaminoglycans in the 1.5 M fraction (Fig. VII,1.) also points in the direction of a rapid increase, while the increase in the 0.75 M fraction supports this conclusion because of the more or less intermediary character of its contents.

From this discussion we may conclude that already prior to the 17th day of gestation sulphated glycosaminoglycans must be synthesized in the dura. On the other hand, calcification starts at a certain moment. Therefore, although it is impossible to indicate from these findings the presence of sulphated glycosaminoglycans as a sufficient prerequisite for calcification, this last process proceeds only when a certain level of sulphated glycosaminoglycans has been attained.

In order to investigate the rate of synthesis of glycosaminoglycans, some precursor incorporation experiments were performed which will be presented in the next chapter.

INCORPORATION OF RADIOACTIVE PRECURSORS DURING INTRAMEMBRANOUS BONE FORMATION

8.1. INTRODUCTION

In the previous chapter it has been established that there is an increase in chondroitin sulphate in rat calvaria relative to age. In order to obtain information about the mechanism behind this gain, it is necessary to know more about the metabolism of glycosaminoglycans in this tissue. The best way to get this information is the use of radioactive precursors that will be incorporated into newly formed glycosaminoglycans.

Labelled substances have been used by many investigators to elucidate aspects of metabolism. In glycosaminoglycan research especially sulphur-35 has been used with great succes. Pioneer work has been done by Dziewiatkowski (1949), and from that time on many publications have been written about the incorporation of this isotope.

Capps and Shetlar (1963) studied the *in vivo* incorporation of labelled glucosamine into glycosaminoglycans of rabbit liver. This publication was followed by an investigation by White et al. (1965) about the incorporation into glycosaminoglycans of rat connective tissue.

Radioactive glucose has been used to clarify many aspects of glucose metabolism. Besides, it has been established (Boström and Rodén, 1961) that glucose is incorporated into hyaluronic acid, both in the glucosamine and glucuronic acid moiety, without scission of the glucose chain.

In this investigation ^{35}S-sulphate, ^{14}C-glucosamine, ^{14}C-galactosamine, and ^{14}C-glucose have been used.

8.2. EXPERIMENTAL SET-UP

For each experiment three groups of 8 pregnant rats each were injected intraperitoneally with each of the different substances as described in 2.3.7. Two groups were injected on the 17th day of gestation. The fetuses (about 9 from 1 mother) were recovered 24 and 48 hours respectively after injection. One group was injected on the 18th day, and 24 hours later the fetuses were recovered. In this way three groups of rats were provided with different incorporation periods: a 17-18th day group, a 17-19th day group and a 18-19th day group.

The material was treated further with the same biochemical procedures as was the unlabelled material. Radioactivity measurements were carried out as indicated in 2.3.7.

Extraction, fractionation and liquid scintillation counting were done in duplicate from the same starting material. The results were recalculated for a sample weight of 20 mg, and the mean is represented in the figures on the following pages. In this way it is possible to compare the ratio of amount of glycosaminoglycans and radioactivity - the specific activity - of different fractions within the same extraction and within the same incorporation experiment. Comparison of the specific activities of experiments with different precursors can only be made with great caution, due to differences in quantities of injected precursors, and to differences in pool size of these precursors.

8.3. FINDINGS

8.3.1. *^{35}S-sulphate incorporation*

The results of ^{35}S-sulphate incorporation experiments are presented in Fig. VIII,1. and in table VIII,1.

Comparing the 17-18th day with the 18-19th day incorporation period, we see that the specific activity of glycosaminoglycans in the 0.75 M and 1.5 M fraction has doubled during the latter period.

On the other hand, the specific activities for these fractions in the 17-19th day period are lower than in the 17-18th day period. For the 0.75 M fraction this is

Table VIII-1 Specific activity of glycosaminoglycans in different fractions.*)

PRECURSOR	PERIOD	0.5 M	0.75 M	1.5 M
^{35}S-sulphate	17-18th day	48	512	619
	17-19th day	151	415	563
	18-19th day	296	1105	1212
^{14}C-glucosamine	17-18th day	11.4	2.4	4.4
	17-19th day	10.6	1.7	3.2
	18-19th day	13.8	2.0	2.8
^{14}C-galactosamine	17-18th day	9.0	1.5	2.6
	17-19th day	7.8	0.8	1.4
	18-19th day	9.2	1.4	2.3
^{14}C-glucose	17-18th day	1.7	3.0	1.7
	18-19th day	1.6	2.5	1.4

*) Expressed as disintegrations per minute per μg glycosaminoglycan.

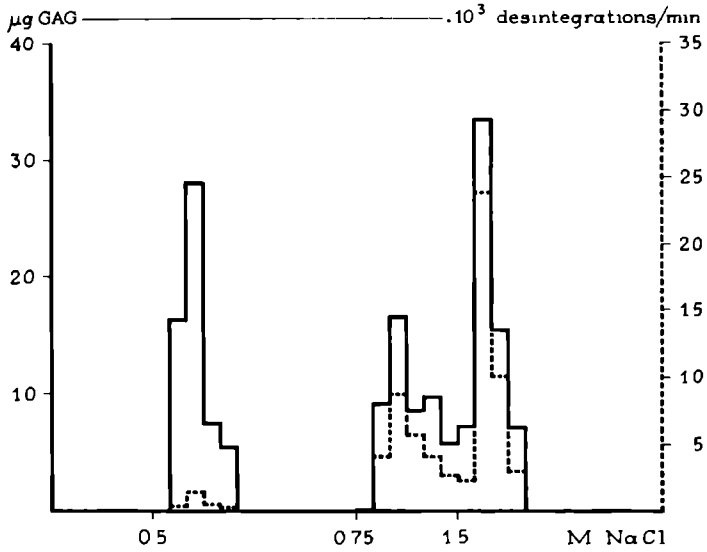
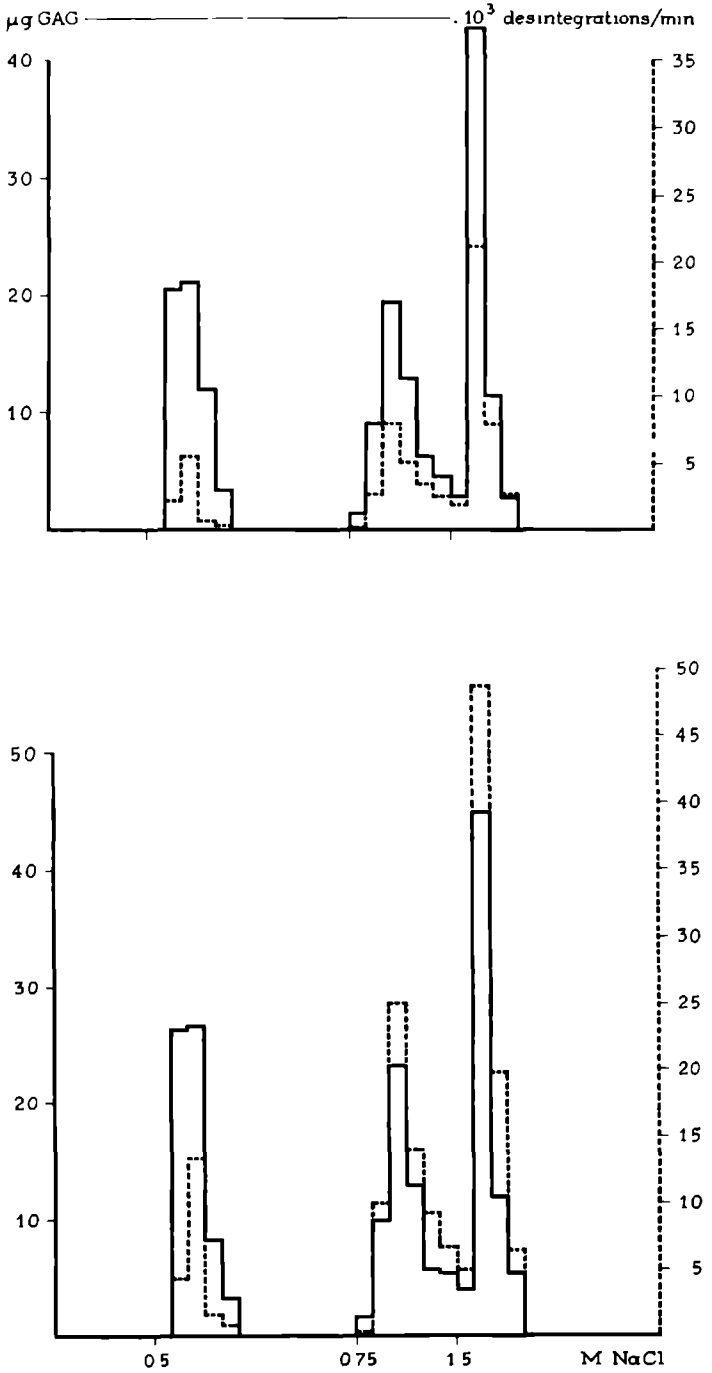


