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Isolation of Mucopolysaccharide from the Precartilaginous Embryonic Chick Limb Bud.* (30072)

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During an investigation of cartilage differentiation in the embryonic chick limb bud, embryos from stage 17 to stage 26(1) were injected with radioactive sulfate and the pattern of sulfate uptake in their limb buds examined by autoradiography(2). An uptake of radioactive sulfate was observed at every stage. An uptake of radioactive sulfate has been reported in the chick embryo as early as stage 4 (primitive streak)(3). The first histological evidence of cartilage in the limb bud of the embryonic chick is found at stage 25(4). An uptake of radioactive sulfate well before the first histological evidence of cartilage has been observed in other vertebrate embryos(5,6,7). It has been proposed that an uptake of sulfate before the first histological evidence of cartilage might be due to compounds other than chondroitin sulfate, perhaps to a chondroitin sulfate precursor(6).

The pattern of radioactive sulfate uptake observed in the autoradiographs and the changes of this pattern with embryonic development suggested that the uptake might be concerned with the differentiation of limb bud cartilage. Before the uptake of radioactive sulfate could be used to study the differentiation of limb bud cartilage, some knowledge had to be gained about the chemical nature of the material to which the incorporated radioactive sulfate was bound.

While this work was in progress, Franco-Browder, De Rydt, and Dorfman reported the presence of chondroitin sulfate A and/or C in the chick embryo at stage 11 to 12 and of the same material in the embryonic chick limb bud at stage 22 to 23(8). This paper verifies and extends that report.

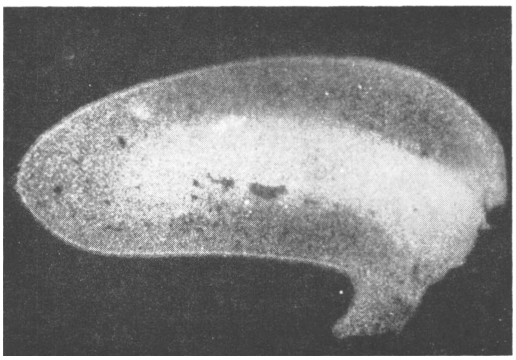

FIG. 1. Stage 23 limb bud which has been allowed to accumulate radioactive sulfate for 5 hr. Photographed in darkfield at 54×. Length of limb is 1.15 mm.

Materials and methods. Fertilized White Leghorn or White Cornish Cross† eggs were incubated for 3½ days at 37.5°. S-35 sulfate (carrier-free, Oak Ridge National Laboratory) was injected through a window in the shell(9) directly onto the *area vasculosa* as was done for the autoradiographs(2). Most of the eggs were injected with radioactive sulfate during stage 22, just before the differential rates of sulfate uptake in the limb bud mesoderm became established. The eggs were then incubated at 37.5° for 5 hours. During this further incubation the embryos were able to reach a stage at which the differential rates were clearly established (Fig. 1). The limbs were removed from the embryos in Tyrode's solution.

In most of the experiments, the limbs were

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† Brown Cornish male × Arbor Acres White Rock female.

fixed in Bouin's fluid for 18 hours. They were then washed for 4 days in repeated changes of 70% ethanol until all of the picric acid had been removed. These limbs were washed with absolute ethanol, xylene, and again absolute ethanol, remaining in each for 2 hours. The limb buds were left in distilled water overnight. This treatment was designed to insure that the radioactive material which would be extracted would be reasonably similar to the radioactive material observed in the autoradiographs. The limbs were then dispersed by sonication for 10 minutes at 10 kc (Raytheon Manufacturing Co. Model D.F. 101) or by incubation in sodium hydroxide.

In a few experiments, as a control for the effect of the above procedure, limb buds were washed twice with distilled water and dispersed in distilled water by sonication for 10 minutes at 10 kc without prior fixation.

