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Studies on the Mode of Action of Excess of Vitamin A

2. A POSSIBLE ROLE OF INTRACELLULAR PROTEASES IN THE DEGRADATION OF CARTILAGE MATRIX

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When cartilaginous limb-bone rudiments from 6- to 7-day chick embryos are cultivated *in vitro*, the cartilage differentiates into a hypertrophic region, proliferative zones of flattened cells and epiphyses as in normal development. If excess of vitamin A is added to the culture medium, the cartilage differentiates at approximately the normal rate but the metachromatic staining properties of the matrix are greatly reduced (Fell & Mellanby, 1952). A similar loss of metachromasia occurs when papain is added to the culture medium (Fell & Thomas, 1960). The injection of papain into rabbits results in a collapse of the ears, accompanied by disappearance of metachromasia from the matrix of the ear cartilage (Thomas, 1956). Similar injections can also cause total disappearance throughout the body of the metachromatic staining properties of cartilage matrix (Spicer & Bryant, 1958; McCluskey & Thomas, 1958) and the appearance in blood and urine of a mucopolysaccharide that resembles chondroitin sulphates A and C (Bryant, Leder & Stetten, 1958). A depletion of the chondromucoprotein of cartilage and decrease of ^{35}S content of cartilage matrix have been observed (Tsaltas, 1958) in rabbits which have received sodium [^{35}S]sulphate and papain; later an increased quantity of ^{35}S in serum and urine and of glucuronic acid in urine occur. The administration of large amounts of vitamin A to rabbits causes histological changes in cartilage that are remarkably similar to those seen in animals treated with small amounts of papain (Thomas, McCluskey, Potter & Weissmann, 1960).

These observations suggest that some of the effect of vitamin A on the cartilage of embryonic-chick rudiments may be mediated through the action of a proteolytic enzyme system present in the cartilage, which degrades the structural components of the matrix with a resultant loss of polysaccharide. In the preceding paper it is reported that, when grown *in vitro* in the presence of excess of vitamin A, the cartilaginous limb-bone rudiments from 6- to 7-day chick embryos have a greater proteolytic effect on the medium than control explants in normal medium (Dingle, Lucy & Fell, 1961). A possible interpretation of this is that the activity of proteolytic enzymes in the tissue is enhanced by vitamin A. In this paper evidence is reported that an enzyme system which will degrade cartilage matrix is present in bone rudiments from normal chick embryos. The enzyme, or group of enzymes, is active after hypo-osmotic treatment of the tissues; the optimum pH of the system is in the acid region.

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MATERIALS AND METHODS

Material. All experiments were performed on limb-bone rudiments from 8–9-day chick embryos. At this stage the limb bones consist of a cartilaginous rod differentiated into the usual three zones of epiphyses, flattened (proliferative) cells and hypertrophic cells; the hypertrophic zone is

covered by a layer of periosteal bone. Embryos of the same age vary considerably in their degree of development; in particular, embryos taken in summer are usually larger and more advanced than those obtained in winter. The rudiments (cartilage, periosteal bone and periosteum) were dissected as free as possible of muscle and connective tissue and were kept at room temperature, in a mixture of equal parts of iso-osmotic salt solution and amniotic fluid, until the beginning of the experiment. Before the rudiments were weighed, excess of fluid was removed with filter paper.

Histology. At the end of the experimental period, bone rudiments were fixed in 3% acetic acid–Zenker's fluid for 30 min. followed by Zenker's fluid without acetic acid for not less than 3 hr. After being washed, dehydrated and cleared in cedarwood oil, the rudiments were embedded in paraffin wax and serially sectioned. Sections were stained for about 30 min. in 0·5% of toluidine blue (G. T. Gurr Ltd., London) in 5% ethanol, rinsed in tap water, dehydrated, cleared and mounted in DePeX (G. T. Gurr Ltd.), in which the metachromatic staining is preserved for years.

In the experiments in which the effect of pH on loss of hexosamine was investigated, 36 limb-bone rudiments from three 9-day eggs were used at each pH. One rudiment was removed from each tube after the bones had been incubated in buffer for 2 hr., and was fixed for histological examination. The error in the estimation of the total hexosamine content of the treated bones caused by the removal of the bone rudiment would be very small, and no allowance was made for this in the determination of the percentage of hexosamine liberated into the buffer.

Effect of pH *on protein breakdown.* Freshly dissected bone rudiments were placed in water at 4° for 1 hr.; they were then incubated for 2 hr. at 37° in 0·1M-sodium acetate–HCl buffer (Vogel, 1948) for pH values 1–5. For pH values 6–8, 0·1M-sodium acetate was adjusted to the desired pH by the addition of 0·1N-NaOH. All pH values were checked, both before and after the incubation, by means of a glass electrode. Two tibiae and two femora were used at each pH. After incubation the supernatant solution was removed from each tube and the bones were washed with several small quantities of buffer. The buffer solutions were dried *in vacuo* over P_2O_5 and KOH. Each extract was hydrolysed for 24 hr. at 110° in HCl of constant boiling point; the residual rudiments were hydrolysed similarly. The hydrolysates were dried *in vacuo* over P_2O_5 and KOH; the hydrolysed material was redissolved in a suitable portion of water and analysed for total α-amino nitrogen with the KCN–ninhydrin reagent of Yemm & Cocking (1955) after the addition of N-sodium acetate buffer, pH 5·1 (1 ml.) (Moore & Stein, 1954). Leucine was used as the standard.