FIGURE VIII,1. Elution and radioactivity patterns of glycosaminoglycans extracted from 20 mg samples after injection with sodium ^{35}S-sulphate.
1. (above) 17–18th day incorporation period. 2. (upper right) 17–19th day incorporation period. 3. (lower right) 18–19th day incorporation period.

about 20%, and for the 1.5 M fraction about 10%. From these findings it can be concluded that ^{35}S-sulphate is incorporated within 24 hours after injection. The lower specific activities in the 17-19th day period are the result of synthesis of unlabelled sulphated glycosaminoglycans from the 18th till the 19th day of gestation, which leads to a dilution of the label. The finding that this dilution is less in the 1.5 M fraction indicates that glycosaminoglycans in the 0.75 M fraction may be precursors of the glycosaminoglycans in the 1.5 M fraction. The change in specific activities found in our experiments are in full agreement with the experiments of Dziewiatkowski et al. (1949) on the utilization of ^{35}S-sulphate for the synthesis of chondroitin sulphate in epiphyseal cartilage. They also observed a clearance of ^{35}S-sulphate from the blood within 24 hours, resulting in a lower specific activity within the next 24 hours.

The increase in specific activity during the 18-19th day incorporation period indicates a rapid synthesis of glycosaminoglycans, or at least a rapid sulphation of glycosaminoglycans with respect to the 17-18th day period.

A rather peculiar finding is the fact that radioactive sulphate shows up in the 0.5 M fraction, in which only hyaluronic acid was found to be present, as stated


μg GAG
10³ desintegrations/min
40
30
20
10
35
30
25
20
15
10
5
50
40
30
20
10
50
45
40
35
30
25
20
15
10
5
0 5
0 75
1 5
M NaCl

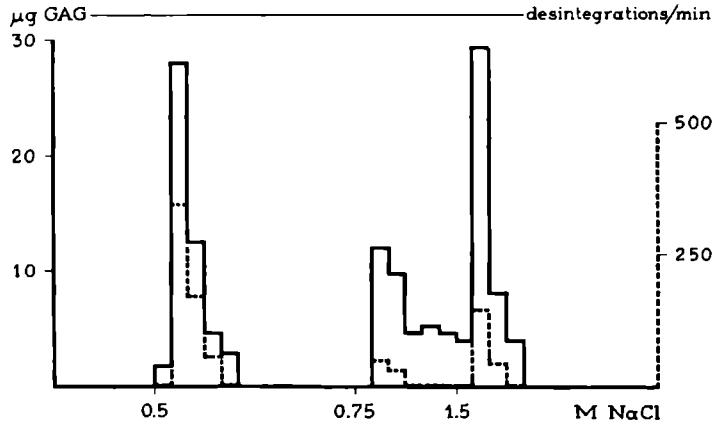


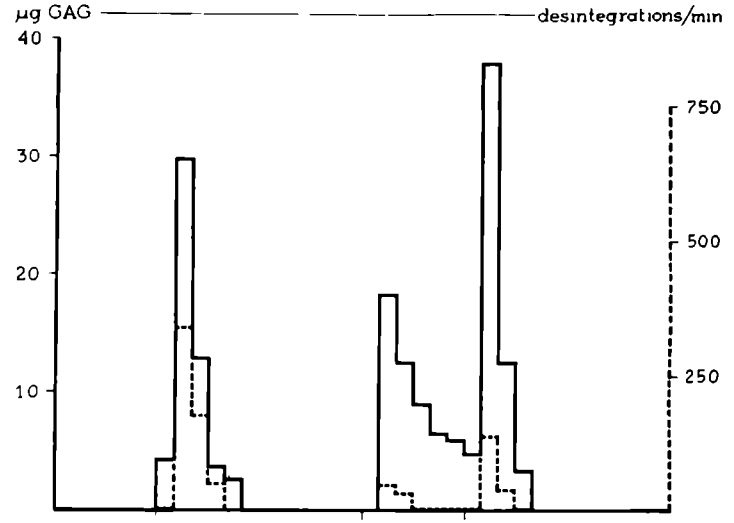
FIGURE VIII,2. Elution and radioactivity patterns of glycosaminoglycans extracted from 20 mg samples after injection with ^{14}C-glucosamine.
1. (above) 17–18th day incorporation period. 2. (upper right) 17–19th day incorporation period. 3. (lower right) 18–19th day incorporation period.

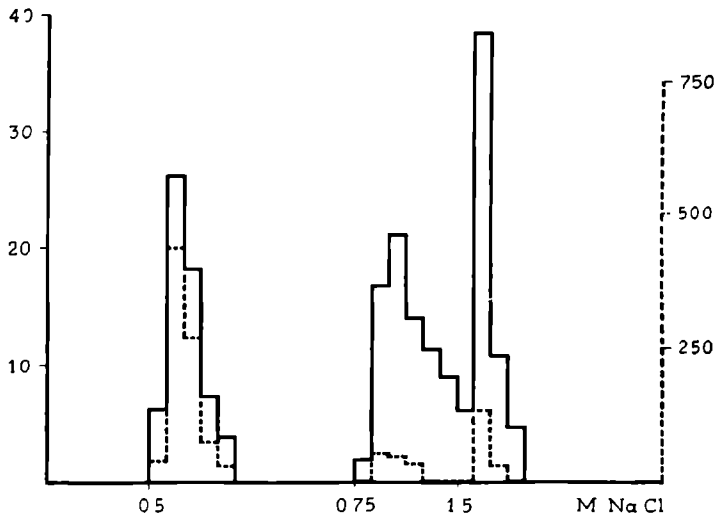
in the previous chapter. The amount of radioactivity in this fraction ranges from 5% of the total amount recovered in the 17-18th day group to almost 15% in both the other groups. There are at least three possibilities to explain the presence of radioactive sulphate in this fraction: 1. hyaluronic acid recovered in this fraction is partially sulphated, 2. chondroitin sulphate of low degree of sulphation with properties different from those in the 0.75 M and 1.5 M fraction is eluted is this fraction; 3. some other substance which incorporates ^{35}S-sulphate is eluted together with hyaluronic acid in this fraction. If we study the elution pattern carefully we see that the distribution of the label does not follow the glycosaminoglycan distribution in this fraction as in the other fractions. This points to the third possibility, i.e. that we are eluting a substance, no glycosaminoglycan but perhaps an oligopeptide, which contains ^{35}S.