Initial experiments indicated that the radioactive material was similar in its behavior to a mucopolysaccharide, but this could not be further investigated because of the extremely small amounts of available material. Non-radioactive polysaccharide-protein complex was prepared from 12-day chick embryos by the procedure of Malawista and Schubert(10). Since it was extracted from the whole embryo at a stage when cartilage is being synthesized in many areas, it was assumed that it would contain material similar to the radioactive material in the 3-day limb bud. The mucopolysaccharide obtained from 12-day chick embryos could be fractionated and the amount of radioactive material which precipitated with each fraction measured. Crude polysaccharide-protein complex from 12-day chick embryos was added just before the final step in the dissolution of the radioactive limbs.

The calcium salts of the radioactive material were fractionated according to the procedure of Meyer, Davidson, Linker and Hoffman (11) for fractionation of mucopolysaccharides.

Digestion with papain (E.C. 3, 4, 4, 10) was carried out in 0.1 M acetate buffer pH 5.0 containing 0.003 M EDTA, 0.005 M cysteine, and 0.5 mg per ml of papain (2× cryst, Nutritional Biochemicals Corp.). Incubation was at 58° to 63° for 3 hours.

Digestion with hyaluronidase (not listed in I.C. report) was carried out in 0.1 M acetate buffer pH 5.0, 0.035 M sodium chloride and 1.0 mg per ml of hyaluronidase (ovine testicular, 508 units per mg, Sigma Chemical Co.). After incubation at 37° for 18 hours, a further 1.0 mg per ml of hyaluronidase was added. The effect of hyaluronidase was determined after 40 hours of incubation by passing the digest through a Sephadex G-50 column 1 × 8 cm and comparing the elution pattern with the elution pattern of undigested extract on the same column.

Radioactivity was determined by spreading on planchets at infinite thinness and counting in a Nuclear-Chicago gas flow counter. This was impossible with those fractions which were insoluble. These were brought into suspension and aliquots were spread on planchets and counted. The counts obtained can be no more than estimates of the total counts present in these fractions. Such counts are indicated by being enclosed in brackets.

Precipitates were collected by centrifuging in an International clinical model centrifuge at top speed for 40 minutes at 5°. This centrifuge was used in order that all fractionations could be carried out in glass centrifuge tubes.

Experimental. The fractionation obtained after Bouin's fixation is shown in Table I.

TABLE I. Fractionation of Ratioactive Limb Buds After Bouin's Fixation and Sonication.

	Total counts
A. Sonicate	(63,700)
B. Precipitate	(51,100)
C. Supernatant	854
D. Remaining insoluble material after treatment with sodium hydroxide	(8,700)
E. Precipitate after addition of ethanol to 66%	(33,700)
F. Supernatant after addition of ethanol to 66%	4,900
G. Dialyzed fraction E	26,700
H. Remaining insoluble material after digestion with papain	(8,900)
I. Supernatant after digestion with papain	15,500
J. 25% ethanol	2,000
K. 40% "	6,900
L. 50% "	2,100
M. Supernatant	278

After sonication and centrifugation very little radioactivity remained in the supernatant (Table I, line C). The precipitant (which contained all of the cell components which had been "fixed," *i.e.*, protein and those cell components which are bound to protein) was very difficult to dissolve. After incubation in 0.5 M sodium hydroxide for 20 hours at 37°, neutralization with acetic acid, and centrifugation, over 10% of the radioactivity remained in the insoluble fraction (Table I, line D). Addition to the supernatant of calcium acetate to 5.0% and ethanol to 66% yielded a precipitate which contained most of the remaining original radioactivity (Table I, line E).