Fractionation of protein and determination of protein breakdown in rudiments. Freshly dissected bones from 9-day eggs were placed in water at 4° for 1 hr. and were then incubated for 2 hr. at 37° in 2 ml. of 0·1M-sodium acetate solutions of different pH values (pH 1–8). Three metatarsals, one femur and one tibia were incubated at each pH. The supernatant buffer was removed from each tube and the bones were washed with several small quantities of buffer; the washings were added to the main supernatant. An equal volume of 10% trichloroacetic acid was added to each buffer supernatant and the solution allowed to stand for 2 hr. in the cold before centrifuging. The scanty, barely detectable precipitates were discarded. Each solution was dried *in vacuo* over P_2O_5 and KOH.

The treated bone rudiments were extracted twice in the cold with two portions (1 ml.) of 5% trichloroacetic acid (10 min. periods), followed by three 15 min. extractions with 5% trichloroacetic acid at 90°. The cold and hot trichloroacetic acid extracts were dried *in vacuo* over P_2O_5 and KOH.

Each of the dried samples described above was hydrolysed in HCl of constant boiling point (2 ml.) for 24 hr., in a sealed tube at 110°; the residual bone rudiments were hydrolysed similarly. The hydrolysates were dried *in vacuo* over P_2O_5 and KOH; final traces of acid were removed by drying under an infrared lamp. Each hydrolysate was redissolved in a suitable volume of water and duplicate portions (2 ml.) were analysed for total α-amino nitrogen as described above.

Amino acid analysis of degraded-protein components. Limb-bone rudiments (354 mg. wet wt.) from sixteen 9-day eggs were placed in water at 4° for 1 hr. and then incubated at 37° for 2 hr. in 0·1M-sodium acetate buffer (4 ml.) at pH 4. The buffer solution was removed and the rudiments were washed with several small quantities of buffer; the washings were added to the main supernatant buffer. An equal volume of 12% trichloroacetic acid was added to the buffer solution. Although the buffer contained a few small pieces of detached tissue, there was no apparent increase in turbidity after the addition of the acid. The solution was centrifuged and the scanty residue discarded. The supernatant solution was dialysed in the cold for 36 hr. against five successive portions (25 ml.) of distilled water. After the total diffusate (125 ml.) had been reduced to a small volume in a rotary evaporator, the solution was heated for 1 hr. at 100° in the presence of a small piece of porous pot to decompose the trichloroacetic acid. The diffusate and the non-dialysable material were dried over P_2O_5 *in vacuo* and then hydrolysed in HCl of constant boiling point (2 ml.) for 24 hr. under N_2 at 110°. Both hydrolysates were dried over P_2O_5 and KOH *in vacuo*, then redissolved in water, and portions analysed for total α-amino nitrogen with ninhydrin, leucine being used as a standard.

A portion of the hydrolysate of the non-dialysable material was applied to a 150 cm. column of Amberlite resin, CG-120 (II), on which the neutral and acidic amino acids were separated by the procedure of Moore, Spackman & Stein (1958). The amino acids in the eluate were quantitatively determined with the KCN–ninhydrin reagent of Yemm & Cocking (1955). Proline was determined in the eluate with the acid–ninhydrin reagent of Chinard (1952), and hydroxyproline was estimated in the eluate by a modification (Lawson & Lucy, 1960) of Neuman & Logan's (1950) procedure in which 1% hydrogen peroxide was used and colour developed at 60–65°.

A portion of the hydrolysed diffusate was de-ionized by passage through a column of Dowex 50 in the H^+ form in order to remove the Na^+ ions present in this fraction from the acetate buffer, pH 4. The solution was then chromatographed on Whatman no. 54 paper in a solvent system of phenol–water–ammonia with an ammonia atmosphere. The chromatogram was run for 10 hr. at room temperature, dried by heat and developed by ninhydrin.

Release of proteolytic activity. The bone rudiments from 16 eggs were placed in water at 4° for 1 hr.; after removal of the rudiments the solution was centrifuged to eliminate any

debris and the proteolytic activity of the supernatant estimated.

Separation of intracellular fractions. The bone rudiments were homogenized in 0·25 M-sucrose at 4° and the homogenate was centrifuged in a Serval Superspeed centrifuge at 4°. The first fraction, consisting mainly of nuclei and debris (Schneider & Hogeboom, 1950), was obtained after 5 min. at 700 *g*. The pellet was washed with sucrose and the supernatant and washings were centrifuged at 10 000 *g* for 20 min. The proteolytic activity of the 'nuclear' fraction and of the 'mitochondrial' fraction was measured after the pellets had been homogenized in water. In the supernatant fraction, which even after dilution contained a considerable concentration of sucrose, the solutions were kept cold to avoid hydrolysis of the sugar by the trichloroacetic acid (Gianetto & de Duve, 1955).

Estimation of proteolytic activity. Proteolytic activity was measured by the method of Anson (1938). Bovine haemoglobin enzyme–substrate (Armour Laboratories) was used at a final concentration of 2% (w/v). The enzyme activity was measured at 37° in 0·1 M-sodium acetate buffer and expressed as the increase (μg.) in acid-soluble 'tyrosine'.