Due to the fact that we were dealing with a very small amount of material in this experiment is was impossible to prove which one of the mentioned possibilities we are dealing with. Electrophoresis of the sample extract, followed by gas flow counting, or liquid scintillation counting of the strip, revealed no other places of activity than the one from chondroitin sulphate.

8.3.2. ^{14}*C-glucosamine incorporation*

The results of ^{14}C-glucosamine incorporation experiments are presented in Fig. VIII,2. and table VIII,1., page 67.


µg GAG
desintegrations/min
40
30
20
10
750
500
250


40
30
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10
750
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250
0 5
0 75
1 5
M Na Cl

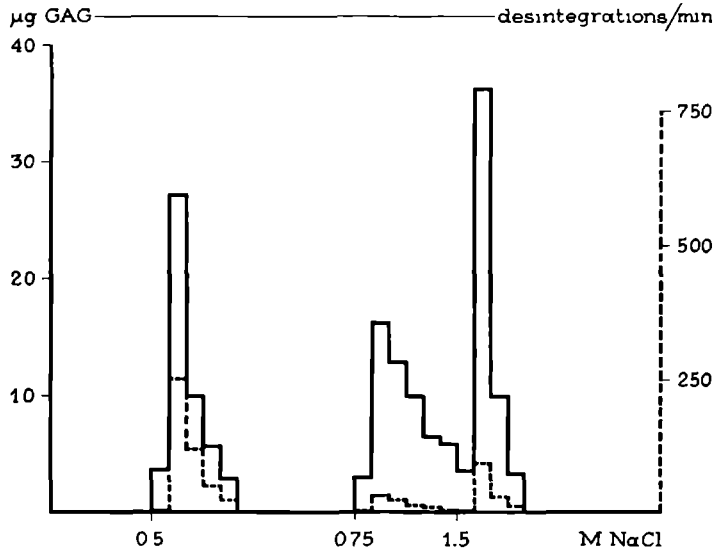
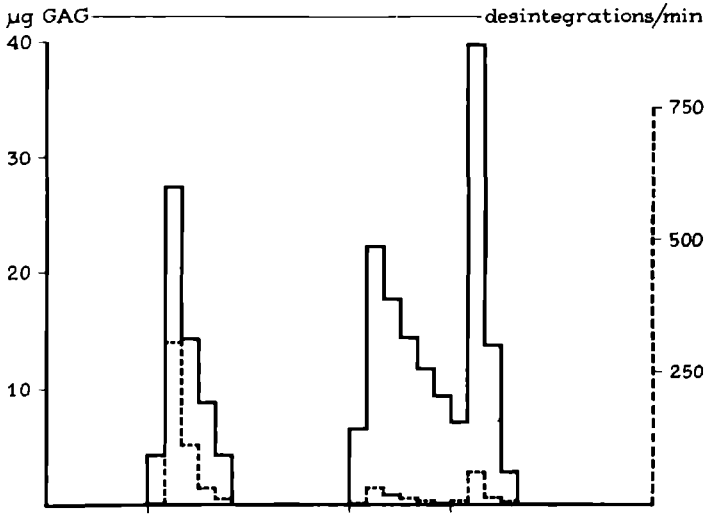


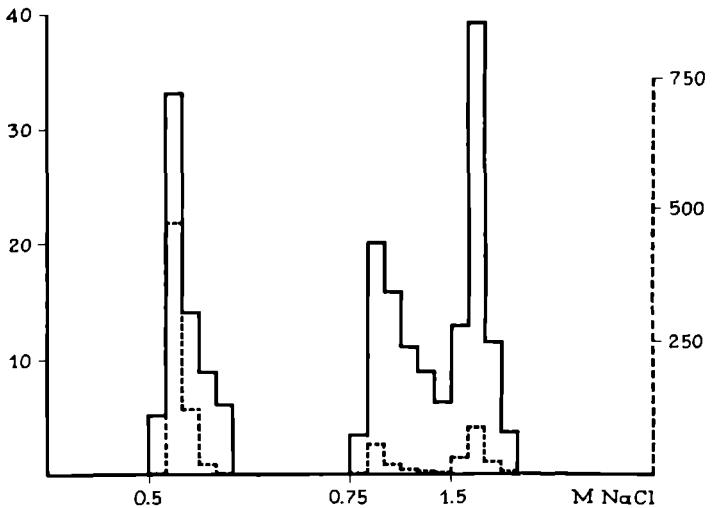
FIGURE VIII,3. Elution and radioactivity patterns of glycosaminoglycans extracted from 20 mg samples after injection with ^{14}C-galactosamine.
1. (above) 17—18th day incorporation period. 2. (upper right) 17—19th day incorporation period. 3. (lower right) 18—19th day incorporation period.

When comparing the 17-18th day to the 18-19th day incorporation period, the specific activity of the 0.5 M fraction has increased, that of the 0.75 M fraction is the same, and that of the 1.5 M has decreased during the latter period. The specific activities for all the fractions are lower in the 17-19th day period than in the 17-18th day period. From this findings we must conclude that ^{14}C-glucosamine is incorporated within 24 hours, just like ^{35}S-sulphate. The differences in specific activities between the 0.5 M fraction and the two other fractions show that ^{14}C-glucosamine is incorporated predominantly in hyaluronic acid. Whether the label present in the 0.75 M fraction is present as glucosamine or as galactosamine was not investigated further.

8.3.3. *^{14}C-galactosamine incorporation*

The results of ^{14}C-galactosamine incorporation experiments are represented in Fig. VIII,3. and table VIII,1., page 67.
Comparing the different incubation periods with each other and with the results of ^{14}C-glucosamine incorporation, it is striking that the specific activities show


µg GAG
desintegrations/min
40
30
20
10
750
500
250


40
30
20
10
750
500
250
0.5
0.75
1.5
M NaCl

practically the same pattern as the specific activities from the corresponding fractions within the corresponding periods.

From ^{14}C-glucosamine incorporation experiments it was expected that the 0.75 M and 1.5 M fractions would be more highly labelled than the 0.5 M fraction in the case of galactosamine incorporation.

