CHONDROITINSULFATES AND CALCIFICATION IN PUPPY RIB CARTILAGE

A Thesis

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Master of Science

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by

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ABSTRACT

PART I

Rib cartilage slices from a litter of dogs killed at various ages were homogenized successively with water, 3% KCl, 0.05 N HCl and 1.25 N KOH and the extracts analysed for calcium, phosphate and chondroitinsulfate (CS) glucuronate. The ribs were divided into 3 groups (uncalcified, beginning calcification and advanced calcification) by the use of arbitrary limits for the inorganic phosphate dissolved by acid extraction and then a statistical analysis of the differences between the results for the three groups was carried out. The results showed that during the initial stages and later in the advanced stages of calcification there was a significant increase in CS in the fractions not extractable with water and this was nearly all accounted for by increases in the amount of CS solubilized in the alkali extract. These changes were accompanied by an initial rise and a later fall in the total CS glucuronate in the cartilage and also by an initial rise and a later large fall in the amount of CS solubilized in the water extracts.

There was a gradual decline in the water content of the ribs as they passed through the various stages of calcification.

Upon incorporation of $Na_2{}^{35}SO_4$ by the rib cartilage the specific activity in all the rib cartilage extracts decreased slowly with increasing age of the puppy and also as the ribs

progressed from the uncalcified state to the advanced stages of calcification. The water-extractable CS was metabolized at a faster rate than that extracted with alkali.

PART II

Rib cartilage powder was homogenized successively with 0.05 M EDTA, water, $CaCl_2$ and water and then incubated with proteolytic enzymes after which the pellet was homogenized further with solutions of 3% KCl, 0.05 N HCl and 1.25 N KOH respectively and the extracts analysed for calcium, protein and CS glucuronate.

During incubation of the pellet with papain it was found that nearly all the calcium, between 75-85% of the non-collagenous protein and 50-75% of the CS was extracted while solubilization of collagen was much less complete (15-30%). Most of the remaining calcium, non-collagenous protein and CS was then dissolved by KCl. During the incubation period the pH of the medium changed from 6.2-6.3 to 4.1-4.6 (unbuffered) and from 5.4 to 4.9 (buffered with 0.02 M sodium acetate-acetic acid). The more acid the pH the greater would be the tendency of CS to form insoluble salts with protein, thus raising the possibility that a portion of the CS formed new insoluble complexes during digestion.

Rib cartilage powder was homogenized with solutions of 0.05 M EDTA, water, $CaCl_2$, water, 3% KCl (dissolved in a 0.2 M Na_2HPO_4-0.1 M citric acid (pH 7.0) instead of KCl

dissolved in distilled water), 0.05 N HCl and 1.25 N KOH respectively. It was found that there was no change in the total amount of CS extracted by the 3% KCl (0.4 M) under the highly buffered conditions. The finding that neither 0.4 M KCl nor a combination of this with buffer will remove the last portion of the CS (that which is dissolved by KOH) indicates that this fraction of the CS must be bound to protein in vivo by non-salt linkages.

In another series of experiments rib cartilage powder was homogenized with solutions of 0.05 M EDTA, water, $CaCl_2$, water, 3% KCl, 0.05 N HCl and 1.25 N KOH respectively and analysed for protein and CS. The removal of solid mineral matter with EDTA did not alter greatly the amount of CS which was not extracted with water or KCl and could only be removed by alkali. This indicates that the insolubility of the alkali-extracted CS is not due to physical binding to the solid mineral matter.

PREFACE

To set the background to this work a brief description of bone and cartilage is given below.

CARTILAGE

Cartilage consists of cells, chondrocytes, embedded in a matrix. This matrix, in turn, is composed of an organized fiber meshwork, chiefly collagen, and of an amorphous ground substance especially rich in sulfated mucopolysaccharides. The chief component of the ground substance is chondromucoprotein, which is a compound containing the polymer chondroitinsulfate linked through a sugar and O-serine to a protein. It may represent up to 44% of the dry weight of hyaline cartilage.

There are 3 generally recognized varieties of adult human cartilage: hyaline, fibrous and elastic. Hyaline cartilage is found covering the articular surfaces of most joints, in the costal cartilages, etc.

BONE

Bone is a calcified connective tissue. Bone tissue is cellular, with the bone cells, osteocytes, enclosed within compartments called lacunae. The organic matrix, which accounts for approximately 38% of the dry weight of the tissue, is almost entirely composed of collagen fibres. There is an additional amorphous ground substance, which contains a small but functionally significant amount of sulfated mucopolysaccharides. The inorganic phase consists almost exclusively

of calcium phosphate salts. All bone is formed by functional osteoblasts. Osteogenesis is a two-phased process; the calcifiable organic matrix, consisting of collagen fibres and ground substance, is formed first, followed by mineralization.

ORGANIZATION OF THE THESIS

The thesis is divided into 2 sections: Part I and Part II. Each part has its own introduction, materials and methods, results and discussion. A final discussion embracing the results of both sections is given at the end of Part II. This is followed by some comments concerning the future expansion of the present work.

ABBREVIATIONS

The following abbreviations were used in this thesis:

chondroitinsulfate	CS
inorganic phosphate	inorganic P or Pi
total phosphate	Pt
week	wk
weight	wt
gram	g or gm
minute	min
hour	hr
chondromucoprotein	CMP
proteinpolysaccharide	PP
non-collagenous protein	NCP
acid mucopolysaccharides	AMPS
optical density	O.D.

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PART I

CALCIUM, PHOSPHATE AND CHONDROITINSULFATE LEVELS AT VARIOUS STAGES OF RIB CALCIFICATION

INTRODUCTION

Neuman and Neuman (1) considered the mechanism of calcification to involve the formation of crystal nuclei upon which epitactic growth of the solid mineral matter takes place. Although the exact nature of the agent or agents responsible for the induction of the crystal nuclei is still unknown, proposals have been made about the nature of the mineralizing site. It has often been suggested that chondroitinsulfate is a necessary factor in calcification but the exact role proposed has varied greatly. For instance, Sobel (2) proposed that it was responsible, together with collagen, for initiating the process of crystal formation whereas Glimcher (3) proposed inhibition of calcification as the main function of CS.

Because of the simpler histological organization and the fact that a transition from an uncalcified to a calcified state can be observed within the same tissue, the relation between CS and calcification can be more easily studied in cartilage than in bone. The results obtained with calcified cartilage, however, may not necessarily apply to bone.

Boyd and Neuman (4) have shown that CS is a principal cation binding constituent of cartilage. Their <u>in vitro</u> experiments showed that cartilage slices bind no phosphate unless the cartilage contains appreciable amounts of calcium, in which case phosphate is taken up by the cartilage until the Ca:P ratio is approximately that of hydroxyapatite.

Eichelberger and Roma (5) showed that during the growth of puppies there is an increase in the calcium content of the rib cartilage even before there is accumulation of phosphate and deposition of solid mineral matter. They found that during the initial 12 weeks of the puppies' life there was a constant amount of calcium and phosphate in the rib cartilage, but from 13-16 weeks of age there was over a 2-fold increase in the calcium content of the ribs while the phosphate content remained fairly constant. They suggested that the excess calcium was associated with the CS of the cartilage, but not with the collagen.

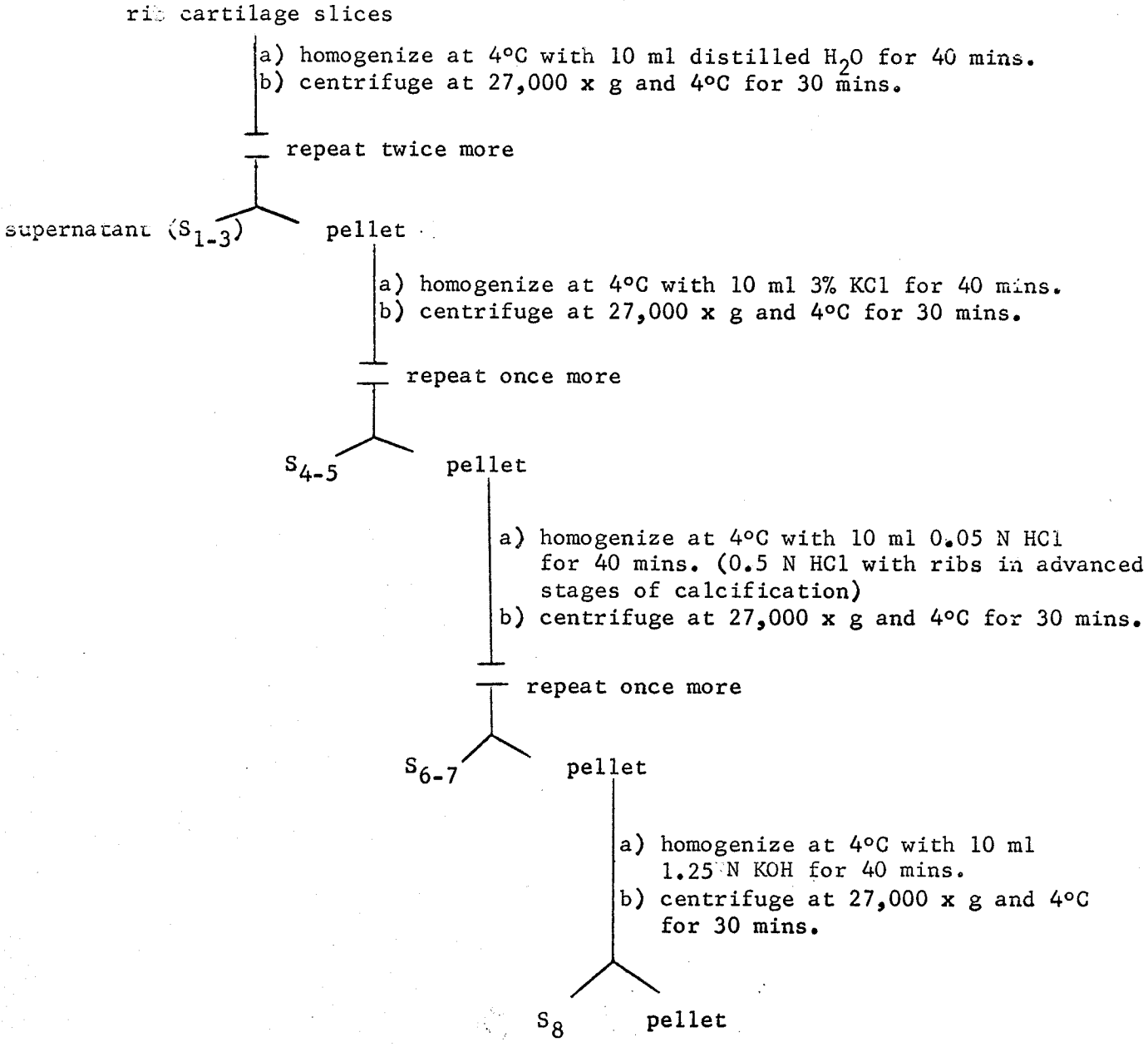
The CS of rib cartilage has been shown to consist of at least two metabolically distinct fractions (6,7,32). The fraction which, with collagen, is not dissolved by homogenization with water or 3% KCl has a lower turnover rate than the soluble CS and has been shown to have the greatest affinity for calcium, being able to retain the major part of the non-precipitated calcium in cartilage even in the presence of a much greater quantity of the soluble CS fraction (8).

The present work was undertaken with the following objectives: 1) to find out which CS fraction, if any, binds the calcium which accumulated in Eichelberger's and Roma's experiment prior to calcification and to see if the calcium is bound to the same material during and after calcification, (2) to find out if there are any changes in the amount and

state of chondroitinsulfates in puppy rib cartilage during calcification.

Chart 1

Flow Chart for the Extraction of Rib Cartilage Slices



MATERIALS AND METHODS

EXTRACTION PROCEDURE

Puppies from the same litter (4-7 animals) were killed with chloroform at various ages up to 27 weeks. The ribs were removed and cleaned of adhering tissue. Thin slices of the cartilage were cut with a scalpel. The ribs were stored in a freezer and utilized as soon as possible after death of the puppy.

In order to remove all water-soluble Ca, inorganic P and CS 0.5 g portions of rib cartilage were homogenized (in a fluted container using a Virtis "23" homogenizer running at top speed) with 10 ml distilled water at $4^{\circ}C$ for 40 mins. The homogenate was centrifuged at 27000 x g and $4^{\circ}C$ for 30 mins, and the supernatant extract poured off and retained for analysis. The precipitate was then homogenized with a further 10 ml water and the procedure repeated to yield a total of three water extracts (S_{1-3}).

At this stage a water-insoluble residue and 3 water extracts (S_{1-3}) had been obtained. To remove the material that might be bound by salt linkages the precipitate was homogenized with 10 ml 3% KCl (0.4 M) and centrifuged as already described. The supernatant was poured off and retained for analysis and the homogenization and centrifugation process was repeated once more to yield a total of two KCl extracts (S_{4-5}).

At this stage most of the insoluble calcium salts from calcified ribs remained in the residue. To solubilize this

inorganic material the precipitate was homogenized with 10 ml 0.05 N HCl (0.5 N HCl with ribs in advanced stages of calcification) and centrifuged as described previously. The supernatant was poured off and retained for analysis and the homogenization and centrifugation repeated once more to yield a total of two acid extracts (S_{6-7}).

Finally to remove nearly all the remaining CS the precipitate was homogenized with 10 ml 1.25 N KOH and centrifuged as above. The supernatant was poured off and retained for analysis (S_8). An insoluble residue remained after the KOH extraction. (See Flow Chart 1, page 5).

UPTAKE OF RADIOACTIVE SULFATE

Puppies, at different ages and from different litters and at various stages of calcification, were injected intraperitoneally with $Na_2{}^{35}SO_4$ in isotonic saline (Atomic Energy of Canada) using 0.25 mC/kg body weight. They were killed with chloroform 25 hours after injection. The rib cartilage was extracted as already described.

The KOH extract was neutralized with HCl and then all the extracts were dialysed against running tap water for 2 hours and subsequently placed in a 20 liter tank of distilled water for 16 hours. Afterwards 0.1 ml of each extract was pipetted onto a separate planchet and spread evenly with the pipette such that each extract covered the same surface area on each planchet. The planchets were placed under an Infra Rediator (Fisher Scientific Co.) until

the extracts had evaporated to dryness. The radioactivity of the extracts was then counted in a Nuclear Chicago 181A scaler, gas flow counter and 111B printing timer. The uronic acid content of all extracts was estimated and the specific activity calculated (counts/min/μmole CS glucuronate).

ANALYTICAL PROCEDURES

The extracts were analysed by standard methods for inorganic phosphate (10), total phosphate (11), uronic acid (12) and calcium (see below). Standard curves were prepared for all the tests (see appendix).

CALCIUM DETERMINATION

Three different methods were tried for calcium. In the first method by Bachra *et al* (13), Cal-red served as the indicator solution. This method was found to have a major weakness in that the readings had to be taken very quickly as the colour tended to fade rapidly and therefore readings depended entirely on the ability of the investigator to detect the rapid change in colour. The glyoxal bis (2-hydroxyanil) estimation procedure (GBHA) of Mager and Farese (14) also exhibited a major weakness in that the test was only accurate for calcium concentrations which were no higher than 40 mg/100 ml of solution. High phosphate concentrations also interfered with the test. The calcein estimation procedure of Eastoe (15) was found to be the best

and was subsequently used in all the tests because there was no detectable interference by CS and/or inorganic P. The least amount of calcium which could be detected was 0.025 µgm atoms/ml and 3.767 µg atoms/ml could be estimated with an accuracy of 2.1%.

The Calcein estimation procedure of Eastoe (15) was slightly modified in that all titrations with Calcein were carried out using a fluorometer as the "end-point guide". The fluorometer was zeroed using a water blank to which all reagents were added including Calcein. The individual unknown samples were then read on the fluorometer and EDTA was added to each until each unknown/or standard sample read zero on the fluorometer. Results using this method of assay were found to have satisfactory reproducibility (Table I) and did not depend on the colour sensitivity of the observer.

MAINTENANCE OF THE PUPPIES

The puppies were fed Pablum and milk twice daily for the initial 12 weeks and then fed a mixture of Dr. Ballard's canned dog food and Gravy Train once daily.

TABLE I

REPRODUCIBILITY AND RECOVERY ESTIMATES FOR CALCIUM USING CALCEIN INDICATOR AND FLUOROMETER

Number of tests	Materials added/ml	EDTA titration (mean ± standard deviation)	Recovery of calcium (% using the mean figures)
10	Ca (0.5 µg atom)	0.103 ± 0.006	-
10	Ca (0.5 µg atom)+CS (2.85 µmole)	0.109 ± 0.006	106
10	Ca (0.5 µg atom)+Pi(0.256 µg/atom)	0.102 ± 0.005	99
10	Ca (0.5 µg atom)+CS(2.85 µmole)+Pi (0.256 µg atom)	0.109 ± 0.006	106
10	Rib extract (RE)(S_1)(0.5 ml)	0.072 ± 0.003	-
10	RE (0.5 ml)+ Ca (0.5 ug atom)	0.174 ± 0.006	99
10	Rib extract (S_7)(0.10 ml)	0.666 ± 0.014	-
10	RE (0.10 ml)+ Ca (0.5 µg atom)	0.762 ± 0.016	93
10	Ca (3.0 µg atom)	0.614 ± 0.014	-
10	Ca (3.0 µg atom) + Pi (9.6 µg atom)	0.612 ± 0.014	99.7

RESULTS

FIRST SERIES

A litter of 4 dogs was used in the first series of experiments. A rib was chosen at random from each of the 4 dogs and homogenized as previously described. The results are shown in Table II. Two thirds of the CS glucuronate was extracted with water as was previously found by Bowness (8). Calcification of the rib cartilage was found to begin between the initial 13½ weeks (dog II) and 16 1/7 weeks (dog III) of the puppies' life and was accompanied by a rapid increase of both calcium and inorganic P in all extracts (S_{1-8}).

SECOND SERIES

A litter of 7 dogs was used in this series of experiments. Specific ribs were homogenized from each dog. In dog III (Table III) all the ribs were homogenized separately and analysed. The results for dog III are given in Table III. Photomicrographs were also taken of each of these ribs and are shown in Figures 1-9 (see appendix). These pictures clearly show the gradual changes in the ribs as they become progressively more calcified.

Not all the ribs from the other dogs were analysed. In dog I (9-9½ wks) the ribs were all very small and were found to be uncalcified. To obtain sufficient material for repeated analyses all the ribs were pooled in this dog. In dog IV, ribs 1 and 2 were analysed together and also ribs 3 and 4. In dog VI, ribs 1 and 2 and ribs 2 and 3 were

TABLE II

EXTRACTION OF RIB CARTILAGE
First Series of Experiments

	Dog I 9 1/3 wks	Dog II 13⅔ wks	Dog III 16 wks 1 day	Dog IV 21 wks 5 days
Ca, S_{1-3}	18.6	13.5	46.4	61.9
Ca, S_{4-5}	16.6	15.2	89.3	110.5
Ca, S_{6-7}	2.4	0.8	400.0	1269.6
Inorganic P, S_{1-3}	6.8	6.5	59.9	34.2
Inorganic P, S_{4-5}	0.4	0.2	21.5	10.4
Inorganic P, S_{6-7}	0.9	0.2	228.0	511.0
Ca/Pi, S_{6-7}	2.69	19.0	1.76	2.48
Pi/Pt, S_{1-8}	0.42	0.36	0.98	0.64
CS glucuronate, S_{1-8}	142.6	149.7	196.4	166.4
CS glucuronate, S_{4-8}	56.0	52.8	62.4	62.4
% CS glucuronate, S_{1-3}	61	65	68	63

Note: Figures are in µg atoms/g wet weight for Ca and P; µmoles/g wet weight for CS glucuronate.