Estimation of acid mucopolysaccharide. After 1 hr. in water at 4° the bone rudiments were incubated at pH 4 in acetate buffer at 37° for 2 hr. The bones were then removed, the fluid was cleared of debris by centrifuging and polymeric acid polysaccharide estimated by the turbidimetric method of Di Ferrante (1956). The buffer extract was adjusted to pH 6 and 1 ml. was treated with 2·0 ml. of 2·5% cetyltrimethylammonium bromide in 2% NaOH. After 5 min. at 37° the extinction was read at 400 mμ. Dilutions of a standard solution of chrondoitin sulphate L. Light and Co. Ltd.) were made at the time of each set of estimations; the method gave a linear relationship between extinction and polysaccharide concentration over the range 20–180 μg./ml.

The mucopolysaccharide in the 0·1 M-sodium acetate buffer extract was treated with testicular hyaluronidase (Benger Laboratories Ltd.), the pH adjusted to 6·4 by the addition of 0·1 N-NaOH, and the extract then incubated at 37°. The remaining polymeric material was estimated turbidimetrically.

RESULTS

Loss of amino sugars from rudiments during treatment with papain. Fell & Thomas (1960) have shown that incubation in a solution of papain protease destroys metachromasia in chick-limb-bone rudiments. As shown in Table 1, the enzyme also causes a rapid release of hexosamine from the rudiments. This finding prompted an investigation into other methods of removing extracellular material from embryonic limb cartilage.

Effect of hypo-osmoticity on metachromasia of cartilage matrix. Initial experiments were made to see if the effect of treatment with excess of vitamin A upon the metachromatic staining could be simulated by extraction of the polysaccharide present in the cartilage. A number of salt solutions that are commonly used to remove chondroitin sulphate from cartilage were tried. The rudiments were extracted for 1–24 hr. with the following solutions: alkaline KCl (Einbinder & Schubert, 1950), neutral $CaCl_2$ (Blix & Snellman, 1945) and water (Shatton & Schubert, 1954). The alkaline reagent had almost no action upon the metachromatic staining properties of the matrix and the neutral salt solution also was ineffective except in the 24 hr. experiment. The water-treatment, however, decreased metachromasia of the cartilage after 3 hr., indicating a loss or depolymerization of sulphated mucopolysaccharide. As is shown in Table 2, water was more effective at 37° than at 4°, whereas incubation at 37° in alkaline KCl had no effect.

Effect of pH *on loss of metachromasia.* After immersion of the rudiments in water at 4° for 1 hr., the chondrocytes became shrunken and their nuclei pycnotic; there was no obvious effect on the metachromatic staining of the matrix. In the following experiments this procedure was adopted as a standard hypo-osmotic pretreatment with the object of disrupting the cells and their organelles and thus releasing the intracellular hydrolytic enzymes.

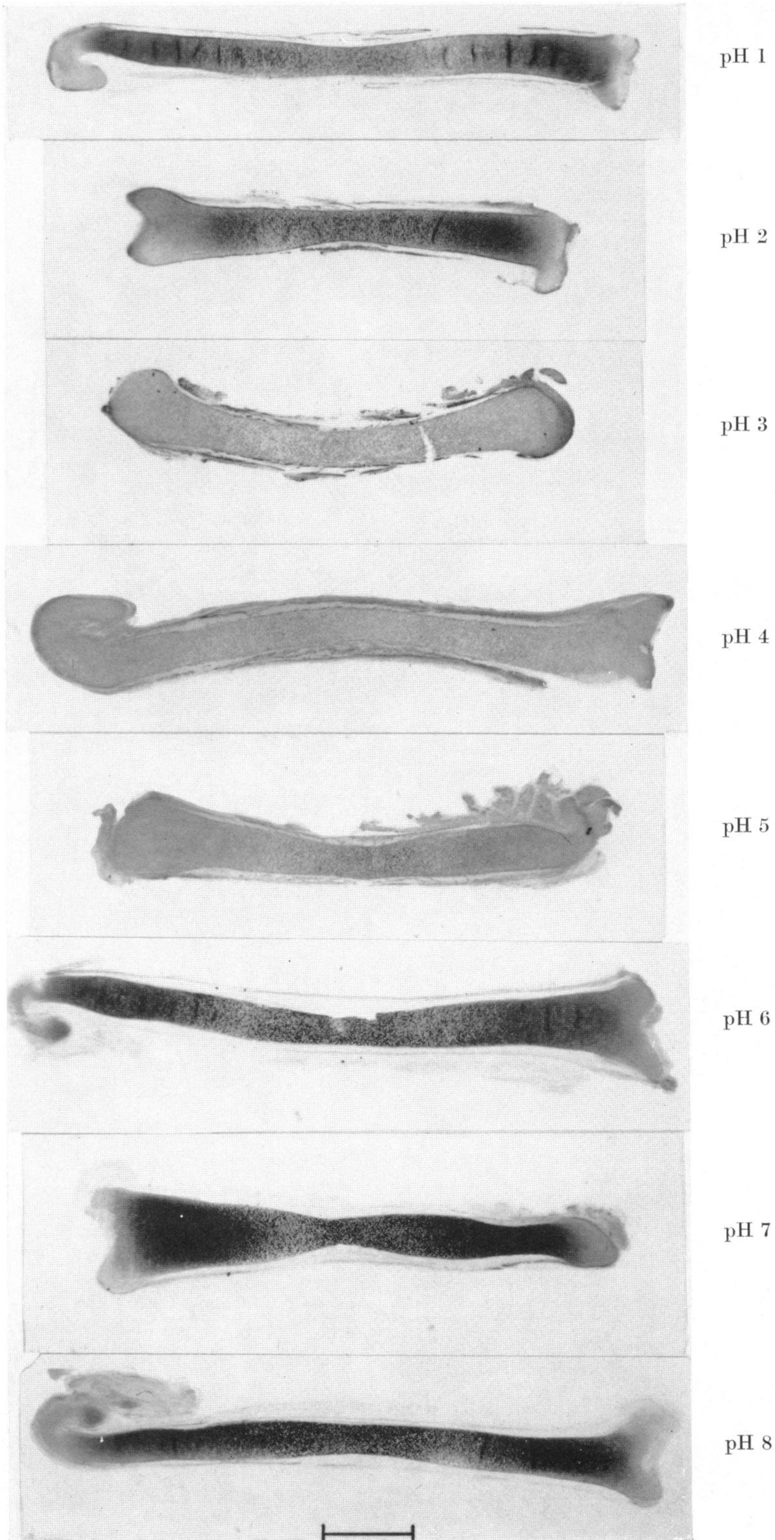
Two more experiments were made (Plate 1 and Table 3) in which the bones, after the usual hypo-osmotic pretreatment, were incubated in buffer solutions of pH from 1 to 8. The greatest loss of metachromasia was at pH 3 and 4, and there was little (Expt. 314) or no (Expt. 292) obvious effect below pH 3 or above pH 5. The rudiments in Expt. 314 were at a more advanced stage than those of Expt. 292 and show an apparently greater histological response; this may be due to the fact that in normal development the matrix becomes less intensely metachromatic with age. In both experiments, however, maximum loss of metachromasia was between pH 3 and 4.