Because of the fact that the injected labelled substances do not reach the place of incorporation directly but are taken up by the blood of the mother and have to

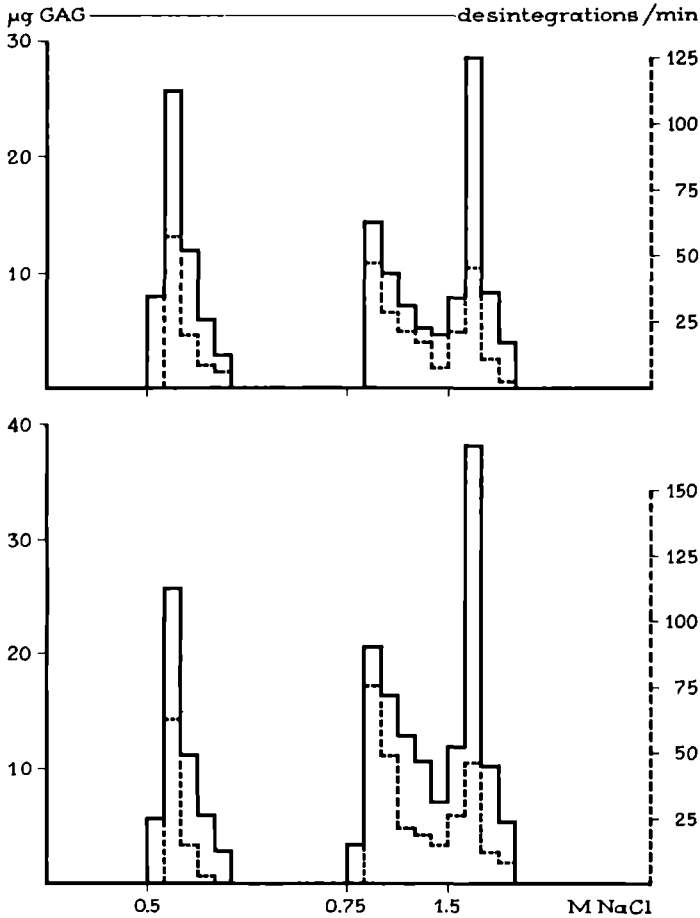


FIGURE VIII,4. Elution and radioactivity patterns of glycosaminoglycans extracted from 20 mg samples after injection with ^{14}C-glucose.
1. (upper) 17–18th day incorporation period. 2. (lower) 18–19th day incorporation period.

pass the placental barrier, it is possible that glucosamine and galactosamine are converted into a common precursor. This could explain the similar outcome of these two experiments.

To investigate whether this labelled common precursor could be ^{14}C-glucose the next experiment was performed.

8.3.4. *^{14}C-glucose incorporation*

The results of ^{14}C-glucose incorporation experiments are represented in Fig. VIII,4. and table VIII,1., page 67.
In this experiment only the 17–18th and 18–19th day incorporation period were used.

Despite a rather low radioactivity of the fractions it is apparent that the specific activities within both incorporation periods are almost the same. The higher value of the specific activities in the 0.75 M fraction again is an indication that the glycosaminoglycans in this fraction are precursors for sulphated glycosaminoglycans.

8.4. DISCUSSION

In order to discuss the findings of the incorporation experiments the reaction scheme as presented in Fig. VIII,5. will be used. In this scheme the different steps in glycosaminoglycan synthesis known up to now are shown.

The incorporation of radioactive sulphate is accomplished by active sulphate, 3'-phosphoadenosine-5'-phosphosulphate, generally abbreviated as PAPS (D'Abramo and Lipmann, 1957). However, there has been some controversy about the stage at which sulphation takes place: monomer or polymer. The discovery of chondroitin, plus the fact that sulphated oligosaccharides do not accept any hexosamine or uronic acid from appropriate nucleotides (Telser et al., 1966) strongly favour the conclusion that sulphation occurs subsequent to polymerization. Therefore, radioactive sulphate is not necessarily a good parameter to measure the rate of synthesis of sulphated glycosaminoglycans.

The incorporation of radioactive glucosamine is started by a hexokinase, probably the same hexokinase as for glucose. After phosphorylation the intermediate steps are as investigated and described for the synthesis of hyaluronic acid (Schiller, 1964). An essentially analogous pattern has been proposed for chondroitin sulphate synthesis from UDP-N-acetylgalactosamine and UDP-glucuronic acid (Perlman et al., 1964). The equilibrium between UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine (67%-33%, Fisher and Weinland, 1965) must be the reason that after ^{14}C-glucosamine incorporation more label is found in

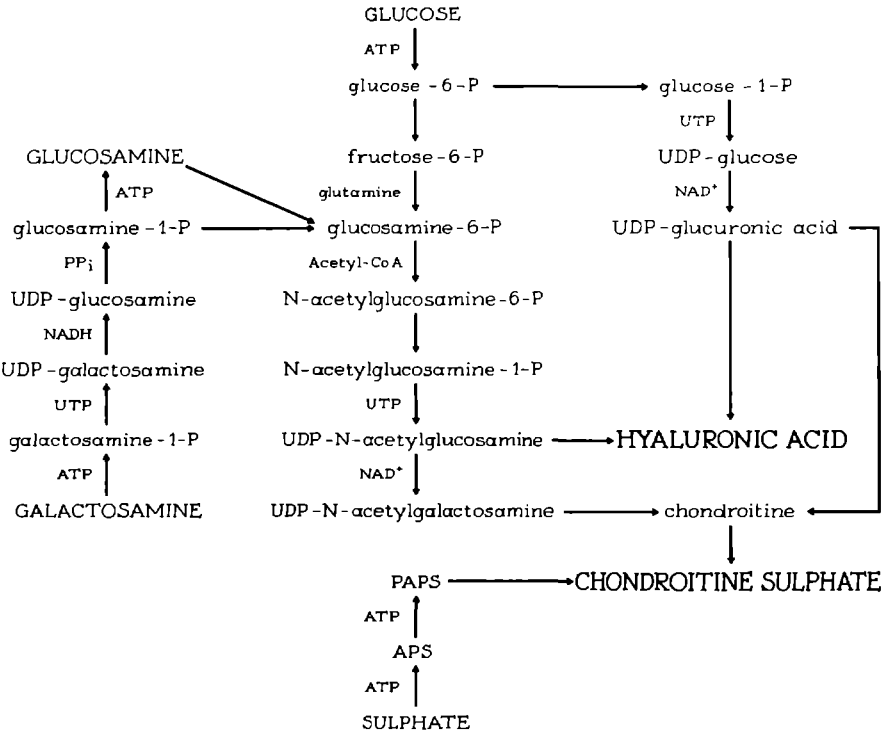


FIGURE VIII,5. Biosynthesis of glycosaminoglycans.

hyaluronic acid than in chondroitin sulphate. These findings are in accordance with experiments of White et al. (1965).

The incorporation of ^{14}C-galactosamine, giving the same results as that of ^{14}C-glucosamine, indicates a conversion to ^{14}C-glucosamine prior to the incorporation in glycosaminoglycans. This could be accomplished via the indicated pathway including the epimerization of UDP-galactosamine into UDP-glucosamine (27%-73%, Fisher and Weinland, 1965) which could take place in the liver of the mother prior to transport through the placental barrier.

We may conclude that radioactive hexosamines are incorporated into hyaluronic acid and chondroitin sulphate, according to the partition that takes place in the hexosamine pool due to the equilibrium between UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine.

The incorporation of ^{14}C-glucose shows a pattern of specific activities quite different from that of ^{14}C-glucosamine and ^{14}C-galactosamine. ^{14}C-glucose is incorporated in relation to the amount of glycosaminoglycans found in the different fractions. This can be explained by the fact that ^{14}C-glucose is a precursor

both for hexosamines and for uronic acid. Apparently the pathway in the direction of UDP-glucose is preponderant over the pathway leading to glucosamine-6-P. This latter pathway could be a rate limiting step in the formation of glycosaminoglycans. The specific activity of the 0.75 M fraction after ^{14}C-glucose incorporation is higher than in both other fractions. This is another indication that glycosaminoglycans found in the 0.75 M fraction are precursors of chondroitin sulphate.