TABLE III

EXTRACTION OF RIB CARTILAGE
Second Series of Experiments

Dog III:Age:13 1/3-13 2/3 wks.	Dog III Rib 1	Dog III Rib 2	Dog III Rib 3	Dog III Rib 4	Dog III Rib 5
Ca, S_{1-3}	21.0	14.4	24.3	34.4	44.0
Ca, S_{4-5}	10.9	10.0	17.2	33.4	40.0
Ca, S_{6-7}	3.1	1.7	3.3	33.3	64.6
Inorganic P, S_{1-3}	2.5	8.1	8.3	19.2	24.5
Inorganic P, S_{4-5}	0.2	0.2	1.4	6.3	8.5
Inorganic P, S_{6-7}	0.2	0.12	0.8	16.0	31.3
Ca/Pi, S_{6-7}	15.5	13.8	4.0	2.08	2.06
Pi/Pt, S_{1-8}	0.18	0.22	0.49	0.74	0.79
CS glucuronate, S_{1-8}	157.2	154.4	179.2	173.5	184.6
CS glucuronate, S_{4-8}	34.3	29.6	36.4	38.0	37.5
Ca S_{1-3}/CS glucuronate S_{1-3}	0.171	0.115	0.170	0.254	0.300
Ca S_{4-5}/CS glucuronate S_{4-8}	0.318	0.338	0.473	0.880	1.067
% CS glucuronate, S_{1-3}	78	81	80	78	80

continued

TABLE III CONTINUED

	Dog III Rib 6	Dog III Rib 7	Dog III Rib 8	Dog III Rib 9
Ca, S_{1-3}	37.0	40.6	42.1	29.9
Ca, S_{4-5}	48.0	42.7	44.7	38.8
Ca, S_{6-7}	71.4	56.7	73.3	27.7
Inorganic P, S_{1-3}	23.5	25.3	29.7	19.4
Inorganic P, S_{4-5}	8.6	10.3	9.7	5.9
Inorganic P, S_{6-7}	39.5	29.7	36.5	14.8
Ca, Pi, S_{6-7}	1.81	1.91	2.00	1.87
Pi/Pt, S_{1-8}	0.80	0.84	0.83	0.77
CS glucuronate, S_{1-8}	213.1	180.1	192.0	173.6
CS glucuronate, S_{4-8}	54.2	38.0	43.4	41.7
Ca S_{1-3}/CS glucuronate S_{1-3}	0.233	0.286	0.283	0.226
Ca S_{4-5}/CS glucuronate S_{4-8}	0.886	1.124	1.030	0.930
% CS glucuronate, S_{1-3}	75	79	77	76

Note: Figures are in µg atoms/g wet weight for Ca and P.
µmoles/g wet weight for CS glucuronate.

analysed together. In all other cases ribs were analysed separately. Those analysed are listed below (rib numbering is anatomical (18), see Figure 1-59).

Dog II - ribs 1-3,6,9

Dog III - all the ribs

Dog IV - ribs 1+2,3+4,6,9

Dog V - ribs 1,3,4,6,9

Dog VI - ribs 1+2,2+3,6,9

Dog VII - ribs 8,9

The results for each of these dogs, except dog VII, will be found in the appendix (Tables VII-X).

Table IV shows the results for rib 9 from each of the 7 dogs.

Results for all ribs which were assayed individually, not in pairs or groups, were put together for a statistical analysis of the difference between calcified and uncalcified ribs in their total CS content and distribution of CS into water extractable and non-extractable fractions (Table V). The ribs were divided into 3 groups (uncalcified (A), beginning calcification (B), and advanced calcification (C)) by the use of arbitrary limits for the inorganic P dissolved by acid extraction (S_{6-7}), which is presumably indicative of the solid mineral matter present. Calcification was considered to have begun if the inorganic P S_{6-7} was > 2 µg atoms/g fresh tissue while the ribs were considered to be in the advanced stages of calcification if the inorganic P

(from "Anatomy of a Dog" by M.E. Miller)

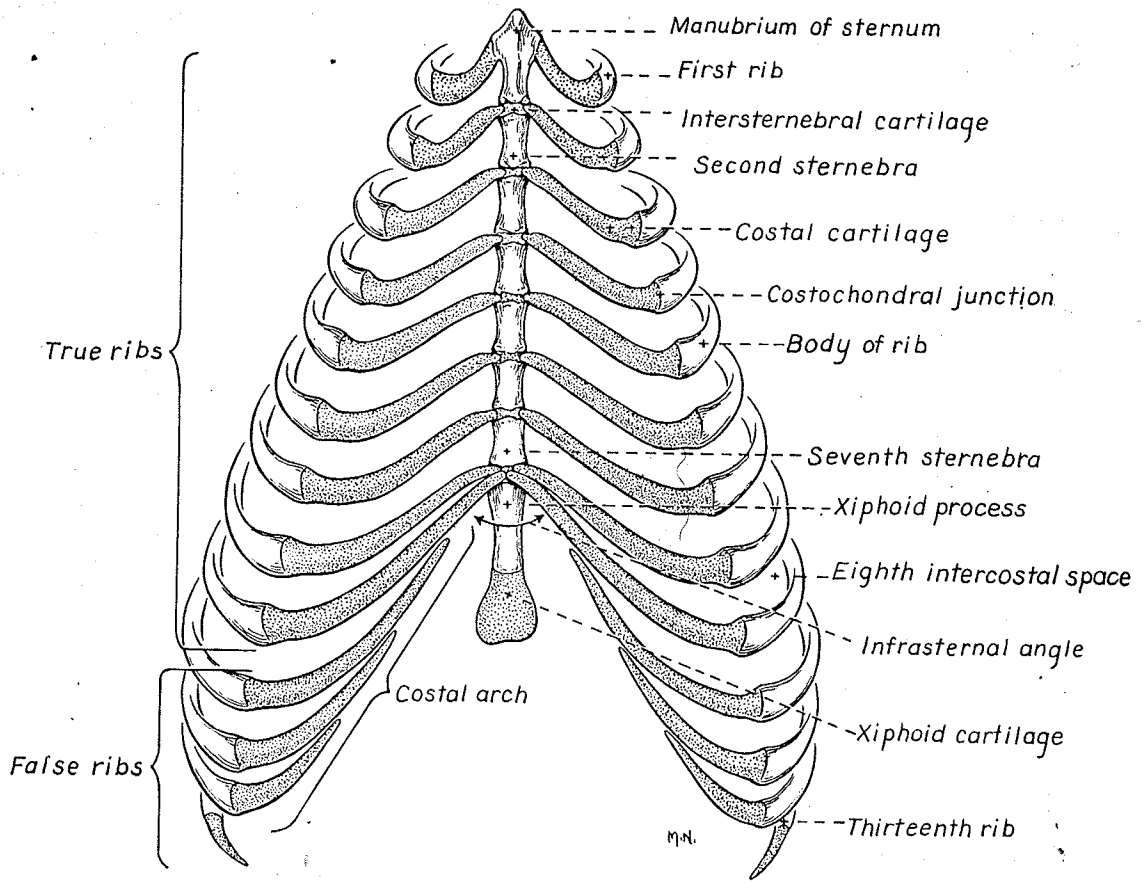


FIG. 1-59. Ribs and sternum, ventral aspect.

TABLE IV

EXTRACTION OF RIB CARTILAGE
Second series of experiments

	Dog I 9-9½ wks	Dog II 13-13½ wks	Dog III 13 1/3-13 2/3 wks
Ca, S_{1-3}	14.6	35.6	29.9
Ca, S_{4-5}	13.1	41.6	38.8
Ca, S_{6-7}	2.1	50.0	27.7
Inorganic P, S_{1-3}	6.1	25.0	19.4
Inorganic P, S_{4-5}	0.18	7.9	5.9
Inorganic P, S_{6-7}	0.16	23.4	14.8
Ca, Pi, S_{6-7}	10.5	2.14	1.87
Pi/Pt, S_{1-8}	0.18	0.85	0.77
CS glucuronate, S_{1-8}	168.6	184.5	173.6
CS glucuronate, S_{4-8}	40.9	46.4	41.7
Ca S_{1-3}/CS glucuronate S_{1-3}	0.114	0.258	0.226
Ca S_{4-5}/CS glucuronate S_{4-8}	0.321	0.898	0.930
% CS glucuronate, S_{1-3}	76	75	76

continued

TABLE IV

EXTRACTION OF RIB CARTILAGE
Second series of experiments
CONTINUED

	Dog IV 13 1/3-13 2/3 wks	Dog V 15 1/3 - 15 2/3 wks	Dog VI 17 1/3 wks	Dog VII 26 1/3-26 2/3 wks
Ca, S_{1-3}	34.2	48.9	54.6	50.7
Ca, S_{4-5}	55.6	64.4	78.7	97.3
Ca, S_{6-7}	160.0	186.8	201.4	1539.6
Inorganic P, S_{1-3}	26.2	42.1	31.3	46.9
Inorganic P, S_{4-5}	10.2	10.1	12.5	13.0
Inorganic P, S_{6-7}	86.8	105.0	110.4	861.2
Ca/Pi, S_{6-77}	1.84	1.79	1.82	1.79
Pi/Pt, S_{1-8}	0.83	0.84	0.88	0.90
CS glucuronate, S_{1-8}	161.6	198.9	182.1	157.7
CS glucuronate, S_{4-8}	46.9	58.1	40.1	69.4
Ca S_{1-3}/CS glucuronate S_{1-3}	0.328	0.347	0.386	0.572
Ca S_{4-5}/CS glucuronate S_{4-8}	1.190	1.110	1.963	1.402
% CS glucuronate, S_{1-3}	71	71	78	56

Note: Rib 9 used in each case.
Figures are in µg atoms/g wet weight for Ca and P;
µmoles/g wet weight for CS glucuronate.

TABLE V

COMPARISON OF THE CS COMPARTMENTS IN RIBS
AT THREE STAGES OF CALCIFICATION
Results are expressed as μmoles/g fresh tissue

CS glucuronate in	RIBS * uncalcified (A)	Calcified (beginning calcification (B)	Calcified (advanced stages) (C)	Difference of means (B-A)	Difference of means (C-B)	% Difference of Means (A and B)	% Difference of Means (B and C)	P Value (A and B)	P Value (B and C)
S_{1-8} (Total)	171.08±12.44 (6)	186.68±17.48 (17)	156.50±7.56 (4)	+15.60	-29.61	9	16	0.10	0.0025
S_{1-3} #	134.02±8.75 (6)	142.52±20.46 (17)	87.83±1.81 (4)	+ 8.50	-54.69	6	38	0.20	0.0005
S_{4-8} ##	37.07±5.07 (6)	44.17±5.98 (17)	68.65±6.59 (4)	+ 7.10	+24.48	19	56	0.01	0.0005
S_{4-5} KCl	23.08±1.65 (6)	23.66±2.99 (17)	26.08±1.73 (4)	+ 0.58	+ 2.42	2.5	10	0.45	0.1000
S_{8} KOH	13.02±3.77 (6)	19.54±5.73 (17)	41.55±6.19 (4)	+ 6.52	+22.01	50	113	0.01	0.0005

*Calcification was considered to have begun if the inorganic P S_{6-7} was 2 ug atoms/g fresh tissue. The ribs were considered to be in the advanced stages of calcification if the inorganic P was 800 ug atoms/g fresh tissue.

**P is obtained from Snedecor's revised form of Fisher's t-test (19,45). P is the probability the two samples are drawn from the same population.

#Water soluble.

##Water insoluble.

Note: Figures in brackets show number of ribs analysed in each group.

S_{6-7} was > 800 µg atoms/g fresh tissue. An examination of histological sections from the ribs stained with von Kossa (33,34) and eosin was also carried out (see appendix, Figs. 1-10). This provided a clear confirmation of the division of the ribs into uncalcified and beginning calcification groups from the phosphate analyses. However, neither the phosphate analyses nor the histological sections provide evidence for any clear break between ribs beginning calcification and those in an advanced stage of calcification. From the point of view of these parameters, the distinction between these two groups is purely arbitrary, but as will be seen the total chondroitinsulfate content of the ribs does suggest that a distinction can be made.

Table VI shows the results of the uptake of radioactive sulfate by one rib from each of the three groups of ribs (uncalcified, beginning calcification and advanced stages of calcification).

TABLE VI

SPECIFIC ACTIVITY (COUNTS/MIN/μMOLES CS GLUCURONATE) IN RIB CARTILAGE EXTRACTS OF PUPPIES KILLED 25 HOURS AFTER INJECTION WITH $Na_2{}^{35}SO_4$ (0.25 mC/kg body wt)

Treatment	RIBS			
	Uncalcified**	Beginning Calcification**	Advanced Calcification***	
Water (S_{1-3})	2990	1735	1336	1254*
3% KCl (S_{4-5})	2230	1220	1048	1069*
1.25N KOH (S_8)	985	700	430	473*
S_8/S_{1-3}	0.329	0.403	0.322	0.379

* water was replaced by 0.05 M EDTA (di-sodium) which was then followed by the latter two treatments.

** ribs are from a 17 week old dog (one rib of each type was analysed).

*** ribs are from a 30 week old dog (one rib was extracted by each method).

DISCUSSION

CHONDROITINSULFATES IN RIB CARTILAGE
(Second series of experiments)

Tables III and IV, VII-X show that the water extracts from uncalcified rib cartilage (S_{1-3}) contain the major portion of the total CS glucuronate extracted from the tissue. More than half of the remaining CS glucuronate was subsequently removed by treatment with 3% KCl while the rest was removed upon treatment with 1.25 N KOH. These water extracts (S_{1-3}) were found to contain about 7-9% of the total calcium content of this tissue and nearly all the inorganic P. These results are similar to those found by Bowness (8).

During the initial stages of calcification a number of changes were detectable in the amounts of the analysed components of cartilage extracted by the solutions used. The increase in calcium and phosphate in the HCl extracts (S_{6-7}) which is presumably due to deposits of solid mineral, is accompanied by increases in calcium and phosphate in the KCl extracts and to a lesser extent in the water extracts. The ratios obtained for calcifying rib cartilage are compared below with those for uncalcified cartilage (Tables III and IV, VII-X in appendix):

	Uncalcified ribs (n)*	Calcified ribs (n)*
Ca S_{1-3}/CS glucuronate S_{1-3}=	0.083-0.171 (8)	0.211-0.459 (21)
Ca S_{4-5}/CS glucuronate S_{4-8}=	0.276-0.510 (8)	0.880-1.963 (21)

n = number of analyses.

	Uncalcified ribs (n)*	Calcified ribs (n)*
Pi S_{1-3}/CS glucoronate S_{1-2}=	0.020-0.065 (8)	0.142-0.312 (21)
Pi S_{4-5}/CS glucoronate S_{4-8}=	0.005-0.038 (8)	0.119-0.454 (21)

These ratios are similar to those reported by Bowness and Lee (16).

It was found that the tables of individual results (Tables III and IV, VII-X) were insufficient alone to develop sound basic conclusions and therefore a statistical analysis of grouped results had to be done. The results from the statistical analysis (Table V) allow some definite conclusions to be drawn with respect to some of the original objectives but not all.

After dividing the ribs into 3 groups (uncalcified, beginning calcification and advanced calcification) on the basis of the acid soluble Pi content, it was found that the mean figure for total CS glucuronate (S_{1-8}) was 9% higher for the group beginning calcification than for the uncalcified group. The P value for the difference was $\langle 0.10 \rangle 0.05$. A large portion of this increase in total CS glucuronate took place in the water-insoluble CS fractions (S_{4-8}). The mean figure for the sum of the CS glucuronate in the S_{4-8} fractions in the group beginning calcification was 19% higher than for the uncalcified group and the P value was $\langle 0.01 \rangle 0.005$. The major portion of this increase took place in the KOH fraction (S_8) where the P value was

$\langle 0.01 \rangle$ 0.005 as compared to the small increase in CS in the KCl fractions (S_{4-5}) where the P value was $\langle 0.45 \rangle$ 0.40. Although the major portion of the increase in total CS glucuronate (S_{1-8}) took place in the water-soluble CS fractions (S_{1-3}) the mean figure for the sum of CS glucuronate in the S_{1-3} fractions in the group beginning calcification was only 6% higher than for the uncalcified group and the P value was $\langle 0.20 \rangle$ 0.15. During this same period there were large increases in calcium and inorganic P, mostly in the HCl extracts, in which the solid mineral matter is dissolved.

In the advanced stages of calcification the mean figure for total CS glucuronate extracted from the ribs (S_{1-8}) was 16% lower than the mean for the group beginning calcification. The P value for the difference was $\langle 0.0025 \rangle$ 0.0005. This drop in total CS glucuronate was due entirely to a decrease in the amount of CS solubilized by water extraction (S_{1-3}) where the mean figure for the ribs beginning calcification was 38% higher than for the ribs in the advanced stages of calcification and the P value was $\langle 0.0005$. This decrease in total CS glucuronate was accompanied by a large net increase in CS solubilized in the water-insoluble CS fractions (S_{4-8}). The mean figure for the sum of the CS glucuronate in the S_{4-8} fractions in the group in the advanced stages of calcification was 56% higher than for the group beginning calcification and the P value was $\langle 0.0005$. Nearly all of this increase took place in the

KOH fraction (S_8) where the P value was < 0.0005 as compared to the small increase in CS in the KCl fractions (S_{4-5}) where the P value was $< 0.10 > 0.05$.

The results, to this point, show that during the initial stages and later in the advanced stages of calcification there is a definite and significant increase in CS in the non-water fractions (S_{4-8}) and this is nearly all accounted for by increases in the amount of CS solubilized in the alkali extract (S_8). These changes are accompanied by an initial rise and a later fall in the total CS glucuronate in the cartilage. This particular change though not the change in the extractability of the CS, has been noted by other workers previously (40-42). Similarly these changes are accompanied by an initial rise and a later large fall in the amount of CS solubilized in the water extracts. These changes are preceded, accompanied or followed by large increases in calcium and phosphate in these same extracts (S_{1-8}).

SPECIFIC ACTIVITY OF CHONDROITINSULFATES IN THE THREE STAGES OF CALCIFICATION

Table VI shows that the specific activity in all the rib cartilage extracts decreased slowly with increasing age of the puppy and also as the rib progressed from the uncalcified state to the advanced stages of calcification.