Relationship between loss of metachromasia and loss of hexosamines. At pH 4 and 5 more than half of the hexosamine content of the rudiments was liberated into the medium during the 2 hr. incubation period after the hypo-osmotic treatment (Fig. 1). In this experiment (Expt. 314 in Table 3, and Plate 1) parallel observations were made on the disappearance of metachromasia and on the loss of hexosamines; the results showed that the two phenomena had the same pH dependence.

The total quantity of amino sugars liberated during a 4 hr. incubation period at pH 3 was no

EXPLANATION OF PLATE 1

Sections of limb-bone rudiments from 9-day chick embryos, incubated at 37° in buffers of different pH values after 1 hr. in water at 4°. The sections are stained with toluidine blue; note complete loss of metachromatic staining of the cartilage matrix at pH 3 and 4 but little effect on the metachromasia at pH 1, 2, 6, 7 and 8.



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(*Facing p.* 502)

greater than the amount removed during 2 hr. This was at first thought to indicate that a specific polysaccharide fraction was completely liberated within 2 hr. Analyses showed, however, that the pH 3 buffer contained both glucosamine and galactosamine after incubation for 2 hr.; in the buffer, glucosamine represented 19 % of the total hexosamine present and in the residual bone rudiments it accounted for 12 % of the total. Since these proportions were not very different it seemed more likely that, if an enzyme caused the observed effects, denaturation of the enzyme at pH 3 was responsible for cessation of liberation of hexosamine within 2 hr.

Loss of high-molecular-weight polysaccharide. In the above-described experiments, loss of hexosamine from the rudiments was estimated after acid hydrolysis. To find whether the polysaccharide liberated at pH 4 after hypo-osmotic treatment of the rudiments was of high molecular weight or

Table 1. *Effect of papain on the hexosamine content of* 8-*day chick-limb-bone rudiments*

Bone rudiments were incubated at 35° for 2 hr. in Tyrode's solution containing 20 μg. of activated papain/ml.

Tissue	Fraction	Hexosamine content (μg.)	Wet. wt. of tissue after treatment (mg.)
Papain-treated (cartilage from one side of two embryos)	Tyrode's solution containing papain	230	—
	Insoluble bone residue	Trace	26
Control (cartilage from other side of two embryos)	Tyrode's solution	Nil	—
	Insoluble bone residue	262	48

Table 2. *Effect of hypo-osmotic treatment on metachromasia from cartilage matrix of* 9-*day rudiments*

The degree to which the various treatments affected the metachromatic staining of the cartilage matrix is roughly indicated by the following code: ? +, possibly a slight effect; +, metachromasia largely gone from the cartilage immediately beneath the articular surface; + +, general loss from the epiphyses; + + +, loss from epiphyses + proliferative zones of flattened cells; + + + +, almost complete disappearance of metachromasia throughout rudiment; + + + + +, complete disappearance.

Treatment	Temperature	Length of treatment (hr.)	Bone rudiment	Effect on metachromasia
Distilled water	4°	3	Femur	Nil
			Femur	+
	4	6	Femur	Nil
			Humerus	Nil
	37	3	Tibia	+ + +
			Humerus	+ +
	37	6	Femur	+ + + +
			Tibia	+ + + +
30 % KCl containing 1 % of K_2CO_3	37	3	Tibia	Nil
			Humerus	Nil

Table 3. *Effect of* pH *on loss of metachromasia from cartilage matrix of* 9-*day rudiments*

All rudiments were pretreated for 1 hr. with distilled water at 4°, then incubated in buffer at 37·0° for 2 hr. For the meaning of +, etc., see Table 2.