The following conclusions can be drawn:

1. Labelled precursors are incorporated in glycosaminoglycans of fetal intramembranous bone within 24 hours after intraperitoneal injection into the mother.

2. Within the next 24 hours there is a dilution of the amount of label by newly synthesized unlabelled glycosaminoglycans, resulting in lower specific activities.

3. ^{14}C-glucosamine and ^{14}C-galactosamine are incorporated in the same ratio, and out of the same hexosamine pool.

4. ^{14}C-glucose is incorporated predominantly in the form of ^{14}C-glucuronic acid.

5. The incorporation of labelled precursors into hyaluronic acid, and the finding that the amount of hyaluronic acid remains the same, points to turnover rather than to net synthesis.

6. The rate of sulphation of glycosaminoglycans increases from the 17th to the 18th day of gestation.

7. The difference between the pattern of incorporation of ^{35}S-sulphate and the pattern of incorporation of ^{14}C-labelled precursors favours the statement that sulphation takes place at a polymer level.

8. During intramembranous bone formation there is an increase in rate of synthesis of sulphated glycosaminoglycans.

SOME REMARKS ON THE RELATION BETWEEN GLYCOSAMINOGLYCANS AND BONE FORMATION

Calcification of skeletal tissues is a process which has intrigued many investigators for a long time, and up to now there is no conclusive answer to all the questions about the different steps in this process. In fact, calcification is only one step in the highly organized process of bone formation. As early as 1867 Gegenbauer suggested that calcifiable tissues contained osteogenic fibers produced by osteoblasts, but in what respect these fibers differ from ordinary connective tissue fibers remained unexplained (Weidmann, 1963).
Numerous theories have been proposed to explain the mechanism of bone mineral deposition, but the importance of the fibers has been ignored for a long time.

Robinson's theory about calcification was the first one that related the function of an enzyme, alkaline phosphatase, to this process, instead of the earlier acceptance of a spontaneous deposition of mineral from tissue fluid components. From this time on the role of glycogen in calcification was studied. It seemed possible to explain the presence of glycogen as a source of phosphate esters, a necessary substrate for the alkaline phosphatase to provide supersaturated tissue fluids at the site of calcification. In this manner it was possible to explain the calcification as a spontaneous precipitation of bone mineral.

After this work attention was directed towards the matrix and to the possibility of a local factor in it, which determined whether a tissue would calcify or not. From histochemical observations it was obvious that areas of calcification showed metachromasia with toluidine blue. This reaction indicated the presence of chondroitin sulphate in the calcification process. The possible role of this substance could be that of ion exchanger. That chondroitin sulphate could be the only local factor responsible, was excluded by the fact that it is also present in high amounts within tissue that does not calcify, for instance hyaline cartilage.

About ten years ago the nucleation concept was presented (see Weidmann, 1963; McLean and Urist, 1968) to explain the calcification mechanism. This implies that calcification begins with the formation of a crystal seed in the organic matrix, thus initiating the crystallization of bone mineral from tissue fluid. Although this concept seems rather simple, a number of substances might be involved in nucleation: 1. a specific form of collagen, 2. a sulphated glycosaminoglycan, 3. enzyme systems, and 4. a system concentrating calcium and phosphate ions.

Another concept is that pyrophosphate inhibits nucleation in tissues. Only on sites where alkaline phosphatase is present to remove this inhibitor nucleation, and therefore calcification, will occur (Fleisch and Bisaz, 1965). This last concept is now investigated in many laboratories.

In the nucleation concept the interaction between chondroitin sulphate and collagen is of major importance (Sobel, 1965). He proposed a mechanism for the nucleation of bone mineral in which it was suggested that collagen interacted with the glycosaminoglycan to form the nucleating entity. The nucleus, once formed, required activation by calcium ions in a manner which resembled a lock and key mechanism. The calcium ion seemed to be specific for this activation.

Mathews (1965) investigated the interaction of glycosaminoglycans and collagen and proposed a model for this interaction. This model shows a parallel-ordered interaction of collagen fibrils with chondroitin sulphate side chains of the chondroitin sulphate-protein macromolecule. In this way chondroitin sulphate might control the organization of collagen fibrils into parallel-ordered bundles. On the other hand, glycosaminoglycans are described as the cementing interfibrillar substance in connective tissues. They are bound in at least three ways to the collagenous fibre (Chvapil, 1967). A small part adheres only physically to the surface of the fibre. The second part, forming approximately 20% of all glycosaminoglycans of collagen structure, participates via a relatively strong chemical bond in the stabilization of the extended helix. The remaining 50% of glycosaminoglycans are an integral part of the collagen ultrastructure.

The results of our histochemical and biochemical studies indicate that glycosaminoglycans are present prior to, and increase significantly during, the formation of intramembranous bone. The specific increase of chondroitin sulphate as shown in 7.2.3. and 8.3.1. may be the argument to state that the principles of the calcification mechanism as suggested in the nucleation concept are also applicable to intramembranous ossification. Our investigation elucidates only a particular aspect of bone formation: the presence and augmentation of sulphated glycosaminoglycans. We have shown that it is feasible to assume that a certain level has to be present before calcification starts.

More research is needed to detect the regulatory mechanism behind calcification. There is no doubt that calcification is under cellular control. Therefore a close co-operation of morphologists, cytologists and biochemists will be necessary to explain the complicated process of calcification.

In this thesis the results of histochemical and biochemical investigations on the amount and composition of glycosaminoglycans (old term acid mucopolysaccharides) in matrix of developing intramembranous bone are described.

In order to form an opinion about the specificity of the histochemical techniques to be used, both fixation (3.1.) and staining methods (3.2.4.) were tested by means of spot tests with commercial preparations of glycosaminoglycans. A description of the specificity of the different staining methods used is presented (3.2.).

In the histochemical investigation the development of the parietal bone and the squamosal bone is followed from the 17th through 19th day of gestation. The squamosal bone shows both calcified and uncalcified matrix during a certain period of time (5.2.).

From the results obtained with the different staining methods it can be concluded that glycosaminoglycans are present prior to calcification; but just prior to and after calcification the matrix shows a completely different staining pattern, giving rise to the impression that glycosaminoglycans are no longer present, or have been transformed into other substances (5.3.). In order to exclude the possibility that glycosaminoglycans have disappeared from the matrix, an autoradiographic investigation was made after the injection of ^{35}S-sulphate. From the results it could be concluded that glycosaminoglycans increase rather than disappear from the matrix (6.4.).

Biochemical techniques were used to extract glycosaminoglycans from fetal calvaria from the same age as those for histochemical investigation and to separate them into different components.

These techniques were tested beforehand for their applicability for this investigation in model experiments. These experiments finally led to the use of the alkaline extraction method in the presence of borohydride (4.1.2.), as well as the separation on DEAE-Sephadex (4.2.2.).