The specific activity of CS in the alkali extract was much lower than that found in the water extracts. Even when the rib in the advanced stages of calcification was extracted firstly with 0.05 M EDTA (di-sodium) instead of water, the specific activity of all the extracts remained more or less unchanged. Previous results show that there are at least 2 metabolically distinct CS fractions (6,7,32); although the KCl fractions might be another metabolic entity it is quite conceivable that the KCl fractions arise from contributions from the other fractions. Bowness found that water-extractable CS of the epiphyseal plate of young puppies (70) and of guinea-pig rib cartilage (7) was metabolized at a faster rate than that extracted with alkali. Gross, Mathews and Dorfman (6), using rat rib cartilage, found that the turnover of CS in the alkali extract was much slower than that found in the water extract. These findings further substantiate the concept that the CS of cartilage is not metabolically homogeneous.

Since the incorporation of $Na_2{}^{35}SO_4$ by the rib cartilage took place over a short time interval (25 hours) it appears likely that the difference in specific activity thus noted at various stages of calcification (Table VI) is probably due to a change in the rate of biosynthesis of CS rather than a breakdown of CS. Previous work on the turnover of CS in rib cartilage (6,7) showed that only a small proportion of labelled CS was broken down in a period of 25 hours. The difference in specific activity of CS fractions from the first two ribs in Table VI could not be due to a difference in size

or specific activity of the inorganic sulfate pool in the blood as both ribs were taken from the same animal. However, it is still possible that the rate of diffusion of sulfate into the calcifying rib is lower than into the uncalcified rib.

CALCIUM AND PHOSPHATE IN RIB CARTILAGE: COMPARISON WITH PREVIOUS WORK

Eichelberger and Roma (5) found that there was an increase in calcium in the rib cartilage of puppies before there is an accumulation of phosphate and deposition of solid mineral matter. Their results were obtained by pooling the previously dried, powdered ribs from one puppy for analysis and then grouping the results obtained with a number of animals by the age of the puppies. The work described in this thesis was done using the wet-weight of individual ribs of a dog at a certain age. Eichelberger and Roma (5) calculated the total water content of the pooled dried and powdered ribs of each dog, and then by grouping the results obtained with a number of animals by the age of the puppies they were able to obtain a mean water value for the age period under investigation. From their data it was simple mathematics to convert their mean dry weight results to a wet weight basis. The comparison of Eichelberger's and Roma's grouped results with the results from individual ribs (second series of experiments) is shown in Table VIa and is made on a wet-weight basis. Four very noteworthy differences were found: 1) Eichelberger and Roma found that there was an increase in calcium before there was an accumulation of phosphate while in our experiments both appeared to increase at the same time although

TABLE VIa

COMPARISON OF THE RESULTS[1] OBTAINED BY EICHELBERGER AND ROMA (5) WITH THOSE FOUND IN THE SECOND SERIES OF EXPERIMENTS

Age of Dog	Calcium[2] E+R Mean±S.D.[4]	Calcium Mean[3]	Total P E+R	Total P	CS E+R (from SO4)	CS (from glucuronate	Ca/P E+R	Ca/P
3- 7 wks	12.03± 1.75	-	12.75± 1.36	-	103.37± 9.86	-	0.95	-
8-12 wks	13.30± 1.70	7.50(1)	13.30± 1.20	6.40(1)	110.64±13.20	168.60(1)	1.00	1.17
13-16 wks	31.50± 11.84	25.00(9)	16.60± 5.85	15.00(9)	123.48± 9.32	179.00(9)	1.90	1.50
17-19 wks	180.98±158.12	87.50(4)	87.40±25.46	50.00(3)	125.96±21.44	188.00(4)	2.07	1.75
22-30 wks	408.00±210.00	425.00(2)	351.00±189.00	250.00(4)	130.20±19.50	158.00(2)	1.16	1.70

[1] results are in µg atom/g wet weight for calcium and total P; µmoles/g wet weight for CS.

[2] E+R refers to Eichelberger and Roma.

[3] In each case one dog only was used. The number of ribs separately analysed is given in brackets.

[4] S.D. = standard deviation.

[5] refers to a reference.

the initial increase in calcium was greater than phosphate, 2) Eichelberger and Roma found that the total CS of the rib cartilage continued to increase with the age of the puppy while our experiments showed that when the rib cartilage had reached the stage of advanced calcification the total CS content of the cartilage had decreased. 3) Eichelberger and Roma found that the Ca/P ratio of their older puppies was 1.16/1 while our experiments showed that the Ca/P ratio approached 1.70/1. It has been found by many investigators that the molar ratios of Ca^{++} to Pi in deposits of calcium phosphate in various parts of the body range from 1 to 1.67 in bone or cartilage and 1.8 in mitochondria (4,17,26). 4) Eichelberger and Roma obtained their results by pooling ribs while our results were obtained using individual ribs. Another factor is that different breeds of dogs and different diets were used in the two sets of experiments. Our dogs were a cross between a German shepherd and collie and were fed a diet (see Materials and Methods) different from that described by Eichelberger and Roma (5).

The significance of Eichelberger's and Roma's results are in doubt due to the fact that such large standard deviations were observed in their calculations. Because of these large deviations it is hard to say whether calcium actually accumulated before phosphate and whether the increase in CS which they observed with the age of the puppies is really significant. The most puzzling result of their experiments is that they found the Ca/P ratio of their older dogs to be 1.16/1 while in rapidly calcifying cartilage this ratio

should approach 1.67/1 as indicated by previous work (4,17,26).

HISTOLOGICAL EXAMINATION OF RIB CARTILAGE

In dog III (Table III) the Pi figures for S_{6-7} show that the first 3 ribs were uncalcified while calcification had begun in all the other ribs, and appeared to be most advanced in rib 6. The sizes of the individual ribs increase from rib 1 to rib 9, so it does not appear that size is the main factor in the degree of calcification (18). Although ribs 1-3 (Figs.1-3, see appendix) were considered to be uncalcified quantitatively, histologically ribs 2 and 3 (Figs.2 and 3) were found to contain very small areas of calcification (dark spots). The dark spots in Figures 2 and 3 might be due to over-exposure when the negatives or prints were taken because the exposure times were not closely controlled. However, neither the phosphate analyses nor the histological sections provide evidence for any clear break between ribs beginning calcification and those in the advanced stage of calcification (Figs.4-10, see appendix). The ribs were divided into 3 groups on the basis of the acid soluble Pi. It therefore appears that the histological findings correlate only approximately, but not completely, with the arbitrary limits placed on the 3 groups.

PART II

SOLUBILIZATION OF CHONDROITINSULFATES, CALCIUM AND COLLAGEN BY ENZYMES AND EDTA

INTRODUCTION

It had been found in the previous series of experiments that during the early stages of calcification there is an increase in the total CS glucuronate in rib cartilage and a large portion of this increase takes place in the water insoluble CS fractions (S_{4-5},S_8). The work described in this section was designed to obtain information on the nature of the CS in the water-insoluble fraction, the reasons for the inability to extract it with water and the possible relationship between this material and calcification.

It has been shown that intravenously injected papain has a very rapid and strong effect on epiphyseal cartilage resulting in the release of CS or chondromucoprotein (CMP) from the matrix (30,31,59,60,67). This effect is looked upon as a proteolytic action on the CMP in the cartilage. According to determinations of hydroxyproline there does not seem to be any effect on the collagen (22). It is also known that testicular hyaluronidase causes the depolymerization of CS and hyaluronate.

Hulth and Westerborn (63,64) have demonstrated that endochondral bone formation was affected by papain administration. The significant changes in the compositional analysis of this tissue produced by alterations in mucopolysaccharides is consistent with the speculation that mucopolysaccharides may be directly involved in calcification processes. In addition, endogenous proteases of lysosomal origin have been implicated in the changes observed in

diseased cartilage and in the processes involved in endochondral bone formation and growth (61,62).

Recently it has been shown by Serafini-Fracassini and Abatangelo (23) that during EDTA decalcification of purified mineralized aortic elastic membranes at pH 7 there is simultaneous extraction of a mucoprotein and that the amount of mucoprotein extracted is directly related to the amount of calcium salt in the tissue, thus indicating an intimate connection between the two.

The next series of experiments were undertaken with the following immediate objectives:

1) to compare the release rates of calcium, CS glucuronate and protein from rib cartilage by treatment with testicular hyaluronidase, papain and collagenase.
2) to see if the CS glucuronate not extractable with water is associated only with the CMP or if collagen also forms an intimate relationship with it.

MATERIALS AND METHODS

EXTRACTION PROCEDURES

Ribs which were in the stage of advanced calcification (from puppies 27 weeks old) were cleaned of adhering tissue, cut up into small pieces, ground to powder with a mortar and pestle and then dried to constant weight under a lamp. The powder was stored in a freezer until needed.

PROCEDURE A

In order to remove all the inorganic material and to see if this would alter the amount of CS solubilized by the other treatments 0.25 g portions of the powder were homogenized (in a fluted container using a Virtis "23" homogenizer running at top speed) with 10 ml of 0.05 M EDTA (di-sodium) at 4°C for 20 mins. The homogenate was centrifuged at 27000 x g and 4°C for 20 mins and the supernatant poured off and retained for analysis. The precipitate was then homogenized with a further 10 ml of 0.05 M EDTA and the procedure repeated to yield a total of three 0.05 M EDTA extracts (S_{A1-3}).

To remove any EDTA which might have been left in the pellet, the precipitate was then mixed with 10 ml distilled water and centrifuged as already described. The supernatant was poured off and retained for analysis and the mixing and centrifugation process was repeated twice more to yield a total of 3 water extracts (S_{B1-3}).

At this stage a calcium-free insoluble residue contain-

ing collagen, non-collagenous protein and CS had been obtained. In order to determine the effect of enzyme treatments on calcium bound to this material the precipitate was mixed with 15 ml $CaCl_2$ (1 mg/ml) and the pH of the mixture was brought up to about 6.0. The mixture was left for 10 mins with occasional stirring and then centrifuged as above. The supernatant was retained for analysis (S_C).

To remove any $CaCl_2$ not tightly bound to the pellet the precipitate was then mixed with 10 ml distilled water, centrifuged and the supernatant was retained for analysis (S_D).

The pellet containing bound calcium was then incubated with either hyaluronidase, papain or collagenase or combinations of these under specified conditions (see Results). At certain time intervals the incubation mixture was centrifuged, an aliquot was removed and retained for analysis and the remaining material re-incubated under the same conditions.

After the incubation period the supernatant was removed by centrifugation and the pellet was homogenized firstly with 10 ml 3% KCl for 20 mins, secondly with 10 ml 0.05 N HCl and lastly with 10 ml 1.25 N KOH. The pellet was completely solubilized after the KOH treatment. After each homogenization the mixture was centrifuged and the supernatant poured off and retained for analysis (see Flow Chart 2).

PROCEDURE B

To check the effect of the EDTA extraction and the

CHART 2

FLOW CHART FOR THE EXTRACTION OF RIB CARTILAGE POWDER

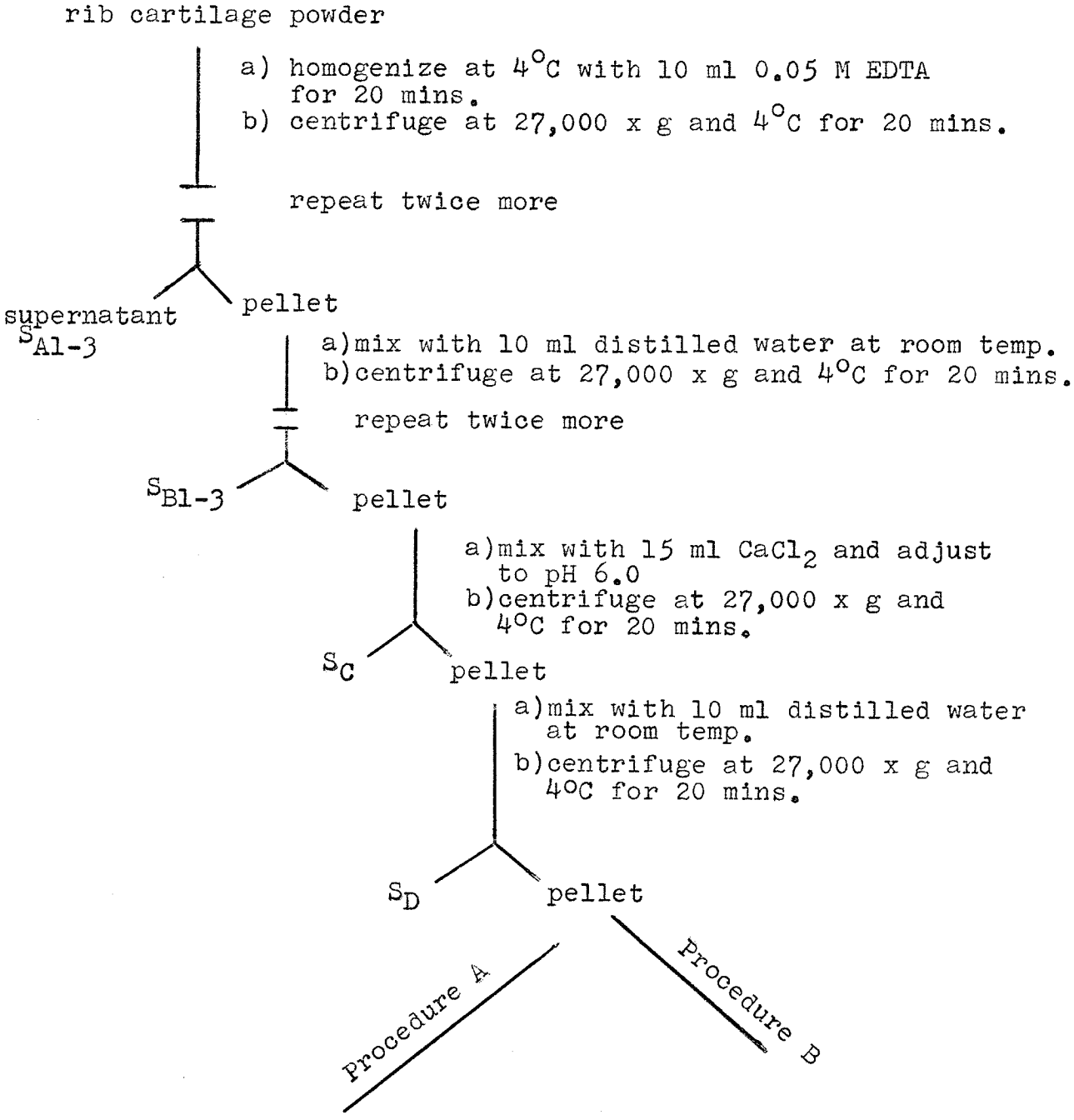
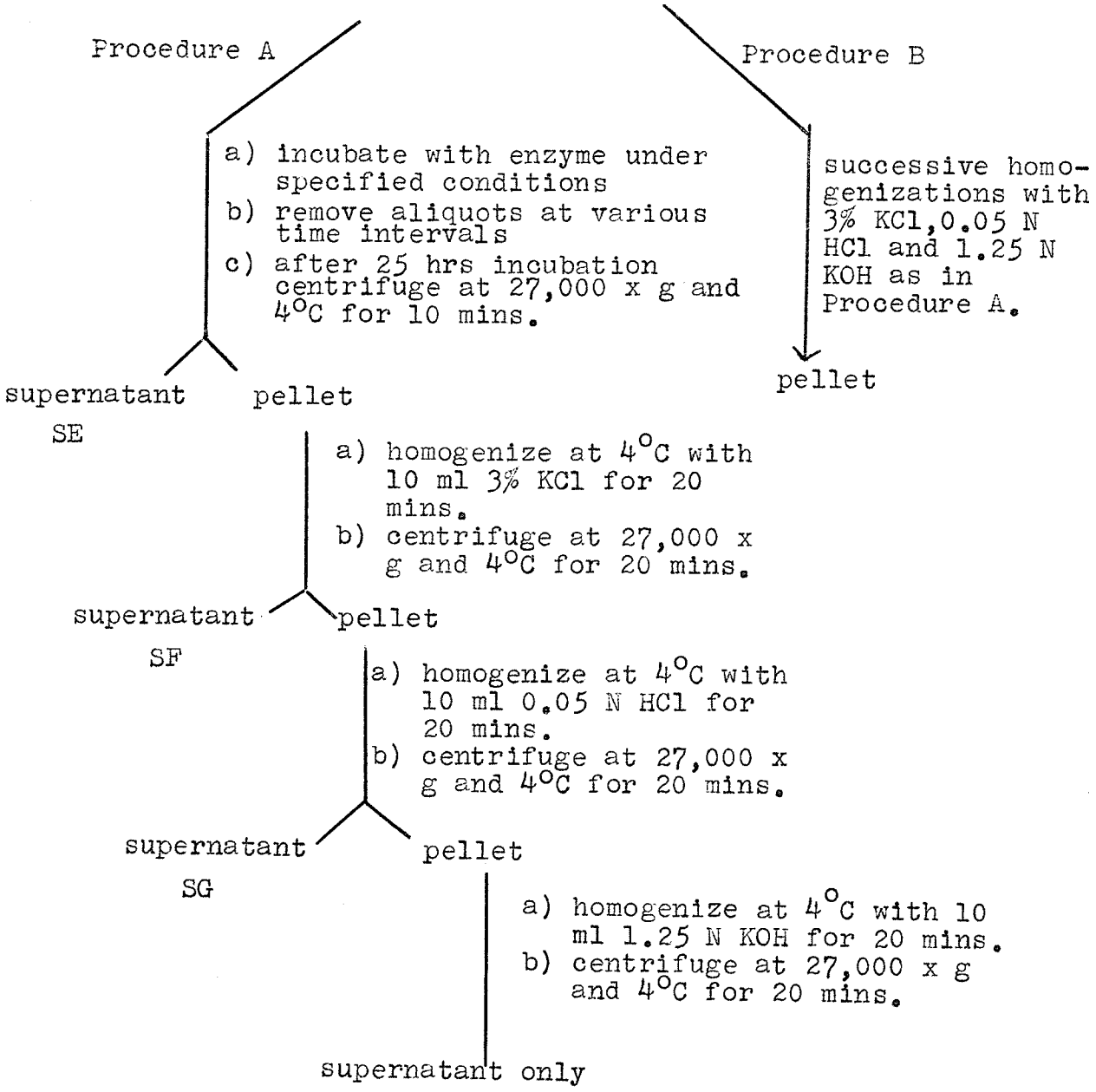


CHART 2 CONTINUED



treatment with $CaCl_2$ on the proportion of CS present in the KOH extract the powder was put through the initial extraction and calcium-binding procedures (S_{A-D}). Then the pellet was homogenized with 10 ml 3% KCl for 20 mins at 4°C. The homogenate was centrifuged at 27000 x g and 4°C for 20 mins and the supernatant poured off and retained for analysis. The KCl extraction procedure was repeated (S_E).

The precipitate was homogenized with 10 ml 0.05 N HCl for 20 mins and centrifuged as already described and then the supernatant was poured off and retained for analysis (S_F).