	Expt. 292		Expt. 314	
pH	Bone	Effect on metachromasia	Bone	Effect on metachromasia
1·1	Femur	Nil	Tibia	+
2·0	Femur	Nil	Femur	+
3·1	Femur	–	Femur	+ + + + +
3·1	Metatarsal	+ + +	–	–
4·0	Femur	+ + + +	Tibia	+ + + + +
5·0	Femur	+ +	Tibia	+ + + +
6·0	Femur	+	Tibia	? +
7·0	Femur	Nil	Femur	? +
8·0	Femur	Nil	Tibia	? +

composed of degraded smaller molecules, the buffer extracts were examined by the Di Ferrante (1956) method at the end of the 2 hr. incubation period. Expressed as chondroitin sulphate, the amount of acid polysaccharide was very similar to that expected from the estimation of amino sugar. Treatment of the buffer extract with testicular hyaluronidase for 15 min. at 37° caused a 32% drop in turbidity, and after longer incubation the turbidity disappeared completely.

The turbidimetric method was also used to follow the rate of release of polysaccharide from the bone rudiments in buffer at pH 4 (Fig. 2). It is interesting to compare the rate of a release with the rate of loss of metachromatic staining. After 10 min. metachromasia had largely gone from the epiphyses; after 20 min. it had disappeared from the proliferative zones also and after 50 min. the matrix was unstained throughout the rudiment; less than a quarter of the polysaccharide was extracted during this period. This result is similar to the observation mentioned in a previous section, namely, that after 2 hr. incubation at pH 4, only 50% of the hexosamine had been removed although metachromasia had completely disappeared.

Loss of protein. Table 4 shows that there is maximum release of ninhydrin-positive material into the buffer at pH 4–5. At pH 7, 12·8% of the total ninhydrin-positive material in the rudiment was extracted and 21·2% at pH 5; this contrasts with 4% of the amino sugars removed at neutrality and 53% at pH 5 (Fig. 1).

In a further experiment rudiments were again treated with water at 4° and then incubated at 37° for 2 hr. in 0·1 M-buffers of differing pH. The trichloroacetic acid-soluble material in the buffers was estimated with ninhydrin after acid hydrolysis; similar estimations were made on three fractions from the rudiments: material soluble in cold 5% trichloroacetic acid, that soluble in hot 5% trichloroacetic acid, and the acid-insoluble fraction.

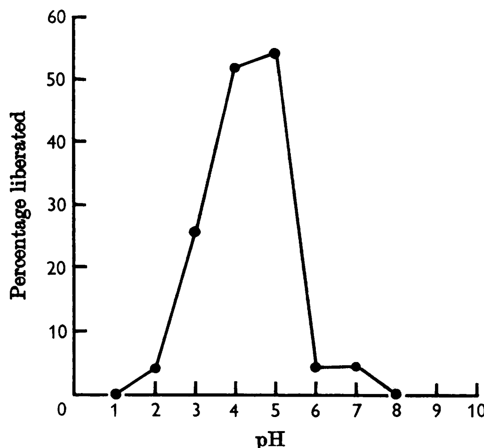

Fig. 1. Liberation of hexosamine from bone rudiments. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1 M-sodium acetate buffers of different pH values for 2 hr. at 37°. The hexosamine in the buffer and that remaining in the rudiments was estimated as indicated in the text.

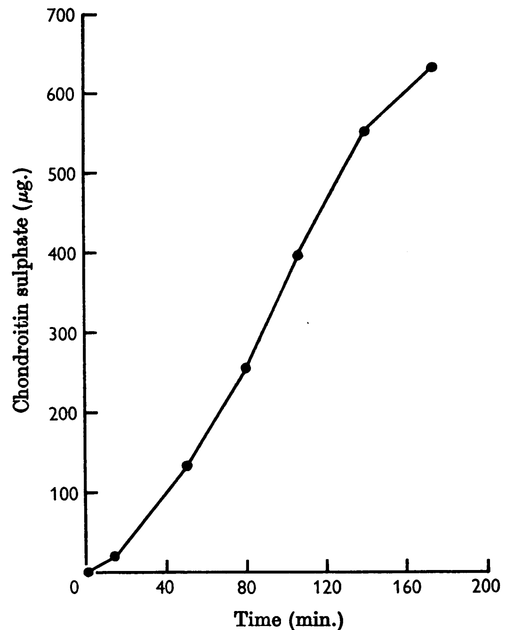

Fig. 2. Rate of polysaccharide release from bone rudiments. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1 M-sodium acetate buffer, pH 4·0, at 37°. The polysaccharide was measured turbidimetrically (in the buffer) by the addition of cetyltrimethylammonium bromide dissolved in 2% NaOH. Results are expressed in µg. of chondroitin sulphate.

Table 4. *Effect of* pH *upon the loss of ninhydrin-positive material from bone rudiments*

After the standard pretreatment with water the rudiments were incubated in acetate buffers of different pH values. The ninhydrin-positive material in the buffer and that remaining in the rudiments was hydrolysed as described in the text. Results are expressed as µg. of leucine equivalents.

	Ninhydrin-positive material		
pH	In buffer (µg.)	In rudiments (µg.)	Percentage extracted
1·1	95·1	405	19·0
2·0	83·5	503	14·2
3·1	110·0	497	18·1
4·0	131·0	524	20·0
5·0	113·0	418	21·2
6·0	104·0	529	16·4
7·0	65·0	442	12·8
8·0	88·8	553	13·8

It has been shown by Fitch, Harkness & Harkness (1955) that extraction of tissues with hot trichloroacetic acid removes collagen almost completely; it has been confirmed in this laboratory that the hot trichloroacetic acid extracts contain most of the hydroxyproline of embryonic chick-limb-bone rudiments (J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb, unpublished observations).