Biochemical investigation revealed the presence of glycosaminoglycans in calvaria at a level of 1% of dry weight (7.2.1.). It appeared to be possible to separate the extracted glycosaminoglycans into two main groups: unsulphated (hyaluronic acid) and sulphated (chondroitin sulphate) glycosaminoglycans (7.2.2.). Furthermore, there is a relation between the relative amounts of these main substances, which changes with time. The change is in favour of the sulphated glycosaminoglycans (7.2.3.).

The metabolism of these identified glycosaminoglycans was investigated by means of ^{35}S-sulphate, ^{14}C-glucosamine, ^{14}C-galactosamine and ^{14}C-glucose as precursors. A high increase in the rate of sulphation of polymer glycosamino-

glycans was found (8.3.1.) from the 17th day of gestation onward. It was established that there is no difference in incorporation of ^{14}C-glucosamine and ^{14}C-galactosamine. Both substances enter the same hexosamine pool and are incorporated according to the equilibrium of the UDP-N- acetylglucosamine-4-epimerase reaction (8.3.2. - 8.3.3.). ^{14}C-glucose, on the other hand, is predominantly incorporated in the glucuronic acid moieties (8.3.4.).

Finally, the relation of glycosaminoglycans to bone formation is described. Different theories on bone formation are reviewed shortly. The role of the detected glycosaminoglycans in intramembranous ossification is discussed (IX).

In dit proefschrift worden de resultaten medegedeeld van zowel histochemische als biochemische onderzoekingen naar de hoeveelheid en samenstelling van glycosaminoglycanen (oude term zure mucopolysacchariden) in de matrix van intramembraneus gevormd botweefsel.

Teneinde een inzicht te krijgen in de specificiteit van de daarvoor benodigde histochemische technieken werden zowel de fixatie (3.1.), alsmede enige kleuringen (3.2.4.) uitgetest door middel van spottests met handelspreparaten van glycosaminoglycanen. Tevens wordt van de gebruikte kleuringsmethoden een beschrijving van de specificiteit gegeven (3.2.).

Voor het histochemische onderzoek werd de ontwikkeling van het os parietale en het os squamosum gevolgd van de 17de dag tot en met de 19de dag na bevruchting. Laatstgenoemd bot geeft gedurende een bepaalde periode zowel verkalkte als onverkalkte matrix te zien (5.2.).

Uit de resultaten van de verschillende kleuringen kan worden geconcludeerd dat glycosaminoglycanen aanwezig zijn vóór de verkalking, maar zowel vlak voor als na verkalking vertoont de matrix een geheel tegengesteld kleuringspatroon, waardoor de indruk ontstaat dat glycosaminoglycanen niet meer aanwezig zijn of omgevormd zijn in andere bestanddelen (5.3.).

Teneinde de mogelijkheid van verdwijnen van glycosaminoglycanen uit de matrix uit te sluiten werd een autoradiografisch onderzoek gedaan na injectie van ^{35}S-sulfaat. Hieruit kon worden afgeleid dat eerder sprake is van toename dan van verdwijnen van glycosaminoglycanen uit de matrix (6.4.).

Biochemische technieken werden toegepast om de glycosaminoglycanen uit foetale schedeldakjes van dezelfde leeftijd als voor histochemisch onderzoek te extraheren en daarna te scheiden in diverse componenten. Alvorens echter deze technieken te gebruiken werden diverse modelexperimenten ondernomen om de bruikbaarheid van deze technieken voor dit onderzoek na te gaan. Hieruit ontstond uiteindelijk de toegepaste alkalische extractie in aanwezigheid van boorhydride (4.1.2.) en de scheiding op DEAE-Sephadex (4.2.2.).

Uit het biochemisch onderzoek is gebleken dat glycosaminoglycanen deel uitmaken van de botmatrix en wel voor circa 1% van het drooggewicht (7.2.1.). Het bleek mogelijk de glycosaminoglycanen te scheiden in twee hoofdgroepen: ongesulfateerde (hyaluronzuur) en gesulfateerde (chondroitine sulfaat) glycosaminoglycanen (7.2.2.). Bovendien bleek er tussen de relatieve hoeveelheden een relatie te bestaan, die met de tijd verandert, en wel ten gunste van de gesulfateerde glycosaminoglycanen (7.2.3.).

Het metabolisme van deze geïdentificeerde glycosaminoglycanen werd nader

onderzocht met behulp van ^{35}S-sulfaat, ^{14}C-glucosamine, ^{14}C-galactosamine en ^{14}C-glucose als precursors.

Duidelijk aantoonbaar vanaf de 17de dag na bevruchting is de sterke toename in de snelheid van sulfateren van reeds polymere glycosaminoglycanen (8.3.1.). Verder is gebleken dat er geen verschil is in de inbouw van ^{14}C-glucosamine en ^{14}C-galactosamine. Beide komen in dezelfde hexosaminepool terecht en worden ingebouwd volgens het evenwicht in de UDP-N-acetylglucosamine-4 epimerase reactie (8 3.2. - 8.3.3.). ^{14}C-glucose daarentegen komt voornamelijk terecht in de glucuronzuurgroepen (8.3.4.).

Tot slot wordt de relatie van glycosaminoglycanen met de botvorming beschreven. Verschillende theorieën over de botvorming worden kort vermeld. De rol van de aangetoonde glycosaminoglycanen bij de intramembraneuze verbening wordt besproken. (IX).

Adam, H., and Czihak, G. 1964. *Arbeitsmethoden der makroskopischen und mikroskopischen Anatomie.* Stuttgart Gustav Fischer Verlag.

Amprino, R 1955 Autoradiographic research on the S^{35}-sulphate metabolism in cartilage and bone differentiation and growth. *Acta Anat.* 24. 121–163

Antonopoulos, C A ; Engfeldt, B.; Gardell, S., Hjertquist, S. O., Solheim, K. 1965. Isolation and identification of the glycosaminoglycans from fracture callus. *Biochim. Biophys. Acta* 101: 150–156.

Antonopoulos, C. A., Gardell, S.; Szirmai, J. A.; and De Tyssonsk, E.R. 1964. Determination of glycosaminoglycans (mucopolysaccharides) from tissues on the microgram scale. *Biochim. Biophys. Acta* 83 1–19.

Balazs, E. A., and Jeanloz, R. W. 1965 *The amino sugars,* Vol. IIa. New York and London: Academic Press.

Bergeron, J. A. 1958. Controlled staining of autoradiographs. *Stain Techn.* 33· 221–223

Bernard, G. W., and Pease, D. C. 1969. An electron microscopic study of initial intramembranous osteogenesis *Amer. J. Anat.* 125 271–290.

Bevelander, G , and Johnson, P. L. 1950. A histochemical study of the development of membrane bone *Anat. Rec.* 108 1–21.

Bitter, T., and Muir, H. M 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4 330–334

Bloom, G., and Kelly, J. W 1960. The copper phthalocyanin dye "Astrablau" and its staining properties, especially the staining of mast cells *Histochemie* 2 48–57

Bostrom, H., and Rodén, L 1961. On the metabolism of mucopolysaccharides. *Biochem. Pharmacol.* 6 100–110.

Brimacombe, J. S., and Webber, J. M. 1964. *Mucopolysaccharides.* B. B. A. Library. Vol. 6. Amsterdam. Elsevier Publ Comp.