Finally the precipitate was homogenized with 10 ml 1.25 N KOH for 20 mins and centrifuged as described above and then the supernatant poured off and retained for analysis (S_G)(see Flow Chart 2). After these treatments, in contrast to those where enzymes were also used, an insoluble residue remained after KOH extraction.

ENZYME PREPARATIONS

Ovine testicular hyaluronidase was obtained from Koch Light and Co., Great Britain. Papain was prepared by Sigma Chemical Co., St. Louis, Missouri from Papaya Latex and 2X crystallised and suspended in 0.05 M sodium acetate buffer at pH 4.5. Collagenase was prepared by Mann Laboratories, New York from Clostridium histolyticum and 1X crystallised.

ANALYTICAL PROCEDURES

Glucuronic acid was estimated by the carbazole

reaction (12). Chemical estimation of calcium was by titration with EDTA using calcein as indicator (15) (see Section I). Protein was determined by the procedure of Lowry et al (24) and hydroxyproline by the method of Leach (25) after the collagen materials were hydrolysed in a sealed tube with 6N HCl for 16 hrs at 105°C.

DETERMINATION OF NON-COLLAGENOUS PROTEIN

Standard Curves

1) Six mg of collagen (rat tail tendon) obtained from Koch Light Laboratories Ltd., Great Britain, was mixed with 10 ml of 0.05 N HCl and homogenized for 20 mins at 4°C. The collagen was then completely solubilized in the acid. Aliquots, ranging from 0.10-1.00 ml, were prepared from the mixture and brought to a volume of 1 ml with distilled water. These solutions were then hydrolysed in sealed tubes with an equal volume of 12 N HCl for 16 hours at 105°C. After this incubation period the solutions were brought to pH 6-8 with NaOH and to a volume of 4 ml with distilled water. One ml of each solution was used for the hydroxyproline assay (25) and a standard curve for collagen hydroxyproline was obtained in this way.

2) A 2 mg sample of the collagen material was mixed with 10 ml of 0.05 N HCl and homogenized for 20 mins at 4°C. The collagen was then completely solubilized in the acid. Aliquots, ranging from 0.10-1.00 ml, were prepared from the mixture and brought to a volume of 1 ml with dis-

tilled water. One ml of each solution was used for the Lowry protein assay (24) and a standard curve for collagen in the Lowry procedure was obtained.

Tests

The amount of collagen in an unknown sample was estimated from the standard collagen hydroxyproline curve (1 above) and then the Lowry reading for this amount of collagen obtained from the standard curve for collagen in the Lowry procedure. This reading was used for subtraction from the observed Lowry reading for the test solution. The difference between the two readings was used to estimate the amount of non-collagenous protein from the standard curve obtained with bovine albumin in the Lowry procedure.

RESULTS

PROCEDURE A

Figure 11. All the calcium was found to have been removed by enzyme hydrolysis but 51.8% of the CS remained after enzyme treatment. Much of this may be due to the acidic nature of the digestion medium in which insoluble salts of CS with collagen would probably form (27). This is supported by the observation that 46.2% of the CS (i.e. 90% of that left after enzyme treatment) was solubilized by KCl.

Figure 12. 81.1% of the originally bound calcium was found to have been removed by enzyme hydrolysis. 14.4% of the calcium was solubilized by KCl while the rest was solubilized by HCl. 57.4% of the CS remained after enzyme hydrolysis. 48.8% (i.e. 80% of that left after enzyme treatment) was removed by KCl.

Figure 13. 82% of the originally bound calcium was removed by enzyme hydrolysis with the remainder removed by KCl. 52.9% of the CS remained after enzyme treatment. 46.0% was removed by KCl. 63.7% of the non-collagenous protein was removed from the pellet by enzyme treatment with the remainder removed by KCl. 84.8% of the collagen remained after enzyme treatment. 63.2% of the collagen (i.e. about 80% of that left after enzyme treatment) was solubilized by HCl, while 12.1% was solubilized by KCl.

Figure 14. 72.7% of the originally bound calcium was

removed by enzyme hydrolysis with 21.7% being removed by KCl and the remainder extracted by HCl. 36.3% of the CS remained after enzyme hydrolysis. 28.3% was removed by KCl. 79.5% of the non-collagenous protein was removed from the pellet by enzyme treatment with the majority of the remainder extracted by KOH. 85.3% of the collagen remained after enzyme treatment with 58.2% being solubilized by KCl and 23.4% by HCl.

Figure 15. 76.1% of the CS was removed by enzyme hydrolysis while 21.3% was removed by KCl. 87.1% of the non-collagenous protein was removed from the pellet by enzyme treatment with the remainder removed by KCl. 64.1% of the collagen remained after enzyme treatment. 45.4% of the collagen (i.e. 70% of that left after enzyme treatment) was removed by HCl while 11.9% was removed by KCl.

Figure 16. 25.2% of the CS remained after enzyme hydrolysis. 21.0% was removed by KCl. 75.0% of the non-collagenous protein was solubilized from the pellet by enzyme treatment with nearly all the remainder being solubilized by KCl. 73.8% of the collagen remained after enzyme treatment with 50.6% (i.e. 70% of that left after enzyme treatment) being removed by HCl, 11.5% by KCl and 11.7% by KOH.

FIGURE 11. Solubilization of calcium and chondroitinsulfate from rib cartilage upon enzyme treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml unbuffered distilled water, adjusted to pH 6.2 and then 2 mg of a testicular hyaluronidase (38) was added and the mixture incubated in a 37°C water bath with slow shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot was removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.5. The pellet was then mixed with 10 ml unbuffered distilled water, adjusted to pH 6.2 and 0.105 mg of papain (35) was added and the mixture incubated in a 37°C water bath with slow shaking. The same procedures were then carried out as was done with hyaluronidase. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.6.

Note: At each time period the points represent the total amount of material extracted up to that time.

O—— O % total bound Ca solubilized upon enzyme treatment.

■——■ % total CS solubilized upon enzyme treatment.

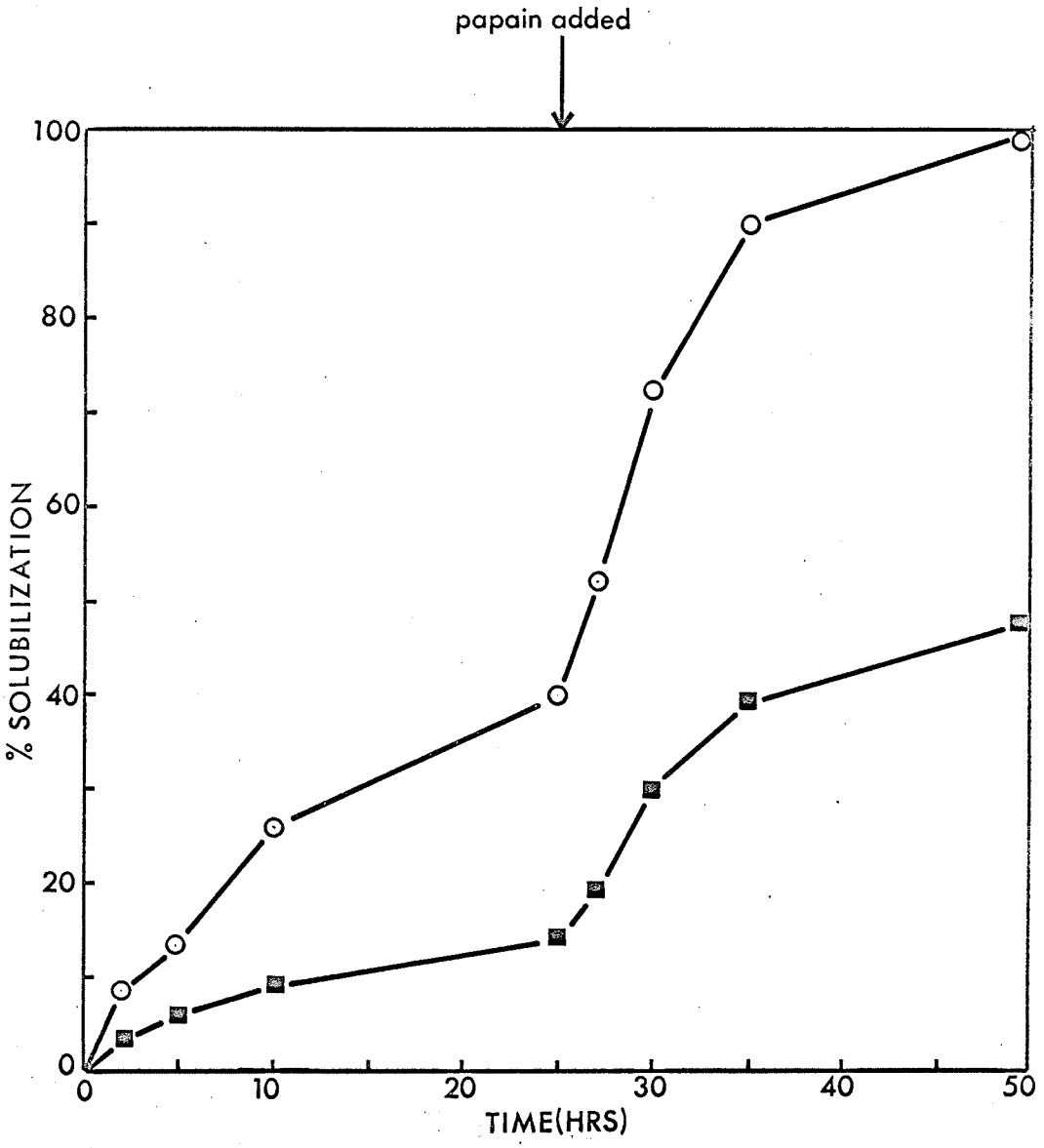


FIGURE 12. Solubilization of calcium and chondroitin-sulfate from rib cartilage upon papain treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml unbuffered distilled water, adjusted to pH 6.3 and then 0.105 mg of papain was added and the mixture incubated in a 37°C water bath with slow shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.6.

Note: At each time period the points represent the total amount of material extracted up to that time.

0——0 % total bound Ca solubilized upon enzyme treatment.

■——■ % total CS solubilized upon enzyme treatment.

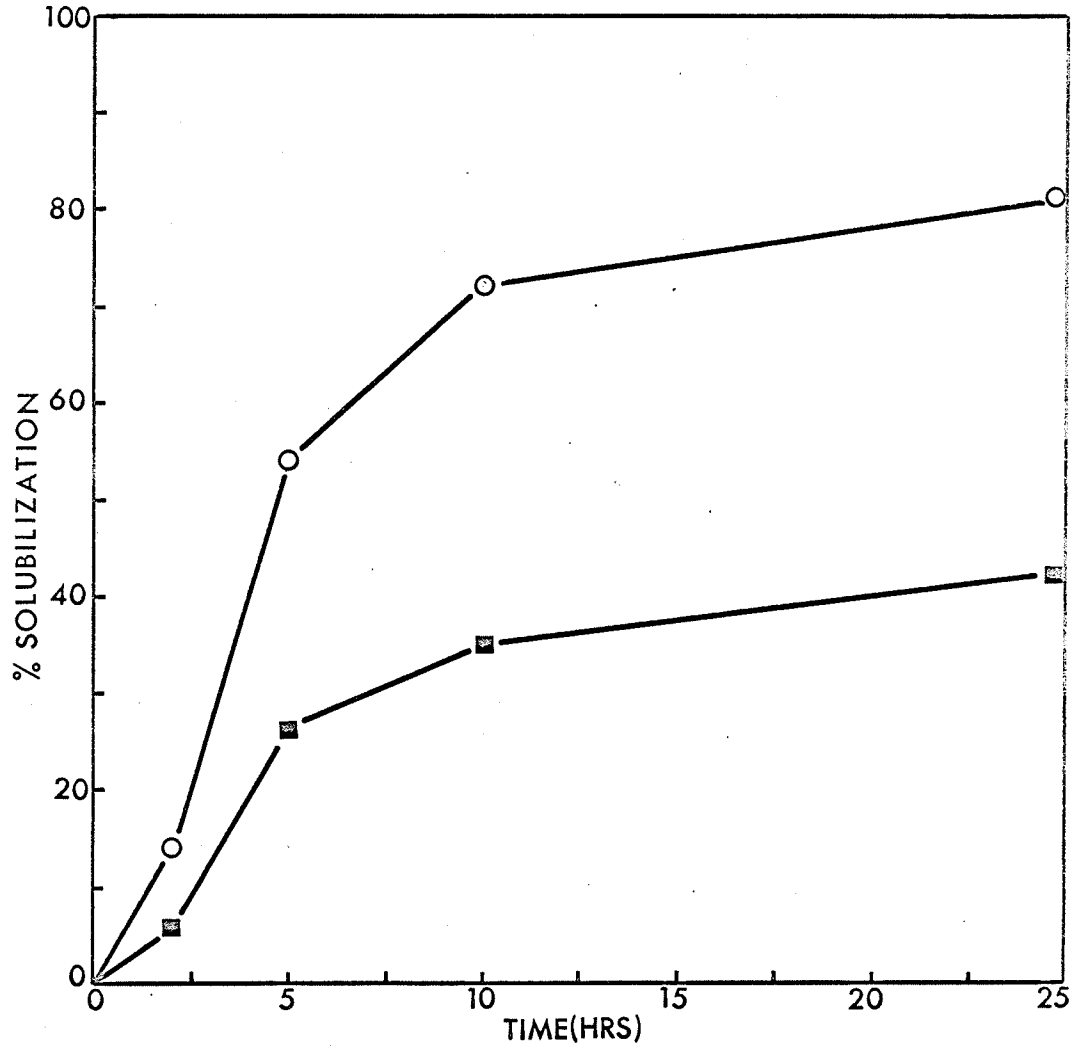

100
80
60
40
20
0
% SOLUBILIZATION
0
5
10
15
20
25
TIME(HRS)

FIGURE 13. Solubilization of calcium, protein and chondroitinsulfate from rib cartilage upon papain treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml unbuffered distilled water, adjusted to pH 6.2 and then 0.105 mg of papain was added and the mixture incubated in a 37°C water bath with slow shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.1.

Note: At each time period the points represent the total amount of material extracted up to that time.

0——0 % total bound Ca solubilized upon enzyme treatment.

■——■ % total CS solubilized upon enzyme treatment.

▼——▼ % total collagen solubilized upon enzyme treatment.

●——● % total non-collagenous protein solubilized upon enzyme treatment.

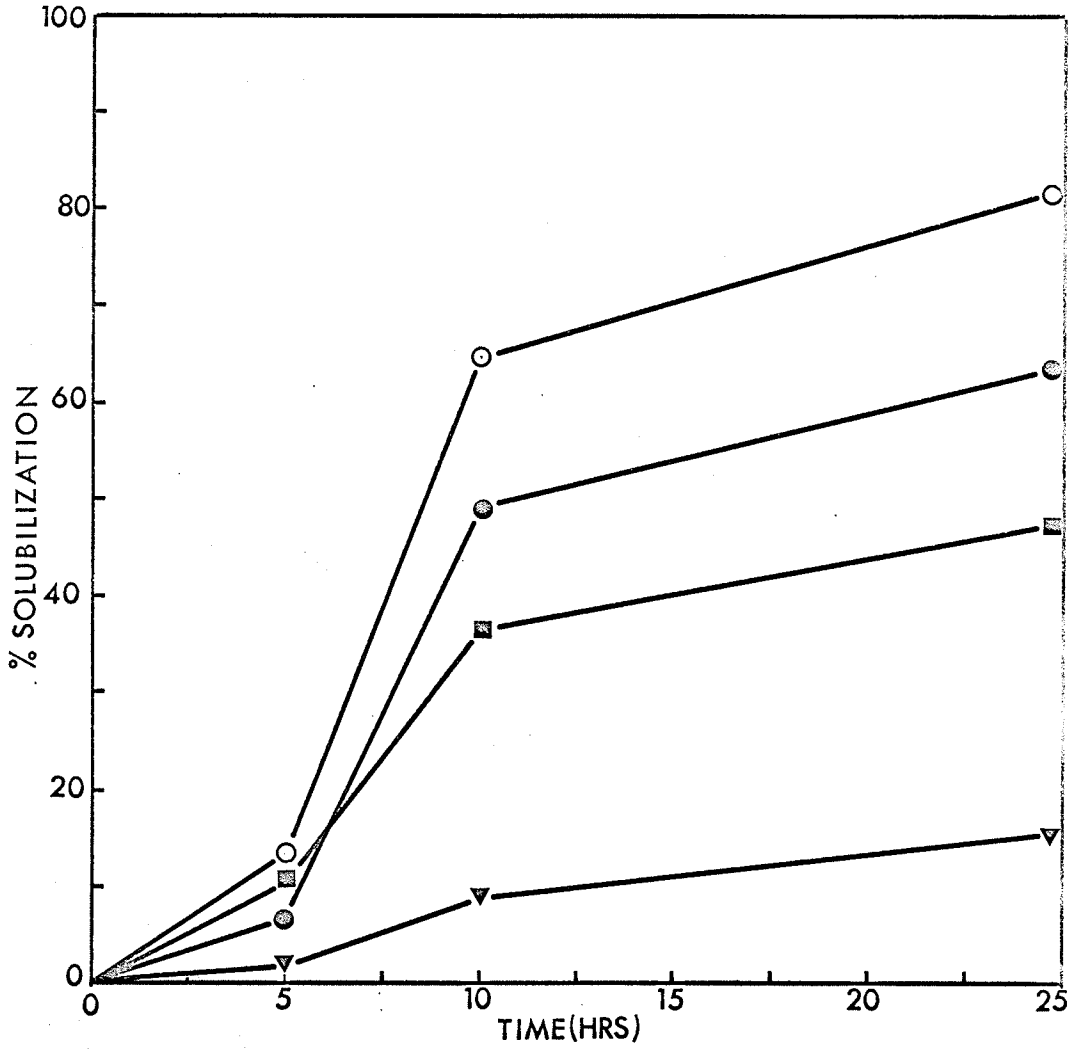

100
80
60
40
20
0
% SOLUBILIZATION
0
5
10
15
20
25
TIME(HRS)

FIGURE 14. Solubilization of calcium, protein and chondroitinsulfate from rib cartilage upon papain treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml unbuffered distilled water, adjusted to pH 6.2 and then 0.105 mg of papain was added and the mixture incubated in a 37°C water bath with slow shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.5.

Note: At each time period the points represent the total amount of material extracted up to that time.

0—0 % total bound Ca solubilized upon enzyme treatment.

■—■ % total CS solubilized upon enzyme treatment.

▼—▼ % total collagen solubilized upon enzyme treatment.

●—● % total non-collagenous protein solubilized upon enzyme treatment.