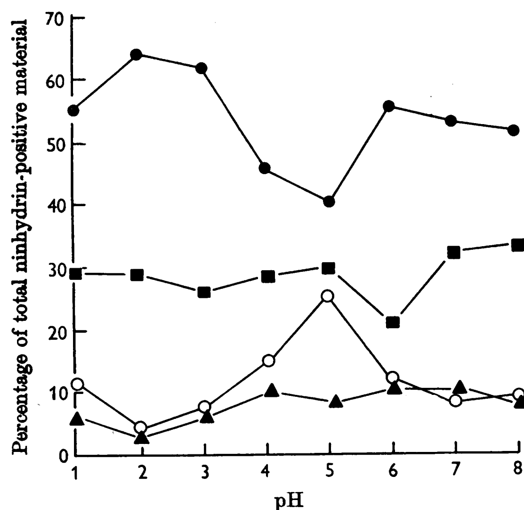

Fig. 3. Distribution of ninhydrin-positive material. Bone rudiments were treated with water at 4° for 1 hr. and then incubated in 0·1 M-sodium acetate buffers of different pH values for 2 hr. at 37°. The rudiments were washed and extracted first with cold 5% trichloroacetic acid and then with hot 5% acid. The ninhydrin-positive material was estimated, after hydrolysis, in these two fractions, in the acid-insoluble material and also in the acid-soluble material in the buffer. ●, Trichloroacetic acid-insoluble; ○, buffer extract (acid-soluble); ■, hot-acid-soluble; ▲, cold-acid-soluble.

In the present experiments there was significant proteolysis in the region pH 4–5. At these pH values, corresponding to those at which polysaccharide is lost, there was a reduction in the content of trichloroacetic acid-insoluble protein of the treated rudiments and an increase in the trichloroacetic acid-soluble material that was liberated into the buffer solution (Fig. 3). By calculation from the data of experiments described above, it was found that hexosamine liberated into the buffer at pH 5 could not account for more than approximately 15% of the ninhydrin-positive material in the buffer; even less hexosamine was probably present, owing to the destruction of amino sugars that occurs during hydrolysis in 6 N-HCl at 110° for 24 hr.

In a subsequent experiment, bone rudiments were incubated at pH 4 for 2 hr., after the usual hypo-osmotic pretreatment. Although the amino acid-containing material liberated into the buffer was soluble in cold 5% trichloroacetic acid, more than 80% of the ninhydrin-positive material (estimated after 24 hr. acid hydrolysis) was non-dialysable. It was thought that either the molecular weight of the material was too low for it to be precipitated by trichloroacetic acid but large enough for the material to be non-diffusible or that the material was attached to a relatively large proportion of non-diffusible polysaccharide. Amino acid analysis of the non-dialysable protein fraction showed that it was unlikely to have been derived from collagen since it contained a low proportion of glycine and very little hydroxyproline; the analysis supported the observations described above, which indicated that the protein component liberated into the buffer was derived from the non-collagenous protein of the rudiments. The partial amino acid analysis of the non-dialysable fraction liberated into the pH 4 buffer is shown in Table 5, in comparison

Table 5. *Partial amino acid composition of non-dialysable material liberated from rudiments at* pH 4

Isolation and analysis of this material was as described in the text. Figures in column 3 are the unpublished results of J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb.

Amino acid	Non-dialysable fraction in buffer, pH 4: g. of N/100 g. of α-amino N	Non-dialysable fraction in buffer, pH 4: Molar ratios (glycine = 1·00)	Non-collagenous protein from 7-day chick tibia (molar ratios, glycine = 1·00)	Human-bone collagen (molar ratios, glycine = 1·00 Eastoe, 1955)
Glycine	6·75	1·00	1·00	1·00
Hydroxyproline	0·29	0·04	0·004	0·31
Aspartic acid	4·60	0·68	1·21	0·15
Threonine	5·02	0·74	0·67	0·06
Serine	6·33	0·94	0·77	0·11
Glutamic acid	5·07	0·75	1·62	0·23
Proline	6·58	0·97	0·74	0·39
Alanine	5·41	0·80	0·98	0·36
Valine	3·19	0·47	0·63	0·07
Methionine	0·61	0·09	0·21	0·02
Isoleucine	1·80	0·27	0·54	0·04
Leucine	2·94	0·44	0·97	0·08

with the composition of the protein that is insoluble in hot trichloroacetic acid obtained from the tibia of the 7-day embryonic chick (J. D. Biggers, K. Lawson, J. A. Lucy & M. Webb, unpublished observations); the non-dialysable fraction had more serine and proline and much less aspartic acid, glutamic acid and leucine.

The dialysable fraction obtained in this experiment was chromatographed on paper after acid hydrolysis. Although all the ninhydrin-staining components were not identified, glutamic acid and aspartic acid were present, and glycine appeared to be absent. There was no evidence of a large quantity of hydroxyproline in the diffusible fraction.

Loss of weight. Loss of wet weight (Fig. 4) by the rudiments, during incubation in buffers of different pH, was determined at the same time as measurements were made of the protein fractions obtained by the use of trichloroacetic acid (Fig. 3). As shown in Fig. 4, the optimum pH for loss of wet weight was similar to that for hexosamines, but there was also an apparently non-specific loss of weight at very low pH values.