Campo, R. D , and Tourtelotte, C D 1967. The composition of bovine cartilage and bone. *Biochim. Biophys. Acta* 141 614–624

Campo, R. D , Tourtelotte, C. D , and Bielen, R. J. 1969. The protein-polysaccharides of articular, epiphyseal plate and costal cartilages *Biochim. Biophys. Acta* 177 501–511.

Capps, J. C , and Shetlar, M R. 1963 In vivo incorporation of d-glucosamine-1-C^{14} into acid mucopolysaccharides of rabbit liver *Proc. Soc. Exp. Biol. Med.* 114 118–120.

Chvapil, M. 1967. *Physiology of connective tissue.* London. Butterworths.

Curran, R C 1964 The histochemistry of mucopolysaccharides. *Int. Rev. Cytol.* 17· 149–212.

Curran, R. C., and Collins, D H. 1957 Mucopolysaccharides in fields of intramembranous ossification in man. *J. Path. Bact.* 74 207–214.

D'Abramo, F , and Lipmann, F. 1957. The formation of adenosine-3'-phosphate-5'-phosphosulphate in extracts of chick embryo cartilage and its conversion into chondroitin sulphate. *Biochim. Biophys. Acta* 25 211–213.

Duterloo, H. S., and Jansen, H. W B 1969 Chondrogenesis and osteogenesis in the mandibular condylar blastema. In *Transactions of the European Orthodontic Society,* pp 109–118.

Dziewiatkowski, D D 1949. Rate of excretion of radioactive sulfur and its concentration in some tissues of the rat after intraperitoneal administration of labeled sodium sulfate. *J. Biol. Chem.* 178. 197–202.

Dziewiatkowski, D. D. 1956. Some aspects of the metabolism of chondroitin sulfate-S^{35} in the rat. *J. Biol. Chem.* 223 239–249.

Dziewiatkowski, D D 1958 Autoradiographic studies with S^{35}-sulfate In *Int. Rev. Cytol.* Vol VII. New York Academic Press.

Dziewiatkowski, D D., Benesch, R.E.; and Benesch, R. 1949 On the possible utilization of sulfate sulfur by the suckling rat for the synthesis of chondroitin sulfate as indicated by the use of radioactive sulfur. *J. Biol. Chem.* 178 931–938.

Fisher, W , and Weinland, F 1965 *Stoffwechsel der Galaktose und ihrer Derivate* Stuttgart Georg Thieme Verlag
Fleisch, H , and Bisaz, S 1965 Further evidence for the inhibitory role of pyrophosphate in calcification In *Proc Sec Eur Symp Calc Tiss*, Luik pp 299–305
Fullmer, H M 1965 In *Int.Rev Connect Tissue Res.* Vol 3 20 New York Academic Press

Good, T A , and Bessman, S P 1964 Determination of glucosamine and galactosamine using borate buffers for modification of the Elson Morgan and Morgan Elson reactions *Anal Biochem* 9 253–262
Greer, R B , Janicke, G H and Mankin, H J 1968 Protein polysaccharide synthesis at three levels of the normal growth plate *Calc Tiss Res* 2 157–164
Greiling, H , Herbertz, T , und Stuhlsatz, W 1964 Chromatographische Untersuchungen uber die Mucopolysaccharidverteilung im hyalinen Knorpel *Hoppe Seyler Z Physiol Chem* 336 149–162
Guri, C D , and Bernstein, D S 1965 Rat epiphyseal cartilage I Chemical analysis and isolation of crude protein polysaccharide *Proc Soc Exp Biol Med* 118 650–652

Hale, C W 1946 Histochemical demonstration of acid polysaccharides in animal tissues *Nature* 157 802
Ham, A W 1965 *Histology* 5th ed Philadelphia J B Lippincot Comp
Helting, T , and Roden, L 1968 The carbohydrate-protein linkage region of chondroitin 6 sulfate *Biochim Biophys Acta* 170 301–308
Hirschman, A , and Dziewiatkowski, D D 1966 Protein polysaccharide loss during endochondral ossification Immunochemical evidence *Science* 154 393–395
Hjertquist, S O 1964a Microchemical analysis of glycosaminoglycans (mucopolysaccharides) in normal and rachitic epiphyseal cartilage *Acta Soc Med Upsal* 69 23–40
Hjertquist, S O 1964b The glycosaminoglycans (mucopolysaccharides) of the epiphyseal plates in normal and rachitic dogs *Acta Soc Med Upsal* 69 83–104
Hjertquist, S O , and Vejlens, L 1968 The glycosaminoglycans of dog compact bone and epiphyseal cartilage in the normal state and in experimental hyperparathyroidism *Calc Tiss Res* 2 314–333
Hoffman, P 1968a In *The chemical physiology of mucopolysaccharides,* ed G Quintarelli, p 217 London J & A Churchill Ltd
Hoffman, P 1968b The chemistry of the protein polysaccharides of connective tissue In *The chemical physiology of mucopolysaccharides* ed G Quintarelli, pp 33–49
Hooghwinkel, G J M , and Smits, G 1957 The specificity of the periodic acid-Schiff technique studied by a quantitative test tube method *J Histochem Cytochem* 5 120–126

Jeanloz, R W 1960 The nomenclature of mucopolysaccharides *Arthritis Rheum* 3 233–237

Kalter, H 1968 How should times during pregnancy be called in teratology? *Teratology* 1 231–234

Lindenbaum, A , and Kuettner, K E 1967 Mucopolysaccharides and mucoproteins of calf scapula *Calc Tiss Res* 1 153–165
Lindner, J 1965 Kolloquium der Gesellschaft fur Histochemie auf den VIII Symposion in Wien 1962 *Acta Histochem* Suppl Band 5 115–136
Lovell, D , Schorah, C J , and Curran, R C 1966 Glycosaminoglycans (acid mucopolysaccharides) in experimental granulomata *Brit J Exp Path* 47 228–234

MacIntyre, I 1957 The flame spectrophotometric determination of calcium in biological fluids and an isotopic analysis of the errors in the Kramer Tisdall procedure *Biochem J* 67 164–172
Mathews, M B 1968 The macromolecular organization of connective tissue In *The chemical physiology of mucopolysaccharides,* ed G Quintarelli, pp 189–197 London J & A Churchill Ltd

McLean, F. C , and Urist, M. R. 1968. *Bone.* 3rd ed. Chicago. Univ. of Chicago Press.
Meyer, K. 1938. The chemistry and biology of mucopolysaccharides and glycoproteins. *Cold Spring Harbor Symposia Quantitative Biology* 6. 91.
Milaire, J. 1968. Histochemical investigations into the development of the extremities and some of their congenital malformations. Symposium 'Histochemistry of morphogenesis', Amsterdam.
Muller, G. 1955. Ueber eine Vereinfachung der Reaktion nach Hale (1946). *Acta Histochem.* 2. 68–70.

Nakada, H. I., Wolfe, J. B., Hochstein, L. I.; and Andreoli, A. J. 1960. A spectrophotometric method for the assay of bacterial mucopolysaccharases. *Anal. Biochem.* 2: 168–177.