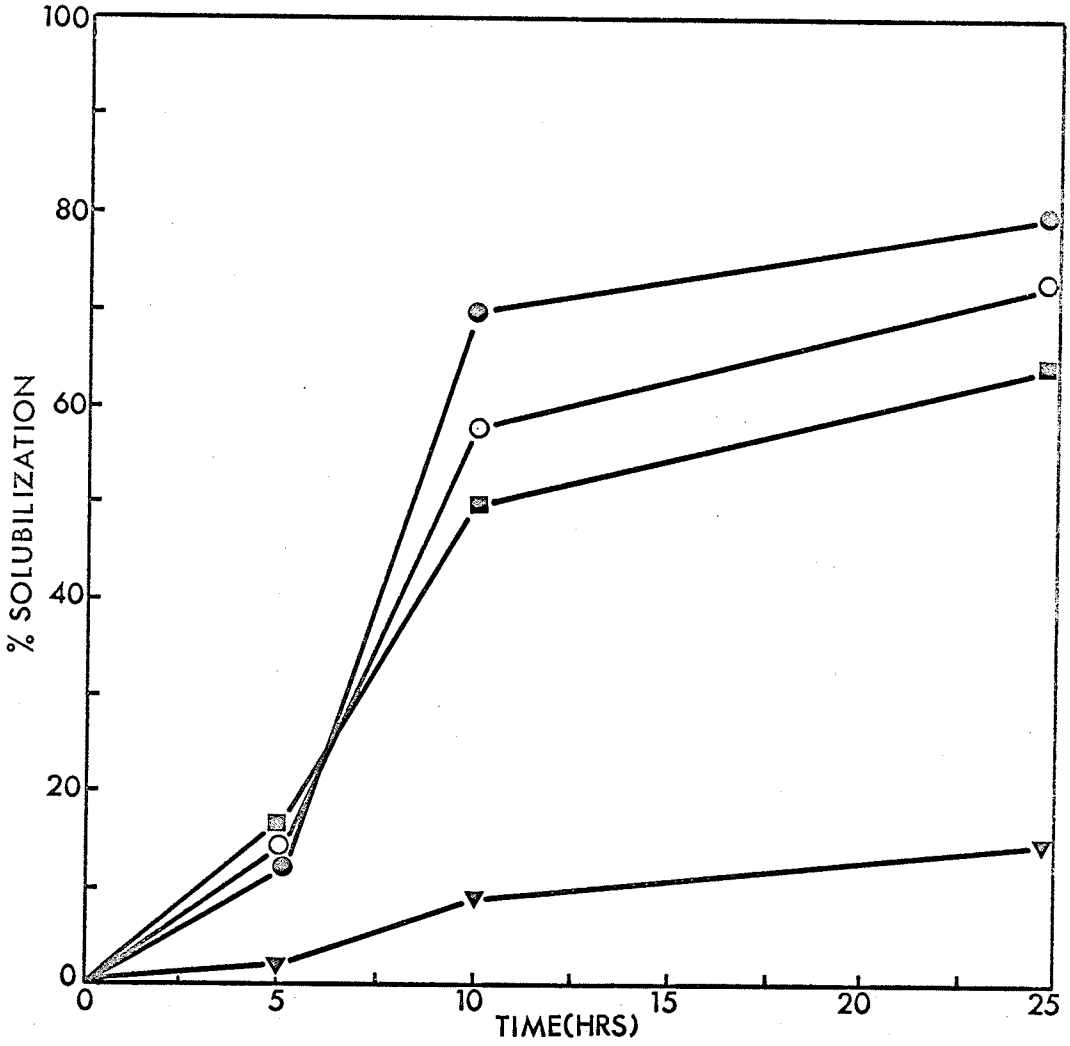

100
80
60
40
20
0
% SOLUBILIZATION
0
5
10
15
20
25
TIME(HRS)

FIGURE 15. Solubilization of protein and chondroitin-sulfate from rib cartilage upon papain treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml 0.02M sodium acetate (35,36) + 0.001 M EDTA + 0.005 M cysteine (adjusted to pH 5.4) and then 0.105 mg of papain was added and the mixture incubated in a 37°C water bath with fairly fast shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 4.9.

Note: At each time period the points represent the total amount of material extracted up to that time.

■—■ % total CS solubilized upon enzyme treatment.

▼—▼ % total collagen solubilized upon enzyme treatment.

●—● % total non-collagenous protein solubilized upon enzyme treatment.

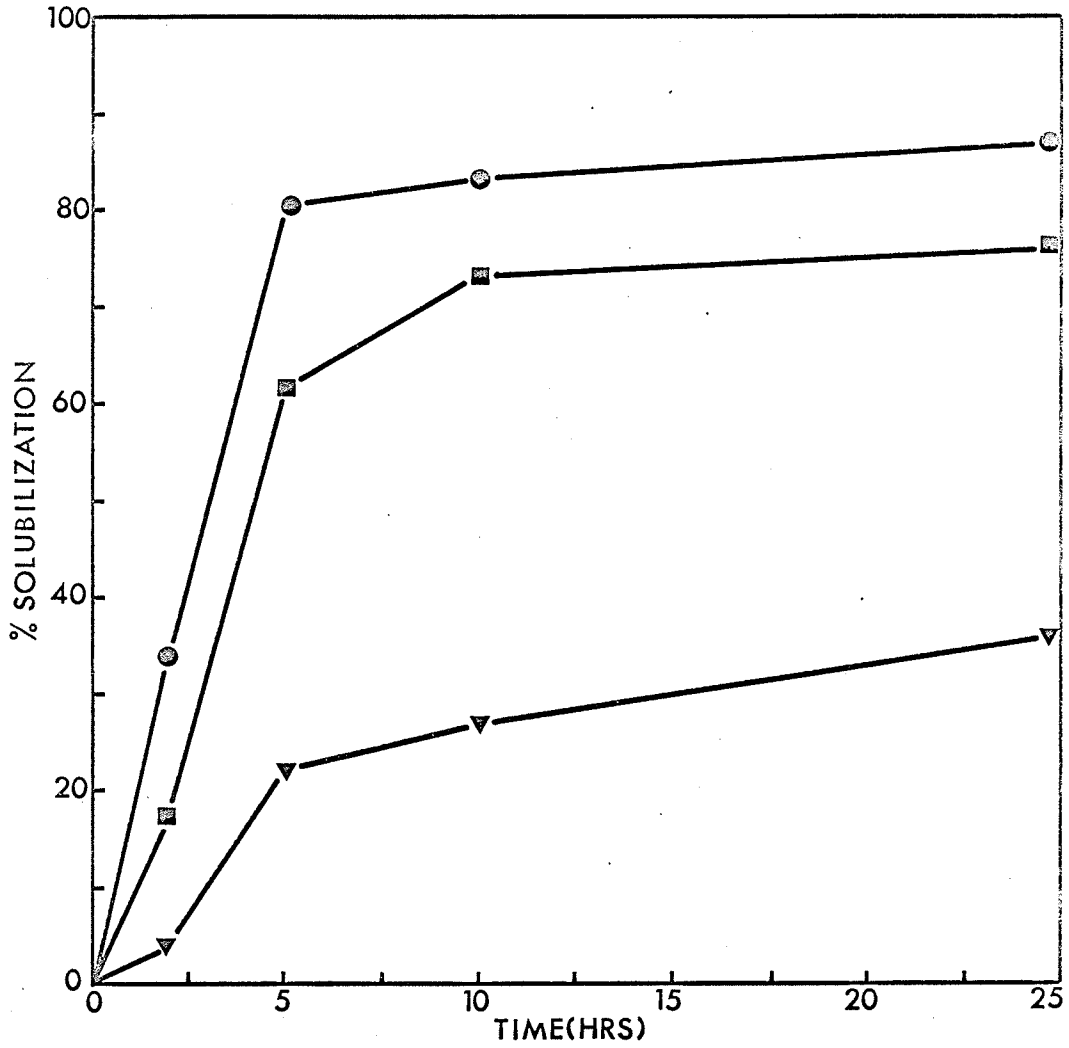

100
80
60
40
20
0
% SOLUBILIZATION
0
5
10
15
20
25
TIME(HRS)

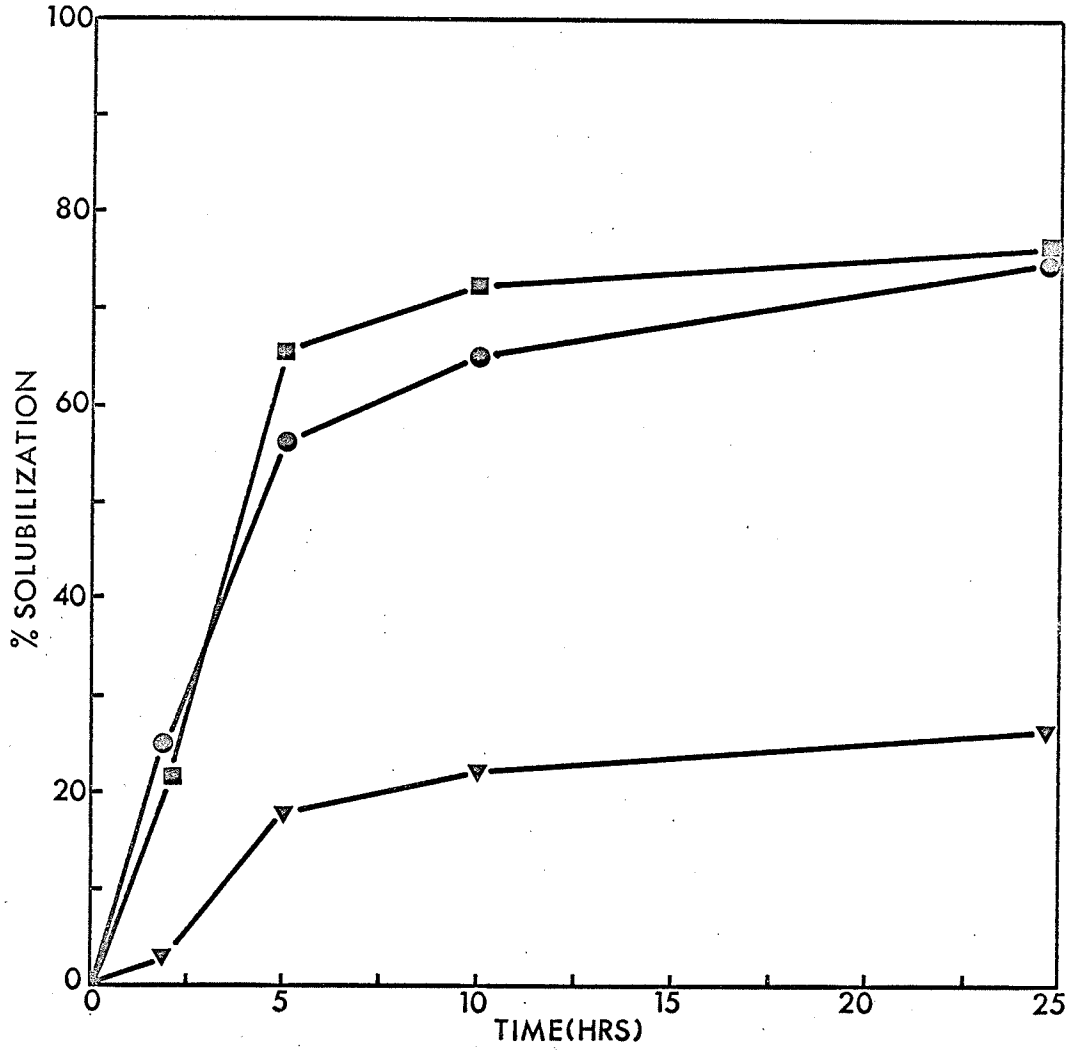
FIGURE 16. Solubilization of protein and chondroitin-sulfate from rib cartilage upon enzyme treatment. After treatments S_{A-D} (page 34) the pellet was mixed with 10 ml of unbuffered distilled water, adjusted to pH 6.0 and then 0.105 mg of papain and 2 mg of collagenase (37) were added and the mixture incubated in a 37°C water bath with fairly fast shaking. At certain time intervals the incubation mixture was centrifuged, an aliquot removed and retained for analysis and the remaining material re-incubated under the same conditions. After 25 hrs of incubation the mixture was centrifuged and the supernatant removed and analysed. The pH was 5.2.

Note: At each time period the points represent the total amount of material extracted up to that time.

■—■ % total CS solubilized upon enzyme treatment.

▼—▼ % total collagen solubilized upon enzyme treatment.

●—● % total non-collagenous protein solubilized upon enzyme treatment.


100
80
60
40
20
0
% SOLUBILIZATION
0
5
10
15
20
25
TIME(HRS)

PROCEDURE B

Tables XI-XIII show the distribution of CS in various extracts in the 3 stages of ribs extracted by procedure B and by the procedure used in Part I. Table XIV is similar to Table XIII except that only the percentage of CS solubilized in the KOH extract is given. Table XV gives the distribution of CS, collagen and non-collagenous protein in various extracts in two stages of ribs. Table XVI shows the loss of water in rib cartilage upon powdering and drying to constant weight under a lamp.

TABLE XI

DISTRIBUTION OF CS IN UNCALCIFIED RIBS BY TWO

DIFFERENT EXTRACTION PROCEDURES

Treatment (Part I)	Amount of CS** extracted µmoles/g fresh wt	% of CS extracted from total (average)	Treatment* (procedure B) Part II	Amount of CS extracted µmoles/g dry wt	Amount of CS extracted µmoles/g fresh wt	% of CS extracted from total
Water (3)	134.0	78.3	0.05 M EDTA(3)	246.0;229.2	82.0; 76.4	54.2;52.6
3% KCl (2)	23.1	13.5	water (3)	12.0; 11.2	4.0; 3.7	2.7; 2.5
0.05 N HCl (2)	1.0	0.2	$CaCl_2$(1)	14.0; 3.6	4.7; 1.2	3.1; 0.8
1.25 N KOH (1)	13.0	8.0	water (1)	- -	- -	- -
Total	171.1	100%	3% KCl (2)	137.6;140.0	45.9; 46.7	30.3;32.2
			0.05 N HCl (1)	4.4; 3.2	1.5; 1.1	1.0; 0.8
			1.25 N KOH (1)	40.0; 48.4	13.3; 16.1	8.7;11.1
			Total	454.0;435.6	151.3;145.2	100%

Note: The brackets represent the number of 10 ml extracts done under each treatment.

* carried out in duplicate.
** results taken from statistical analysis Table V.

TABLE XII

DISTRIBUTION OF CS IN RIBS BEGINNING CALCIFICATION BY TWO DIFFERENT EXTRACTION PROCEDURES

Treatment (Part I)	Amount of CS** extracted µmoles/g fresh wt	% of CS extracted from total (average)	Treatment* (procedure B) Part II	Amount of CS extracted µmoles/g dry wt	Amount of CS extracted µmoles/g fresh wt	% of CS extracted from total
Water (3)	142.4	76.4	0.05 M EDTA (3)	197.0;204.5	78.8; 81.8	51.7;48.3
3% KCl (2)	23.6	12.7	water (3)	11.8; 14.2	4.7; 5.7	3.1; 3.3
0.05 N HCl (2)	0.9	0.5	$CaCl_2$(1)	11.3; 2.2	4.5; 0.9	3.0; 0.6
1.25 N KOH (1)	19.4	10.4	water (1)	0.5; -	0.2; -	0.1; -
Total	186.3	100%	3% KCl(2)	117.3;142.2	46.9;56.9	30.8;33.6
			0.05 N HCl(1)	2.8; 3.8	1.1; 1.5	0.7; 0.8
			1.25 N KOH(1)	40.3; 56.8	16.1;22.7	10.6;13.4
			Total	381.0;423.7	152.3;169.5	100%

Note: The brackets represent the number of 10 ml extracts done under each treatment.

* carried out in duplicate.
** results taken from statistical analysis Table V.

TABLE XIII

DISTRIBUTION OF CS IN THE ADVANCED STAGES OF CALCIFICATION BY TWO DIFFERENT EXTRACTION PROCEDURES

Treatment (Part I)	Amount of CS** extracted µmoles/g fresh wt	% of CS extracted from total (average)	Treatment * (procedure B) Part II	Amount of CS extracted µmoles/g dry wt	Amount of CS extracted µmoles/g fresh wt	% of CS extracted from total
Water (3)	87.8	56.1	0.05 M EDTA (3)	167.0;178.2	83.5; 89.1	49.8;50.4
3% KCl (2)	26.1	16.7	water (3)	3.0; 2.5	1.5; 1.3	0.7; 0.8
0.05 N HCl(2)	1.0	0.6	$CaCl_2$(1)	6.8; 8.0	3.4; 4.0	1.7; 2.2
1.25 N KOH(1)	41.6	26.6	water(1)	- -	- -	- -
Total	156.5	100%	3% KCl (2)	67.0; 69.8	33.5; 34.9	19.9;19.6
			0.05 N HCl(1)	5.0; 3.2	2.5; 1.6	1.1; 0.9
			1.25 N KOH(1)	91.0; 92.2	45.5; 46.1	26.8;26.1
			Total	339.8;353.9	169.9;176.9	

Note: The brackets represent the number of 10 ml extracts done under each treatment.

* carried out in duplicate.
** results taken from statistical analysis Table V.

TABLE XIV

PERCENTAGE OF CS GLUCURONATE IN KOH EXTRACTS USING VARIOUS TREATMENTS OF RIBS IN ADVANCED STAGES OF CALCIFICATION

Treatment	Total CS glucuronate in all extracts (μmoles/g fresh wt)	% CS glucuronate in KOH extract
1. Extraction of fresh ribs with water (3), KCl (2), HCl (2), and KOH (Results from Table V, Part I).	156.5	26.6
2. Extraction of dried rib powder (A) with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(2), HCl and KOH (Results from Table XIII, Part II).	169.9	26.8
3. Extraction of dried rib powder (B) with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(3), HCl and KOH (Results from Table XIII)	176.9	26.1
4. Extraction of dried rib powder (C) with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(2) (phosphate-citrate buffer pH 7.0), HCl and KOH (procedure B, Part II)	172.5	28.1
5. Extraction of dried rib powder (C) with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(2), HCl and KOH (procedure B, Part II)	166.7	28.8
6. Extraction of fresh rib 4 with H_2O(3), KCl(2), HCl and KOH (Part I).	158.2	32.2
7. Extraction of fresh rib 4 with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(2), HCl and KOH (procedure B, Part II)	163.4	29.0

continued

TABLE XIV CONTINUED

Treatment	Total CS glucuronate in all extracts (umoles/g fresh wt)	% CS glucuronate in KOH extract
8. Extraction of fresh rib 7 with water (3), KCl(2), HCl and KOH (Part I).	174.2	26.8
9. Extraction of fresh rib 7 with EDTA(3), H_2O(3), $CaCl_2$, H_2O, KCl(2), HCl and KOH (procedure B, Part II)	169.5	27.6

Note: The brackets represent the number of 10 ml extracts, if more than one, done under each treatment.