Proteolytic activity of the aqueous extract. Since the above-described experiments indicated that the rudiments probably contained an enzyme or enzymes that were released by hypo-osmotic treatment, an attempt was made to obtain these enzymes in soluble form.

It was found that the initial distilled-water treatment for 1 hr. at 4° released an active protease into the water. This had a pH optimum against haemoglobin of between 2 and 3 (Fig. 5); heating the extract to 100° for a few seconds resulted in a complete loss of activity at pH 4 with haemoglobin. Homogenization of the bone rudiments in water at 4° gave a much more active extract; with this method it is possible to measure the activity released from bone rudiments weighing only a few milligrams.

Proteolytic activity after homogenization in iso-osmotic sucrose. Though the proteolytic activity was easily released by hypo-osmotic treatment of the rudiments, homogenization in iso-osmotic sucrose released little. Fractionation of the sucrose homogenate by centrifuging, followed by homogenization of the fractions in water, showed that 51 % of the activity against haemoglobin at pH 4 was contained in the 'mitochondrial' fraction, 33 % in the 'nuclear' fraction and 16 % was in the supernatant. The pH optimum of all three fractions was about pH 3. It would appear that the proteolytic enzyme or enzymes of cartilage with a low pH optimum are mainly in an inactive particulate form, but are easily activated by hypo-osmotic treatment.

DISCUSSION

It has been demonstrated that treatment of chick-bone rudiments with distilled water, followed by incubation at pH 4–5, produces rapid changes in both the histological appearance and the chemical composition of the tissue. During incubation for 2 hr. at pH 4, the metachromatic staining properties of the cartilage matrix are greatly reduced,

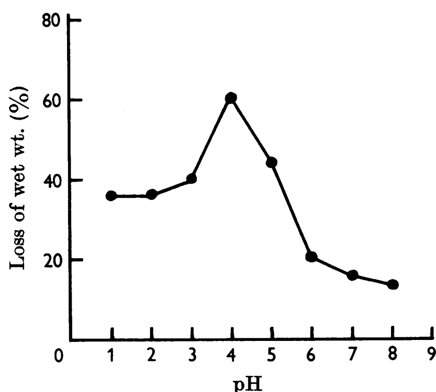

Fig. 4. Loss of wet wt. Weighed bone rudiments were treated with water for 1 hr. at 4° and then incubated at 37° for 2 hr. in 0·1 M-sodium acetate buffers of different pH values. At the end of this period the rudiments were removed, blotted dry on filter paper and reweighed.

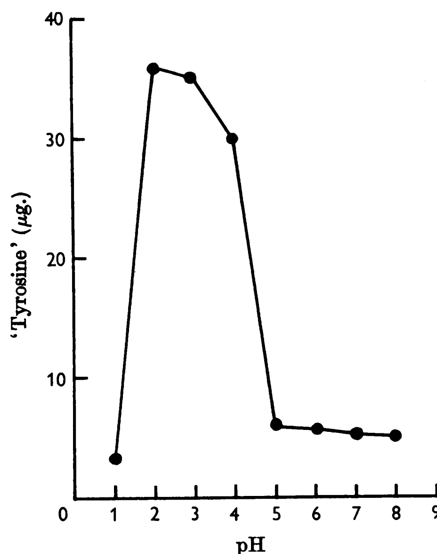

Fig. 5. Optimum pH for enzyme in the aqueous extract of bone rudiments. Bone rudiments from 16 eggs were treated with water at 4° for 1 hr.; after removal of the rudiments the solution was centrifuged and the proteolytic activity measured with 2 % haemoglobin as substrate in 0·1 M-sodium acetate buffers of different pH values. Activity is expressed as μg. of acid-soluble 'tyrosine'.

and half of the hexosamine content of the rudiments is removed. That this effect is not peculiar to chick cartilage has been demonstrated in experiments, to be reported elsewhere, on late foetal-mouse bones pretreated with distilled water and then incubated in buffers of different pH; in the terminal cartilage of these bones, metachromasia was completely removed at pH 3 and 4 but was almost unchanged at pH 2 or 6.

In the chick rudiments the loss of amino sugars occurs primarily by removal of polysaccharide of high molecular weight, whereas acid-soluble products are lost into the buffer from the protein constituents of the tissue. The strong pH-dependence indicates enzymic degradation of the tissue, since it is unlikely that a chemical-extraction process would be so sharply dependent upon pH. The much more rapid loss of metachromasia in distilled water at 37° than at 4° also suggests an enzymic mechanism. Einbinder & Schubert (1950) state that enzyme action might have been responsible for the fact that only 11% of the chondroitin sulphate was extracted by potassium chloride solution from fresh wet cartilage, but 72% after storage of the wet cartilage at 0° for 4–6 weeks.

Both papain (Bryant *et al.* 1958) and plasmin (Lack & Rogers, 1958) remove polymeric polysaccharide from cartilage. In the present experiments, protein was degraded at pH 4 and 5 after hypo-osmotic treatment of the chick rudiments and it seems possible that proteolytic action was the primary cause of the loss of polymeric polysaccharide. This hypothesis is supported by the demonstration that treatment of the rudiments with water causes the release of a proteolytic enzyme, with an acid pH optimum, that is active against haemoglobin.