Pearce, R. H.; Mathieson, J. M.; and Grimmer, B J. 1968. Fractionation of anionic glycosaminoglycans by ion-exchange chromatography. *Anal.Biochem.* 24 141–156.
Pearse, A. G. E. 1955 Copper Phthalocyanins as phospholipid stains. *J.Path.Bact.* 70: 554–557.
Pearse, A. G. E. 1961. *Histochemistry*. 2nd ed. London: J. & A. Churchill Ltd.
Pearse, A. G E. 1968. *Histochemistry*. 3rd ed. Vol. 1. London J. & A. Churchill Ltd
Perlman, R. L., Telser, A., and Dorfman, A 1964. The biosynthesis of chondroitin sulfate by a cellfree preparation. *J.Biol.Chem.* 239· 3623–3629.
Pioch, W 1957 Ueber die Darstellung saurer Mucopolysaccharide mit dem Kupferphthalocyaninfarbstoff Astrablau. *Virchow.Arch.* 330. 337–346.
Pritchard, J. J. 1952. A cytological and histochemical study of bone and cartilage formation in the rat. *J.Anat.* 86· 259–277.

Quintarelli, G., and Dellovo, M. C. 1965. The chemical and histochemical properties of alcian blue. IV. Further studies on the methods for the identification of acid glycosaminoglycans. *Histochemie* 5. 196–209.
Quintarelli, G.; Scott, J E., and Dellovo, M. C. 1964a. The chemical and histochemical properties of alcian blue. II. Dye binding of tissue polyanions *Histochemie* 4 86–98.
Quintarelli, G., Scott, J. E., and Dellovo, M. C. 1964b. The chemical and histochemical properties of alcian blue. III. Chemical blocking and unblocking. *Histochemie* 4 99–112.

Ray Sarkar, B. C., and Chauhan, U. P. S. 1967. A new method for determining micro quantities of calcium in biological materials. *Anal. Biochem.* 20. 155–166.
Ringertz, N R , and Reichard, P. 1960. Chromatography on ECTEOLA of sulfate containing mucopolysaccharides *Acta Chem.Scand.* 14: 303–311.
Romeis, B. 1968. *Mikroskopische Technik.* 16. Aufl. Munchen, Wien· R. Oldenbourg Verlag.

Saunders, A. M 1964. Histochemical identification of acid mucopolysaccharides with acridine orange. *J.Histochem.Cytochem.* 12 164–170
Schiller, S 1964. Synthesis of hyaluronic acid by a soluble enzym system from mammalian tissue. *Biochem.Biophys.Res.Commun.* 15 250.
Schiller, S., Slover, G A., and Dorfman, A. 1961. A method for the separation of acid mucopolysaccharides. its application to the isolation of heparin from skin of rats. *J.Biol. Chem.* 236. 983–987.
Schmidt, M. 1962. Fractionation of acid mucopolysaccharides on DEAE-Sephadex anion exchanger. *Biochim.Biophys.Acta* 63 346–348.
Scott, J. E 1955 The solubility of cetylpyridinium complexes of biological polyanions in solution of salts. *Biochim.Biophys.Acta* 18. 428–429.
Scott, J E 1960. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues *Meth.Biochem.Anal.* 8 145–197
Scott, J E. 1968 Patterns of specificity in the interaction of organic cations with acid mucopolysaccharides In *The chemical physiology of mucopolysaccharides*, ed G. Quintarelli, pp 219–231. London. J. & A Churchill Ltd.
Scott, J. E., and Dorling, J. 1965 Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. *Histochemie* 5 221–233.

Scott, J E , Quintarelli, G , and Dellovo, M C 1964 The chemical and histochemical properties of alcian blue I The mechanism of alcian blue staining *Histochemie* 4 73–85
Searls, R L 1965 Isolation of mucopolysaccharide from the precartilaginous embryonic chick limb bud *Proc Soc Exp Biol Med* 118 1172–1176
Sobel A E 1965 Multiple substances and mechanisms of nucleation In *Proc Sec Eur Symp Calc Tiss* , Luik pp 291–298
Solheim, K 1965 The glycosaminoglycans of human semilunar cartilage *J Oslo City Hosp.* 15 127–132
Solheim, K 1966 Distribution of glycosaminoglycans, hydroxyproline and calcium in healing fractures *Acta Soc Med Upsal* 71 1–13
Steedman, H F 1950 Alcian blue 8 GS A new stain for mucin *Quart J Micr Sci* 91 477–479
Stefanovich, V , and Gore, I 1967 A micromethod for the determination of acid mucopolysaccharides in vascular tissue *J Chromatogr* 31 473–478
Svejcar, J and Robertson, W V B 1967 Micro separation and determination of mammalian acidic glycosaminoglycans (mucopolysaccharides) *Anal Biochem* 18 333–350
Sylven, B 1947 Cartilage and chondroitin sulphate II Chondroitin sulphate and the physiological ossification of cartilage *J Bone Joint Surg* 29 973–976

Telser, A , Robinson, H C , and Dorfman, A 1966 The biosynthesis of chondroitin sulfate *Arch Biochem* 116 458–465

Van den Hooff, A 1964 Polysaccharide histochemistry of enchondral ossification *Acta Anat* 57 16–28

Weidmann, S M 1963 Calcification of skeletal tissues In *Int Rev Connect Tissue Res* Vol I London Academic Press
White, B N , Shetlar, M R , Shurley, H M and Schilling, J A 1965 Incorporation of 1-^{14}C-glucosamine into mucopolysaccharides of rat connective tissue *Biochim Biophys Acta* 101 97–105
Williams, G , and Jackson, D S 1956 Two organic fixatives for acid mucopolysaccharides *Stain Techn* 31 189–191

Youssef, E H 1966 The chondrocranium of the albino rat *Acta Anat* 64 586–617
Youssef, E H 1969 Development of the membrane bones and ossification of the chondrocranium in the albino rat *Acta Anat* 72 603–623

Zugibe, F T , and Fink, M L 1966 A new ion association technique for demonstrating polyanions in tissue sections *J Histochem Cytochem* 14 147–152

STELLINGEN

I

De frequent in histologische literatuur gebruikte term 'glycolgroep' geeft weliswaar een bruikbare aanduiding van een bepaalde configuratie, maar is als chemische term onjuist.

II

Astrablau is wellicht toe te passen als vitale kleurstof voor botmarkering.

III

Loodacetaat, intraveneus toegediend aan proefdieren in een dosis van 4 mg per kg lichaamsgewicht, is een zeer bruikbare vitale kleurstof voor botmarkering in combinatie met lichtmicroscopie van ontkalkte coupes.

IV

Bestudering van normale biologische processen is moeilijker dan van pathologisch of experimenteel opgeroepen verschijnselen.

V

Toevoeging van fluoride aan drinkwater is niet alleen van belang ter voorkoming van tandcaries op jeugdige leeftijd, maar bovendien ter voorkoming van osteoporose op latere leeftijd.

VI

Modelexperimenten voor het onderzoek van krachten, die door middel van vaste orthodontische apparatuur aangrijpen op gebitselementen, kunnen een beter inzicht verschaffen over de materialen die voor deze apparatuur bij voorkeur gebruikt dienen te worden.

VII

Voor een academische carrière is het aantal publicaties belangrijker dan de inhoud ervan.

VIII

Bij de bouw en inrichting van laboratoria wordt als regel te weinig rekening gehouden met de eisen die voor het gebruik van dergelijke ruimten gesteld worden.

IX

Dat vooral de vrouw haar fotografisch portret meestal maar matig weet te waarderen is voornamelijk terug te voeren op het feit dat zij haar spiegelbeeld te goed kent.

Henk W. B. Jansen

Nijmegen, 1 april 1971