TABLE XVI

DRY WEIGHT/WET WEIGHT RATIOS FOR RIBS AT VARIOUS STAGES

Ribs	Wt of ribs after powdering and drying under a lamp to constant wt / fresh wt of rib
Uncalcified	0.33
Beginning calcification	0.40
Advanced calcification	0.50

TABLE XV

DISTRIBUTION OF CS, COLLAGEN AND NON-COLLAGENOUS PROTEIN IN VARIOUS EXTRACTS OF TWO GROUPS OF RIBS BY PROCEDURE B, PART II

Treatment	RIBS[1]					
	Uncalcified			Advanced calcification		
	mg CS	mg collagen	mg non-collagenous protein	mg CS	mg collagen	mg non-collagenous protein
0.05 M EDTA (3)	36.17	3.48	4.56	23.03	3.24	4.67
3% KCl (2)	20.23	1.30	3.20	13.35	0.96	1.95
1.25 N KOH (1)	5.68	46.40	0.60	7.98	16.50	7.40
				3.00*	21.60*	1.80*
pellet (1)	-	-	-	3.03	52.20	0
Total	62.08	51.18	8.36	47.39	72.90	14.02

Note: The brackets represent the number of 10 ml extracts done under each treatment. Only the EDTA, KCl and KOH extracts are shown in the table ($CaCl_2$, H_2O and HCl extracts are not shown).

The pellet after KOH treatment was homogenized with 6 N HCl until solubilization and then analysed.

* Another rib in the advanced stages of calcification. Only the KOH extract was analysed.

[1] 250 mg of dried, powdered cartilage used in each case.

DISCUSSION

PROCEDURE A

CALCIUM AND CHONDROITINSULFATE SOLUBILIZATION

In Figures 11-14 the curves showing percent of calcium solubilized from rib cartilage after various incubation times were very similar in shape to those for CS glucuronate but the solubilization of CS was less complete than calcium. Nearly all the calcium and about 50% of the CS was removed by enzyme treatment. Practically all the remaining CS and calcium was removed by KCl.

During the incubation period the pH of the medium changed from 6.2-6.3 to 4.1-4.6 (unbuffered). At lower pH conditions CS tends to form insoluble salts with protein (27,43) whereas calcium could be removed from binding to CS (28,29). According to Dunstone (29) binding of calcium takes place best between pH 6.8-7.2. This might account for the fact that 50% of the CS still remained in the pellet after enzyme treatment whereas nearly all the calcium was solubilized. It would be expected, however, that CS bound to collagen only by salt formation could be extracted with KCl. Thus, although the evidence is not conclusive, the observations would fit in with the hypothesis that the calcium added was bound to CS.

CHONDROITINSULFATE AND PROTEIN SOLUBILIZATION

The shape of the curves (Figs.13-16) for solubilization

of CS, non-collagenous protein and collagen are very similar although at all times a much smaller proportion of collagen was solubilized than of the other two. 75-85% of the non-collagenous protein was solubilized upon 25 hr enzyme treatment (except in Fig.13 where only 64% was solubilized) while at the same time 65-75% of the CS was being solubilized (except in Fig. 13 where only 47% was extracted.) The solubilization of collagen upon 25 hr enzyme treatment was only 15-30%, varying with the enzyme and incubation procedure used.* The papain-acetate buffer (pH 5.4) combination was the most effective system in causing the solubilization of all three materials from the pellets. In these experiments the pH of the medium changed from 6.0-6.2 to 4.1-5.2 (unbuffered) and from 5.4 to 4.9 (buffered with 0.02 M sodium acetate-acetic acid).

Most of the remaining non-collagenous protein and CS was removed by KCl treatment. This, as already pointed out, would be expected if insoluble salts of CS had formed during incubation.

PROCEDURE B

EFFECT OF KCl WITHOUT ENZYME TREATMENT ON CHONDROITINSULFATE SOLUBILIZATION

Rib cartilage powder was extracted according to procedure B except that the pellet was treated with KCl dissolved in

*See note p.57.

a 0.2 M Na_2HPO_4-0.1 M citric acid buffer (pH 7.0) (39) instead of KCl dissolved in distilled water. There was no change in the total amount of CS extracted by KCl under highly buffered conditions. The pH of the EDTA extracts was 4.8 which would increase the tendency for CS to form insoluble salts with protein at the expense of calcium which solubilizes at this pH (27,28). The fact that buffered 3% KCl (0.4 M), which would be expected to break up any salt linkage, did not remove the last portion of the CS (that which is dissolved by KOH) indicates that the CS must be bound to protein in vivo and that the CS-protein complex, in the EDTA and $CaCl_2$ insoluble fractions of cartilage, is not a salt linkage (30,31,68).

COMPARISON OF WATER AND EDTA IN EXTRACTING CHONDROITIN-SULFATES

Tables XI-XIII show that the removal of solid mineral matter with EDTA did not alter greatly the amount of CS which was not extracted with water or KCl solution and could only be removed with alkali. Table XIV shows that regardless of the extraction procedure used, the percent CS which could only be extracted with alkali, from ribs in the advanced stages of calcification, was approximately the same. It was further found that almost all the calcium had been removed by the EDTA. It therefore appears that the CS remaining behind and extracted only by KOH must have been rendered insoluble

by virtue of the organic matter, namely collagen and non-collagenous protein, rather than insoluble mineral matter (see Table XV).

Note: It should be mentioned here that after papain digestion the collagen becomes HCl or KCl soluble. Before treatment with enzyme it is not. This is the reason why the pellet, after prior incubation with papain, becomes completely solubilized after KOH treatment. This suggests that the papain has broken certain linkages in the collagen fibril which alters it such that the collagen becomes acid soluble.

FINAL DISCUSSION

FINAL DISCUSSION

(PARTS I AND II)

RE-DISTRIBUTION OF RIB CHONDROITINSULFATE: A FORE-RUNNER OR CONSEQUENCE OF CALCIFICATION ?

In Part I it is shown that during the initial stages and later in the advanced stages of calcification in puppy ribs there is a large increase in the proportion of the tissue CS which is not extractable with water; this is accompanied by an initial rise and a later fall in the total CS in the cartilage. There are two possible explanations for these changes. The first explanation is that the changes are a physical consequence of the deposition of mineral matter or removal of water. The second explanation is that the redistribution of the rib CS fractions is a consequence of changes in the rate of metabolism of the CS fractions of the rib or of changes in the binding of the CS fractions to other organic tissue constituents. If the second explanation is correct this redistribution of CS fractions may be one of the causes of the development of calcified from uncalcified ribs.

Regarding the first explanation there are three points:

1) The redistribution of the CS in the tissue might be due to a portion of the water soluble CS being rendered insoluble by the solid mineral matter.

2) There is a gradual decline in the water content of the ribs as they pass through the various stages of calcification.

3) According to Eichelberger (49) it seems likely that this

water is being replaced by deposited hydroxyapatite.

According to the third point, the solid mineral matter would increase the density of the tissue by replacement of water so that the concentration of the total CS expressed on a fresh weight basis would drop. Subtraction of the weight of calcium phosphate in the 26 week and older ribs and calculation of the CS glucuronate concentration in the mineral free rib shows that this can account for most, if not all, of the drop in total CS concentration in the older ribs. It cannot account for the initial increase in total CS concentration which occurs during the early stages of calcification, but according to the second point, a portion of this could be due to a decline in water content without corresponding deposition of solid mineral. In the experiments described in Part II (Tables XI-XIII) it was found that removal of the solid inorganic material with EDTA did not greatly alter the amount of CS which was not extracted with water or KCl solution and could only be removed by alkali. This also eliminates the possibility that the increase in the proportion of water-insoluble CS in the ribs is due to binding to solid mineral matter.

This leaves the second explanation for the redistribution of the rib CS fractions which is that it is due either to changes in the rate of metabolism of the CS fractions or to a change in the binding of the CS fractions to organic constituents in the tissues; either one of these is a possibility. The results from Table VI indicate that there are

at least two metabolically distinct CS fractions, the water-soluble (S_{1-3}) fractions and the alkali fraction (S_8) and that the specific activity of these fractions decreased slowly as the rib progressed from the uncalcified state to the advanced stages of calcification. These results may be related to the change in total CS concentration of the ribs (Table V). In regard to the redistribution of the CS amongst the fractions, however, there is little change in the ratio

$$\frac{\text{specific activity water insoluble CS (S8)}}{\text{specific activity water soluble CS } (S_{1-3})}$$

as calcification progresses in the rib which indicates that the relative rates of metabolism of these fractions is unaltered. Therefore, an alteration in the binding of CS to collagen or non-collagenous protein is the most likely explanation for the observed changes.

BINDING OF CHONDROITINSULFATE

In the enzyme experiments described in Part II (Figs. 11-16) it was found that the pH of the medium changed from 6.2-6.3 to 4.1-4.6 (unbuffered) and from 5.4 to 4.9 (buffered). Between 15-25% of the non-collagenous protein, 25-50% of the CS and 75-85% of the collagen remained in the pellet after enzyme treatment while nearly all the calcium had been solubilized. Einbinder and Schubert found that as hydrogen ions combined with the carboxylate anions of collagen, the cationic

groups of the collagen became free to bind anions of CS. Binding was best for CS between pH 2.5 and 5.0 and this tapered off at pH 7.0. At lower pH conditions calcium is removed from binding with CS (28,29). Therefore, the more acid the pH the greater would be the tendency of CS to form insoluble salts with protein,thus raising the possibility that a portion of the CS formed new insoluble complexes during digestion.

By using procedure B (Part II) to extract the rib cartilage it was found that 0.4 M KCl, which would be expected to break up any salt linkage, did not extract all the CS thus indicating that some CS must be bound to protein in vivo and that the CS-protein complex, in the EDTA and $CaCl_2$ insoluble fractions of cartilage, is not a salt linkage (30,31,68).

According to several investigators (71-74) the chondromucoprotein or proteinpolysaccharide (PP) which can be extracted by high speed homogenization of cartilage with water can be separated into two fractions: PP-L and PP-H by centrifugation and that the PP-H consists in part of a mixture and in part of a combination of PP and collagen. The PP-L sediments as a single component and has a sedimentation constant at infinite dilution of 10.5 S while the PP-H sediments completely at 10,000 x g. Gerber et al (71) found that the non-collagenous protein (NCP)/CS ratio of their PP-L after alkali treatment was 0.176 while the protein/CS ratio of the PP-H was 1.000.

The results thus far have indicated that the binding of CS to protein in the insoluble residue after homogenizing ribs with EDTA, H_2O and KCl is not a salt linkage. Recently it has been found that the bond between CS and protein in the water-soluble chondromucoprotein is formed through the hydroxyl group of serine (75,76). The results in Table XV suggest that if there is PP-H or PP-L in the insoluble residue after EDTA and KCl extraction of uncalcified ribs then there is not enough NCP present to bind all the CS since the ratio NCP/CS is only 0.102. However, the alkali extract of one of the ribs in the advanced stages of calcification contains more than enough NCP to bind all the CS solubilized if PP-L was present and just about enough if PP-H was present. The ratio NCP/CS is 0.930. The analyses on the pellet remaining after KOH extraction of ribs at the advanced stage indicate that only collagen is available to bind the remaining CS. This is an indication that the insolubility of at least a small portion of the CS is due to association with collagen.

It is very unlikely that PP-L would be found in the insoluble residue after extraction with EDTA, water and KCl as it is known to be soluble in water and not sedimented at 27,000 x g (68,71,72). However, PP-H is insoluble in water (after ethanol precipitation) and/at sediments 10,000 x g (68,71) and at least according to Serafini-Fracassini and Smith (73), consists in part of a mixture and in part of a combination

of PP and collagen. Thus it is quite possible that the CS in the insoluble residue exists in a form related to the as yet ill-defined PP-H material.

WATER CONTENT OF RIBS

There is a gradual decline in the water content of the ribs as they pass through the various stages of calcification (Table XVI). Eichelberger (49), using rib cartilage of puppies, also found the same result. According to Eichelberger it seems likely that this water is being replaced by deposited hydroxyapatite.

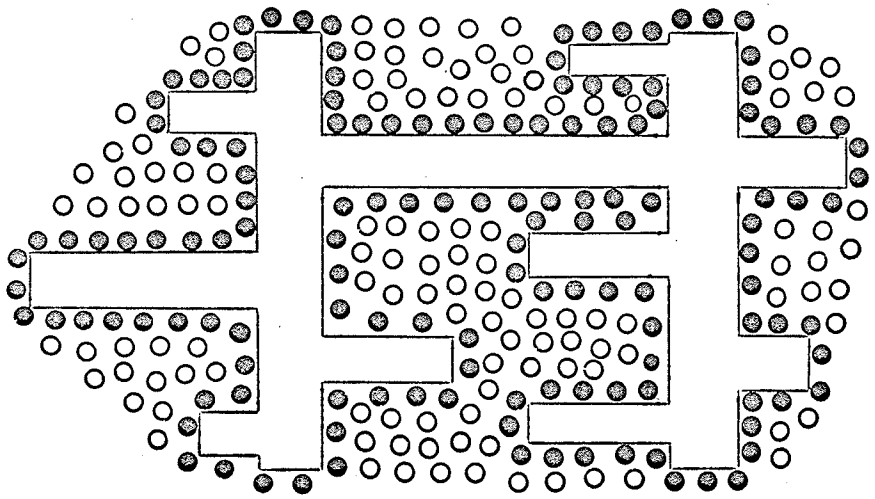
Schubert (58), using bovine nasal cartilage, has found that the PP-L seems to be responsible for the water held by the tissue. He attributed this to the diffuse nature of the PP-L molecule and its tendency to occupy a large domain because of the mutual repulsion of nearby CS chains. This tendency to occupy a large domain is imagined to be exerted against the collagen fibrils among which the PP-L is trapped.

The question finally arises as to the possible part water plays in this protein-CS relationship?

There is no direct evidence on how the presence or absence of acid mucopolysaccharides(AMPS)can regulate the ossification process of cartilage *in vivo*. Recent findings (43,50-52) suggest the participation of endogenous proteolytic enzymes, e.g. cathepsin B, in the degradation of cartilage

matrix. The controlled removal of AMPS protein complexes (proteinpolysaccharide (PP) of Schubert (58)) may also cause a removal of water and make space available for occupation by apatite crystals since according to Schubert (58) the PP seems to be responsible for the water held by the tissue. The large amounts of AMPS in the resting zone of the cartilage may also act as inhibitors of calcification because of their ability to restrict the movement of fluids and ions needed for mineralization and/or because of their ability to mask reactive groups on the collagen (43).

According to Klotz (53) water may also act as an inhibitor of calcification. The presence of protein chains increases the tendency of the surrounding water to form ice-like structures (see following diagram).



Schematic diagram of ice-like character of hydration sheath around nonpolar groups in protein molecules. Small circles represent water molecules.

● ice-like H_2O

○ free H_2O

This may result in the effective "masking" of active groups on the protein since the diffusion of substrates towards the active groups is restricted. The presence of water surrounding hydrophilic groups in proteins causes the formation of "hydrophilic bonds" between such groups (54). As calcification proceeds the water content (see previous diagram) of the rib cartilage declines. According to Tristram (55) there are a number of unusual linkages in collagen and these may be affected by or depend upon local dehydration. There is now evidence to suggest that soluble AMPS are responsible for the water held by the cartilage (56-58,69). It is therefore possible that a removal of soluble CS (or chondromucoprotein) from cartilage may initiate changes in the cross-linkage of collagen and thus of the binding of CS to collagen, through a change in the water content.

FUTURE WORK

There are many problems concerning calcification in rib cartilage which merit further investigation. A few of those which arise from the present works are given below:

1) One problem which was attempted and could not be solved was whether or not calcium actually accumulated before phosphate and the deposition of solid mineral matter. Perhaps other animals or other tissues where calcification takes place in discrete stages might be found and used to

investigate this problem.

2) The radioactive work should be extended to cover measurements of specific activity over much longer periods of time in an effort to determine more definitely the causes of the changes in ^{35}S uptake in the 3 groups of ribs.

3) An attempt should be made to characterize the CS fractions removed by KCl and KOH treatments by means of density gradients, or other means, in an effort to see if the KCl fractions, themselves, actually represent a separate metabolic entity.

4) The binding of CS in the insoluble rib fractions will have to be further investigated with respect to the type of bond formation and also the characterization of non-collagenous protein(s) (65,66).

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APPENDIX

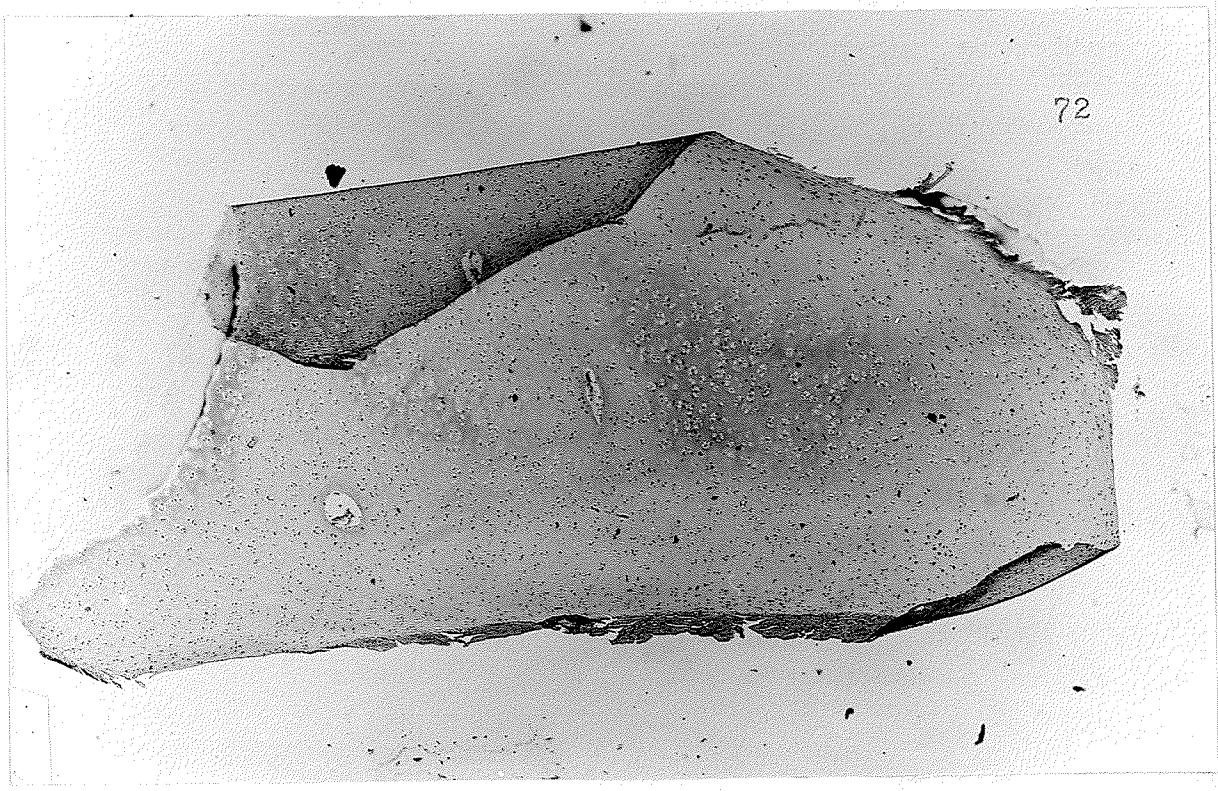

Fig.1-Rib cartilage section from rib 1 dog 3(thickness:7μ, von Kossa's stain[33,34] and eosin,magnification:30x).No calcium deposit observed.