Although components soluble in trichloroacetic acid that were ninhydrin-positive were released from the rudiments at pH 4, more than 80% of this material was non-dialysable. The amino acid composition of the non-dialysable material indicates that it was probably derived from the non-collagenous protein of the rudiments rather than from collagen. This evidence, combined with the observed breakdown at pH 4 and 5 of the protein of the rudiments insoluble in trichloroacetic acid, shows that maximum liberation of polysaccharide was associated with breakdown of the non-collagenous protein of the tissue. It is thought that these observations may be related to those of Shatton & Schubert (1954), Partridge & Davis (1958) and Muir (1958), who found a polysaccharide–protein complex in mammalian cartilage, which was composed of a non-collagenous protein bound to chondroitin sulphate. Mathews (1956) also found a protein, in chondroitin sulphate preparations, that had a hydroxyproline content of only 0·1%; proteolytic enzymes have been shown to degrade these mucoproteins of cartilage, with a consequent loss of viscosity (Shatton & Schubert, 1954; Muir, 1958).

The amino acid-containing material liberated from the rudiments at pH 4, that was non-dialysable, differed significantly in amino acid composition from the non-collagenous protein of whole chick-limb-bone rudiments in having considerably less glutamic acid, aspartic acid and leucine, and more serine and proline relative to glycine (Table 5). On the other hand, the diffusible fraction contained glutamic acid and aspartic acid but apparently no glycine. One possible interpretation of these observations is that the degradation of the non-collagenous protein is the result of enzyme action at the linkage between glycine and glutamic acid. The non-dialysable fraction may be associated in some manner with polysaccharide in the cartilage, since at pH 4 the two components are liberated simultaneously from the bone rudiments. Further study of this system may throw light on the mode of linkage of protein and polysaccharide in cartilage, and also on the enzymic mechanism by which the polysaccharide may be liberated. More serine is present relative to glycine in the non-dialysable component than in the non-collagenous protein of whole rudiments; Muir (1958) has reported that serine was the only amino acid that was not greatly decreased by papain digestion of the polysaccharide–protein complex obtained from pig tracheal cartilage.

Gianetto & de Duve (1955) found that the cathepsin activity of rat liver is contained within cytoplasmic granules and that full cathepsin activity occurs when the enzyme is released by exposure of the lysosomes to hypo-osmotic media (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). A possible interpretation of the experiments described in this paper is that the proteolytic enzyme liberated from the cartilage cells by treatment with distilled water subsequently attacks the protein to which the polysaccharide of the cartilage is attached. The enzyme concerned may be a cathepsin, and in connexion with this suggestion, Press, Porter & Cebra (1960) have recently isolated a proteolytic enzyme from bovine spleen which has been named cathepsin D since it will not hydrolyse any of the typical substrates of cathepsins A, B and C. Cathepsin D has a pH optimum at 4·2 with denatured albumin, and at pH 3 with denatured haemoglobin; it does not require cysteine for activation.

These studies were undertaken to see if the cartilaginous limb-bone rudiments of the chick possess enzymes that are capable of producing effects similar to those observed when the rudiments are cultivated in a medium containing

excess of vitamin A. The results described show that the limb cartilage contains a proteolytic enzyme that may release polysaccharide by degradation of structural protein. The reduction of metachromatic staining and loss of hexosamines from the cartilage matrix as a result of incubation in the buffer solutions are similar to the changes observed in explants exposed to excess of vitamin A. It is probable that chondroitin sulphate is the principal polysaccharide of the cartilaginous bone rudiments and that it is freed under the conditions used, since galactosamine comprises 80% of the hexosamine liberated at pH 3. Glucosamine also is liberated at pH 3 and more than one polysaccharide must therefore be lost from the rudiments since chondroitin sulphate contains little or no glucosamine. Hypo-osmotic treatment of cartilage cells may release a number of enzymes, each of which can degrade a particular component of cartilage matrix.

Chick-limb-bone rudiments grown in culture in the presence of excess of vitamin A produce more lactic acid than the corresponding rudiments in control cultures (Dingle *et al.* 1961) and this increased acidity in the vitamin A-treated cartilage would favour degradation of the matrix, since this is much more rapid at pH 5 than at pH 7.

The results described in this paper provide some additional support both for the hypothesis (Fell & Thomas, 1960) that vitamin A acts on cartilage matrix by enhancing the enzymic activity of the chondrocytes and also for the suggestion (Dingle, 1961) that the vitamin may increase proteolytic activity by altering the permeability of the lysosomes. Further investigations are needed, however, to isolate and characterize the enzyme or enzymes responsible for the changes in the rudiments at pH 4, and to find whether these changes are in fact directly related to those produced by excess of vitamin A.

SUMMARY

Experiments were made to find whether normal cartilage contains an enzyme that is capable of producing an effect on the matrix like that of vitamin A.

2. Normal cartilaginous rudiments were pretreated with distilled water to disrupt the cells and their organelles and then incubated in buffer solutions of pH 1–8. Between pH 3 and 5 the metachromatic staining of the matrix was greatly reduced, half the hexosamine content was lost (mostly as polysaccharide of high molecular weight) and protein components were liberated into the buffer. Above and below pH 3–5 there was little effect on the cartilage matrix.

3. An extract having proteolytic activity at a pH optimum of 3 has been obtained from cartilage. The proteolytic activity is located in cytoplasmic particles, from which it can be liberated by water; it is destroyed by heating at 100°.

4. It is concluded that normal chondrocytes contain an enzyme or a group of enzymes capable of producing an effect on cartilage matrix that closely resembles that produced by excess of vitamin A.

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