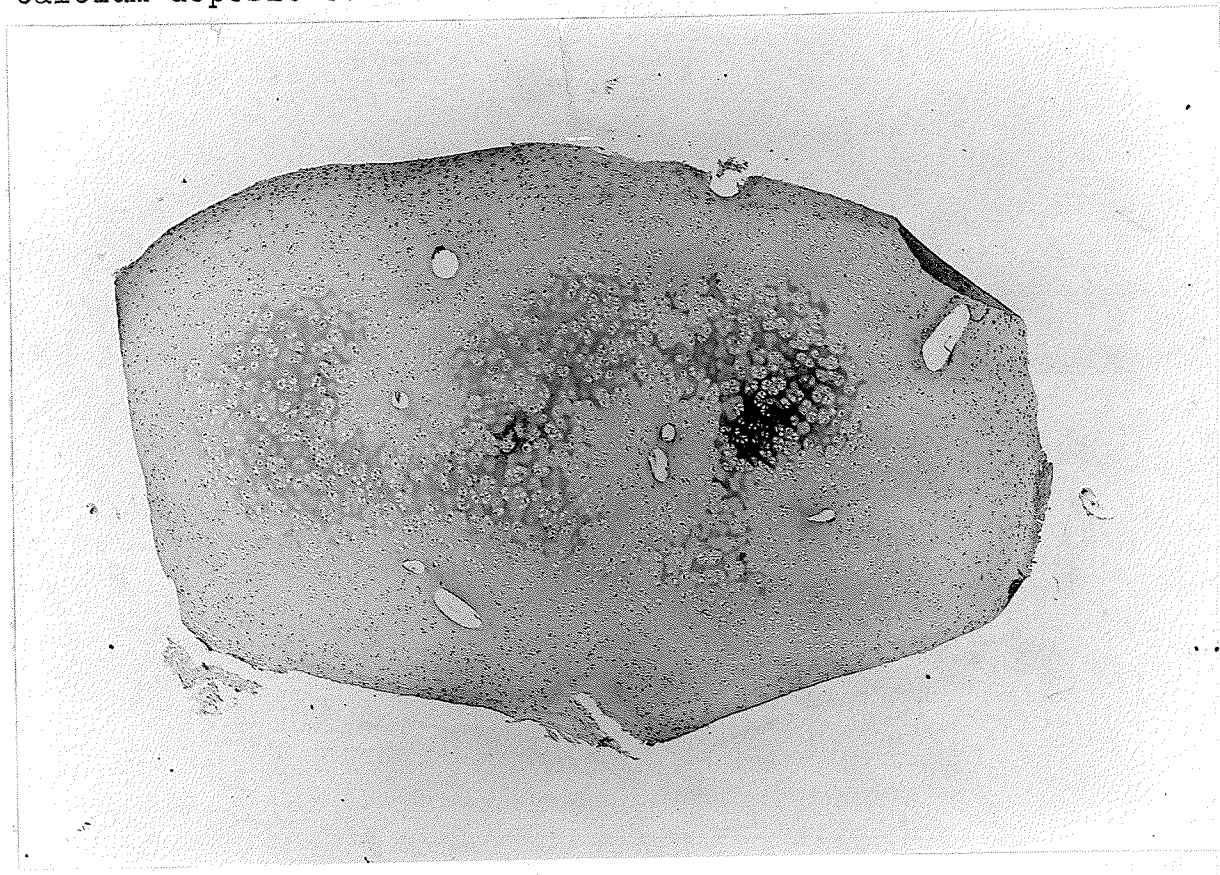

Fig.2-Rib cartilage section from rib 2 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Very slight calcium deposit observed(dark area).

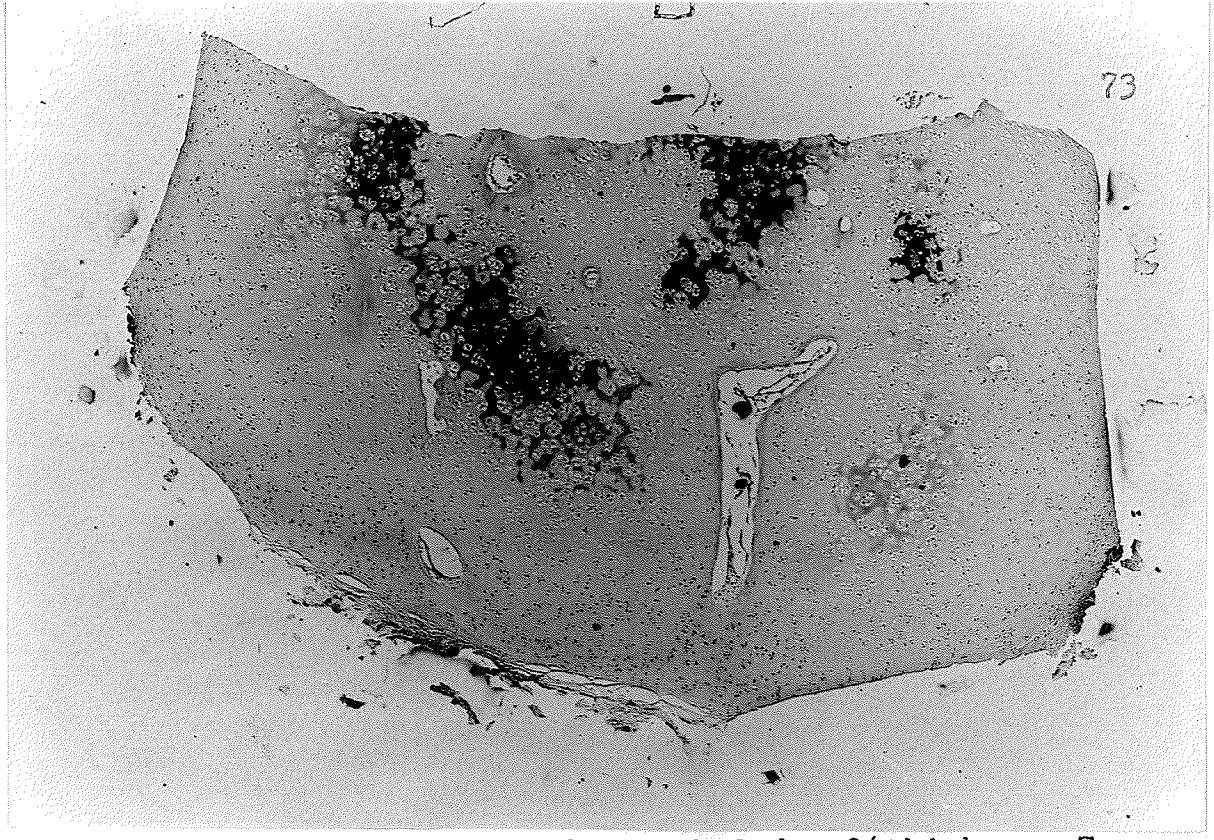

Fig.3-Rib cartilage section from rib 3 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Slight calcium deposit observed.

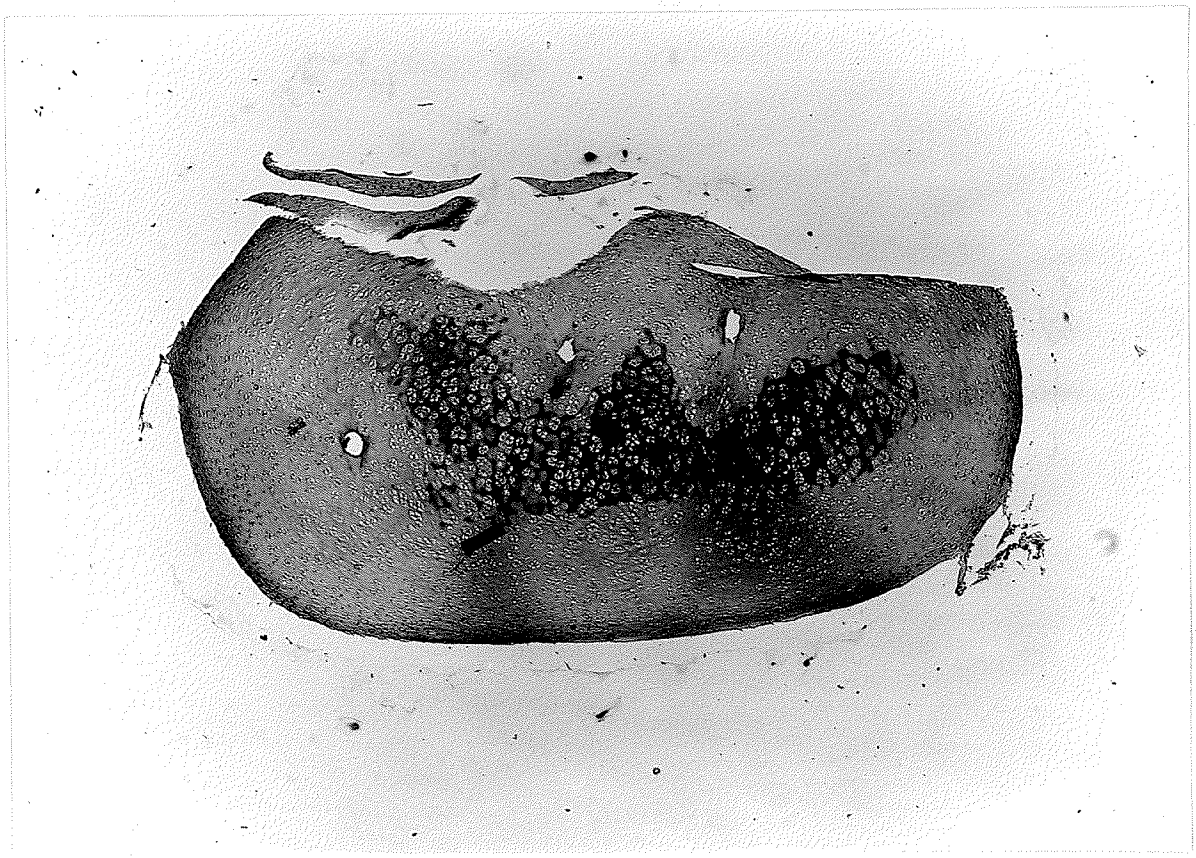

Fig.4-Rib cartilage section from rib 4 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Many calcium deposits observed.

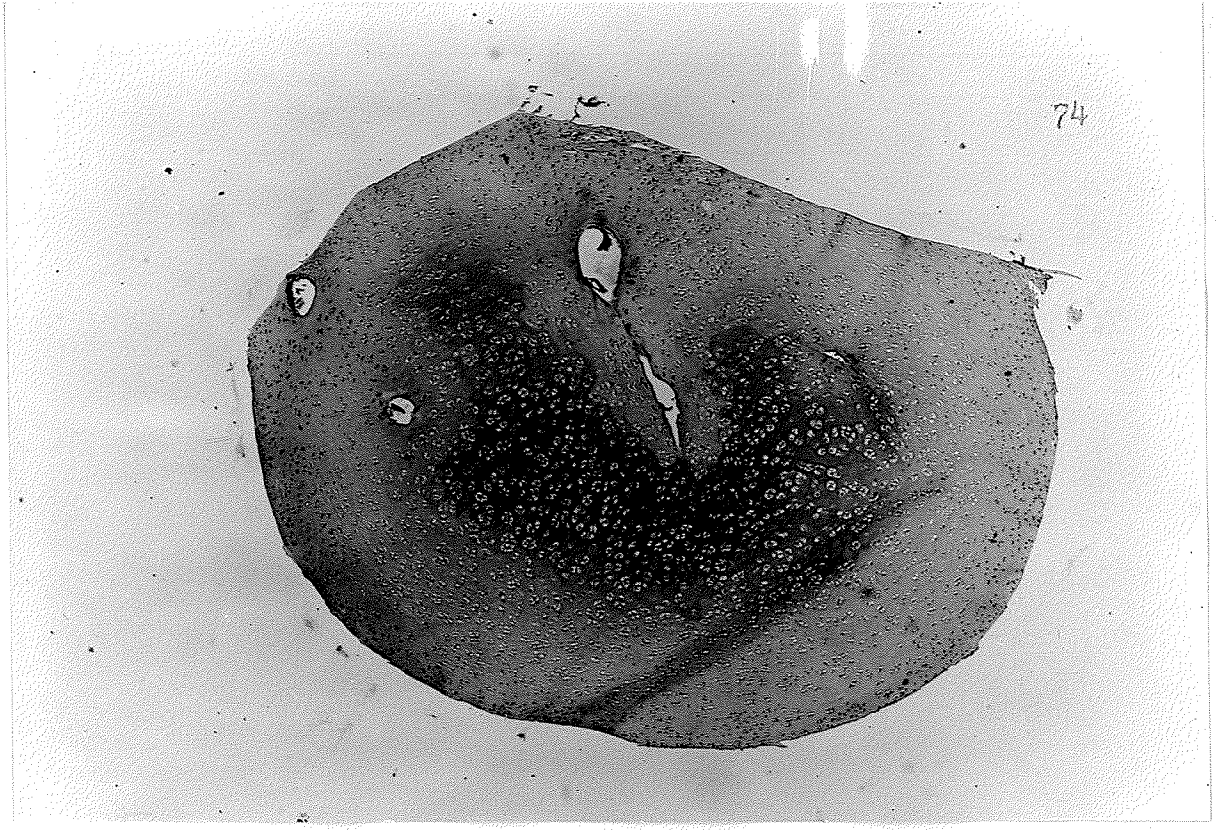

Fig.5-Rib cartilage section from rib 5 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Many calcium deposits observed.

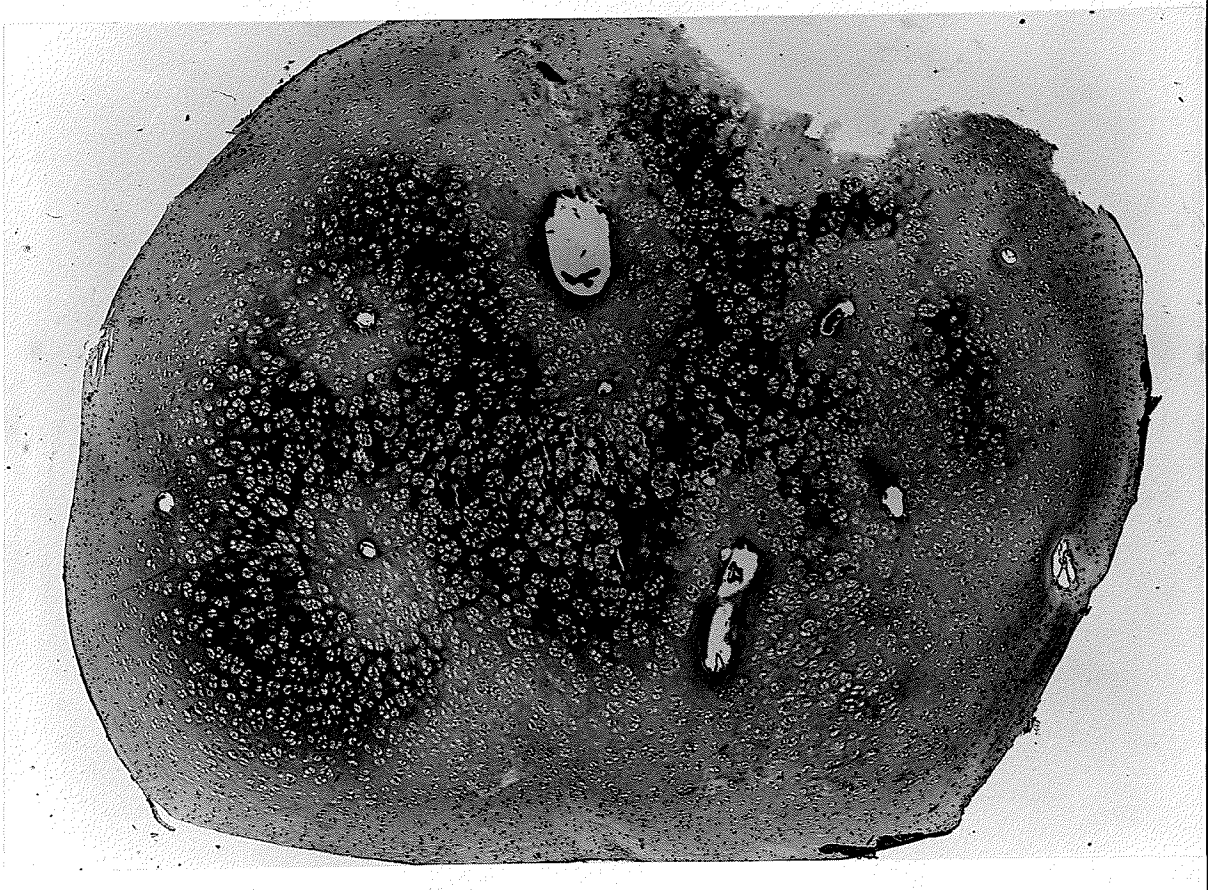

Fig.6-Rib cartilage section from rib 6 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).More calcified than rib 5.

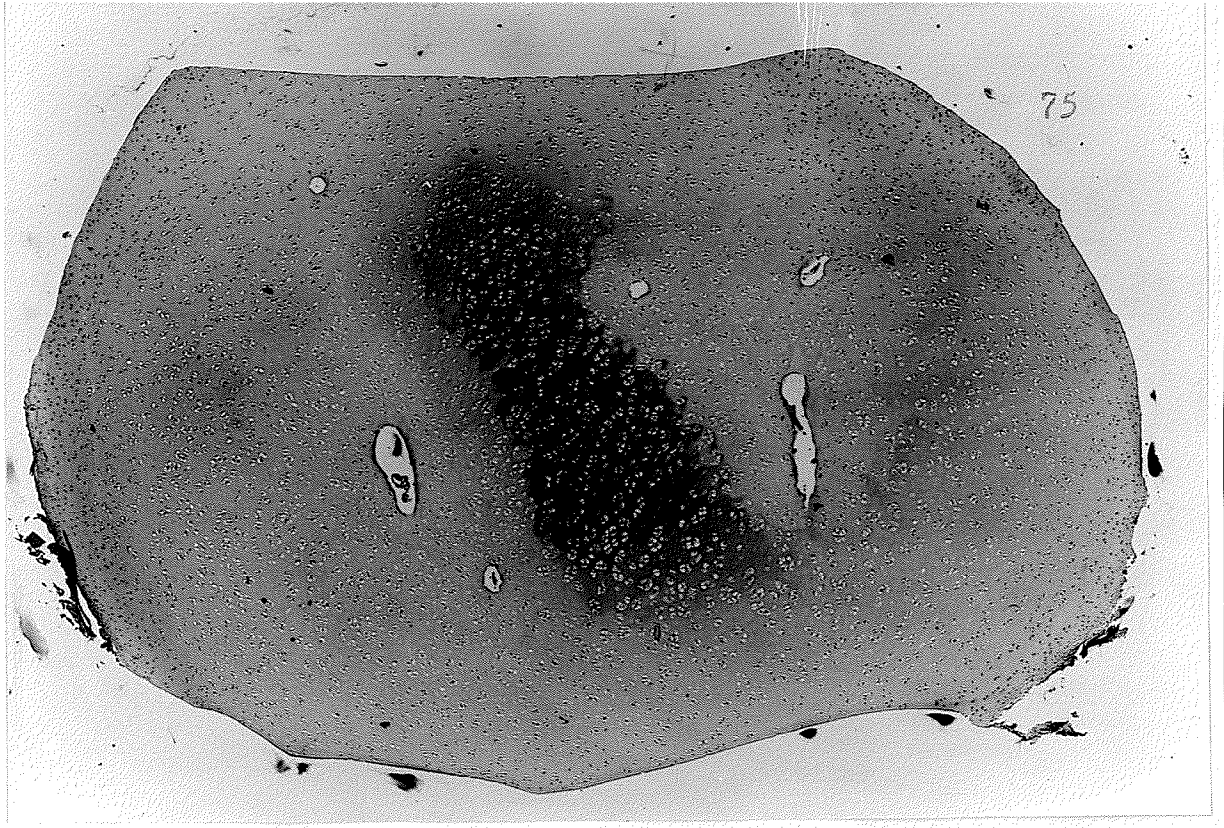

Fig.7-Rib cartilage section from rib 7 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Not as strongly calcified as rib 6.

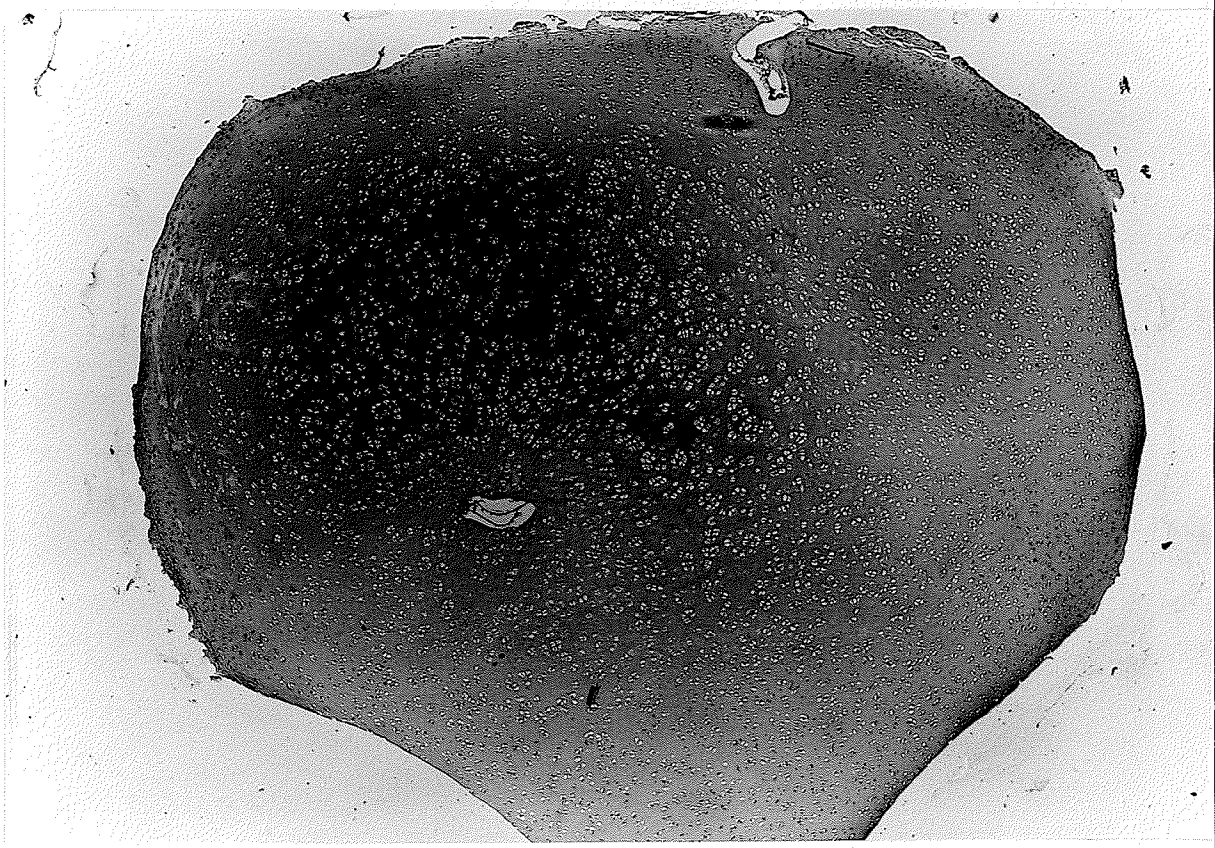

Fig.8-Rib cartilage section from rib 8 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Areas of calcification are not strongly defined.

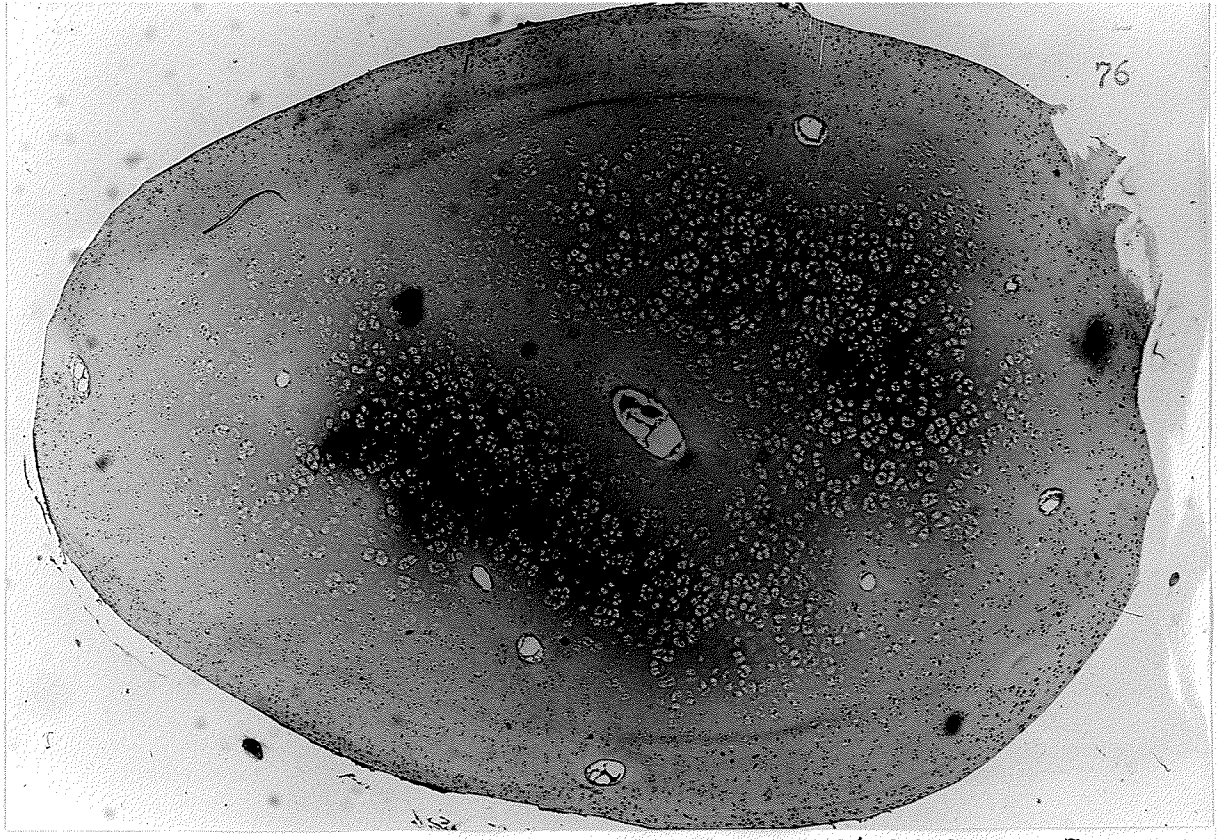

Fig.9-Rib cartilage section from rib 9 dog 3(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Not as strongly calcified as rib 7.

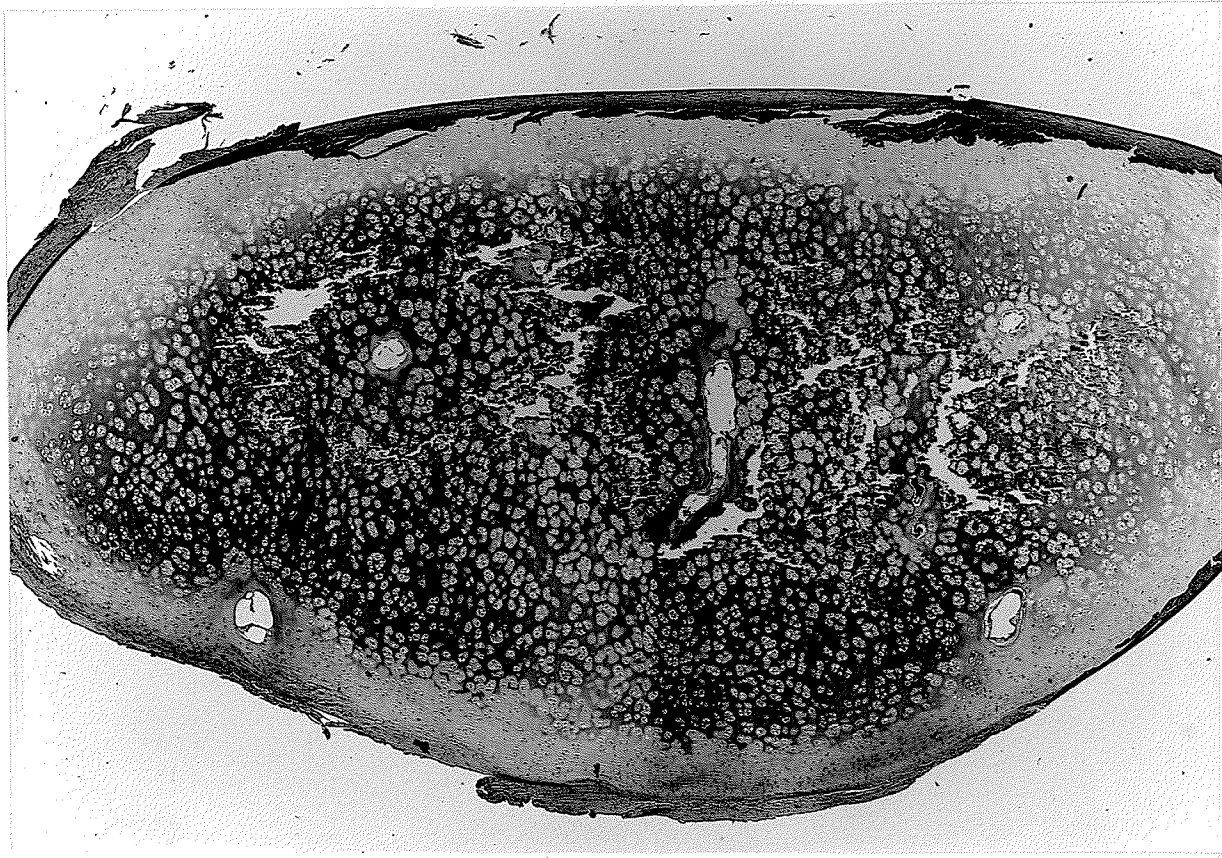

Fig.10-Rib cartilage section from rib 9 dog 7(thickness:7μ, von Kossa's stain and eosin,magnification:30x).Calcification has proceeded to the advanced stages.

READINGS FOR STANDARD CURVES

a) Calcium (15)

mg Ca used in test	titration with 0.005 M EDTA (mls)
0.01	.023
0.02	.041
0.05	.103
0.07	.142
0.10	.213
0.20	.406
0.40	.804

b) Uronic acid (12)

mg glucurono-lactone used in test	O.D. at 525 mμ
0.01	.078
0.02	.159
0.03	.224
0.04	.304
0.05	.362
0.06	.430
0.07	.504
0.08	.600
0.09	.654
0.10	.730

c) Total P (11)

mg P used in test	O.D. at 650 mμ
0.002	.056
0.004	.090
0.008	.167
0.012	.245
0.016	.325
0.020	.395
0.024	.490
0.028	.560
0.032	.646
0.036	.729
0.040	.790

d) Inorganic P (10)

mg P used in test	O.D. at 650 mμ
0.0004	.028
0.0012	.078
0.002	.139
0.004	.270
0.006	.405
0.008	.545
0.010	.660
0.012	.818

e) Collagen standard

	Protein (24)	Hydroxyproline (25)
µgm collagen	O.D. at 650 mµ	O.D. at 555 mµ
10	.021	-
25	.044	.032
50	.076	.067
75	.102	.105
100	.130	.138
125		.158
150	.184	.208
175		-
200	.235	-

f) Bovine albumin standard (24)

µgm bovine albumin	O.D. at 650 mµ
10	.030
25	.065
50	.120
75	.200
100	.250
125	.310
150	.370
175	.430
200	.510

TABLE VII

EXTRACTION OF RIB CARTILAGE

Second Series of Experiments

Dog II. Age 13-13½ wks.	Dog II Rib 1	Dog II Rib 2	Dog II Rib 3	Dog II Rib 6	Dog II Rib 9
Ca, S_{1-3}	15.0	23.3	46.7	52.0	35.6
Ca, S_{4-5}	10.7	14.7	47.6	55.3	41.6
Ca, S_{6-7}	1.32	2.00	99.9	294.6	50.0
Inorganic P, S_{1-3}	4.4	6.9	26.6	29.7	25.0
Inorganic P, S_{4-5}	0.2	0.5	8.3	12.5	7.9
Inorganic P, S_{6-7}	0.25	0.5	54.1	163.9	23.4
Ca/Pi, S_{6-7}	5.3	4.0	1.85	1.75	2.14
Pi/Pt, S_{1-8}	0.22	0.30	0.79	0.86	0.89
CS glucuronate, S_{1-8}	171.8	181.9	187.3	180.0	184.5
CS glucuronate, S_{4-8}	38.7	38.6	45.2	40.4	46.4
Ca S_{1-3}/CS glucuronate S_{1-3}	0.133	0.163	0.329	0.372	0.258
Ca S_{4-5}/CS glucuronate S_{4-8}	0.276	0.400	1.053	1.369	0.898
% CS glucuronate S_{1-3}	77	79	76	78	75

Note: Figures are in µg atoms/g wet weight for Ca and P; µmoles/g wet weight for CS glucuronate.

TABLE VIII

EXTRACTION OF RIB CARTILAGE

Second Series of Experiments

Dog IV: Age 13½-13 2/3 wks	Dog IV Ribs 1+2	Dog IV Ribs 3+4	Dog IV Rib 6	Dog IV Rib 9
Ca, S_{1-3}	12.3	41.0	50.0	34.2
Ca, S_{4-5}	17.3	44.4	58.7	55.6
Ca, S_{6-7}	2.1	101.0	503.0	160.0
Inorganic P, S_{1-3}	3.3	23.5	37.8	26.2
Inorganic P, S_{4-5}	0.2	8.6	14.4	10.2
Inorganic P, S_{6-7}	0.1	55.4	268.9	86.8
Ca/Pi, S_{6-7}	21.0	1.82	1.87	1.84
Pi/Pt, S_{1-8}	0.16	0.80	0.85	0.87
CS glucuronate, S_{1-8}	134.4	162.8	159.5	161.6
CS glucuronate, S_{4-8}	44.3	38.3	38.4	46.9
Ca S_{1-3}/CS glucuronate S_{1-3}	0.136	0.330	0.413	0.328
Ca S_{4-5}/CS glucuronate S_{4-8}	0.390	1.160	1.529	1.190
% CS glucuronate, S_{1-3}	67	76	75	71

Note: Figures are in µg atoms/g wet weight for Ca and P; µmoles/g wet weight for CS glucuronate.

TABLE IX

EXTRACTION OF RIB CARTILAGE
Second Series of Experiments

Dog V: Age 15 1/3 - 15 2/3 wks	Dog V Rib 1	Dog V Rib 2	Dog V Rib 3	Dog V Rib 4	Dog V Rib 6	Dog V Rib 9
Ca, S_{1-3}	11.4	19.6	31.6	53.7	78.7	48.9
Ca, S_{4-5}	16.4	20.5 (approx)	47.2	60.0	54.7	64.4
Ca, S_{6-7}	3.1	#	45.0	200.0	317.3	186.8
Inorganic P, S_{1-3}	3.9		21.5	37.5	37.6	42.1
Inorganic P, S_{4-5}	0.2		6.1	11.5	13.1	10.1
Inorganic P, S_{6-7}	0.2		22.7	109.9	173.8	105.0
Ca/Pi, S_{6-7}	15.5		2.00	1.82	1.83	0.79
Pi/Pt, S_{1-8}	0.20		0.74	0.82	0.86	0.84
CS Glucuronate, S_{1-8}	182.0		201.2	196.7	226.2	198.9
CS Glucuronate, S_{4-8}	44.8		51.4	47.2	41.5	58.1
Ca S_{1-3} /CS glucuronate S_{1-3}	0.083		0.211	0.359	0.426	0.347

continued

TABLE IX CONTINUED

Dog V: Age 15 1/3-15 2/3 weks	Dog V Rib 1	Dog V Rib 2	Dog V Rib 3	Dog V Rib 4	Dog V Rib 6	Dog V Rib 9
Ca S_{4-5}/CS glucuronate S_{4-8}	0.366		0.918	1.271	1.318	1.110
% CS glucuronate, S_{1-3}	75		75	75	80	71

machine broke down, sample lost.

Note: Figures are in µg atoms/g wet weight for Ca and P; µmoles/g wet weight for CS glucuronate.

TABLE X

EXTRACTION OF RIB CARTILAGE

Second Series of Experiments

Dog VI: Age 17 1/3 wks	Dog VI Ribs 1+2	Dog VI Ribs 2+3	Dog VI Rib 6	Dog VI Rib 9
Ca, S_{1-3}	23.3	59.3	58.0	54.6
Ca, S_{4-5}	26.6	56.6	68.0	58.7
Ca, S_{6-7}	7.32	184.0	598.6	201.4
Inorganic P, S_{1-3}	7.3	29.6	33.8	31.3
Inorganic P, S_{4-5}	1.2	9.2	19.3	12.5
Inorganic P, S_{6-7}	1.4	87.3	327.2	110.4
Ca/Pi, S_{6-7}	5.2	2.11	1.83	1.82
Pi/Pt, S_{1-8}	0.47	0.72	0.77	0.79
CS glucuronate, S_{1-8}	205.8	194.5	168.9	182.1
CS glucuronate, S_{4-8}	52.2	45.9	42.5	40.1
Ca S_{1-3}/CS glucuronate S_{1-3}	0.152	0.399	0.459	0.386
Ca S_{4-5}/CS glucuronate S_{4-8}	0.510	1.233	1.600	1.963
% CS glucuronate, S_{1-3}	75	75	75	78

Note: Figures are in µg atoms/g wet weight for Ca and P; µmoles/g wet weight for CS glucuronate.