This precipitate would not dissolve in distilled water, buffers at various pH's or in saline solutions. It was finally dissolved by dialysis against 0.001 M sodium hydroxide after first being dialyzed against 0.001 M EDTA; 0.1 M acetate buffer pH 5.0, to remove the calcium salts. This behavior suggested that the radioactive material was still associated with denatured protein. The extract was digested with papain and then dialyzed for 18 hours against 0.1 M acetate buffer pH 5.0. A precipitate was still present after this time (Table I, line H); but the remaining radioactive material could be fractionated with ethanol. The fractions which were obtained could be dissolved in distilled water or in acetate buffer.

The resistance of the radioactive material to alkaline degradation is not due to the action of Bouin's fluid, since fractions obtained from fresh limbs, dispersed and fractionated in the same manner, behave in the same way. The radioactive material does not precipitate from the sonicate after addition of ethanol to 70%, but does precipitate after addition of potassium acetate to 0.1 M, as would be expected of a polysaccharide-protein complex (10). Unlike a polysaccharide-protein complex, this precipitate will not redissolve in buffers, and is not made soluble by incubation in 0.5 M sodium hydroxide at 37° for 20 hours(10). The radioactive material can be brought into solution only after digestion with papain.

The insolubility of the material obtained after sonicating for 10 minutes at 10 kc had caused sizable losses at each step in the further purification of the material. An attempt was made to recover and purify most of the initial radioactivity (Table II). The limb buds were incubated in Bouin's fluid and washed as in Table I. Crude non-radioactive polysaccharide-protein complex was added and the mixture was suspended in 0.5 M sodium hydroxide and left at 37° for 18 hours. Acetic acid was added to neutralize the sodium hydroxide and the precipitate was centrifuged out. This precipitate was resuspended in 0.25 M sodium hydroxide and left for 24 hours at 37°. The solution was again neutralized with acetic acid and the remaining precipitate was centrifuged out. The supernatants were combined, ethanol was added to 70% and the precipitate which formed was collected by centrifugation (Table II, line B).

TABLE II. Fractionation of Ratioactive Limb Buds After Bouin's Fixation and Extraction with Sodium Hydroxide.

A. Residue after extraction with sodium hydroxide	(7,200)
B. Precipitate after addition of ethanol to combined supernatants	(344,000)
C. Supernatant after addition of ethanol to combined supernatants	27,700
D. Supernatant after treatment with papain	2,000
E. Remaining insoluble material after treatment with papain	(8,200)
F. 25% ethanol	57,400
G. 40% "	143,000
H. 50% "	96,000
I. 70% "	7,000
J. Supernatant	0
Refractionation of:	
F. 25% ethanol	28,700
40% "	23,100
G. 40% "	110,500
50% "	18,300
H. 40% "	30,400
50% "	59,400

The precipitate was resuspended in 0.1 M acetate buffer pH 5.0; 0.002 M EDTA and digested with papain at 56° for 5 hours. Calcium acetate was added to 2.5%, acetic acid to 0.25 M and ethanol to 70%. After centrifugation, almost all of the precipitate was soluble in 5.0% calcium acetate; 0.5 M acetic acid. It was then fractionated by addition of ethanol. In the fractions which precipitated between 0. and 50% ethanol, 77% of the original counts were recovered. Of this,

19% precipitated at 25% ethanol, 49% at 40% ethanol, and 32% at 50% ethanol. Each of these fractions was refractionated under the same conditions. After recombining the fractions which precipitated at the same concentrations, 73% of the original counts were recovered and of these 10.5% precipitated at 25% ethanol, 60% at 40% ethanol, and 29.5% at 50% ethanol.

The sensitivity of each of these fractions to hyaluronidase was tested (Fig. 2). Each of these fractions was excluded by Sephadex G-50 prior to digestion with hyaluronidase. This implies that the molecular weight of each was in excess of 8,000. After treatment with hyaluronidase, only 32% of the fraction which precipitated at 25% ethanol was excluded, less than 10% of the fraction which precipitated at 40% ethanol was excluded, and practically none of the fraction which precipitated at 50% ethanol was excluded. Thus, over 90% of the original extract was sensitive to hyaluronidase.

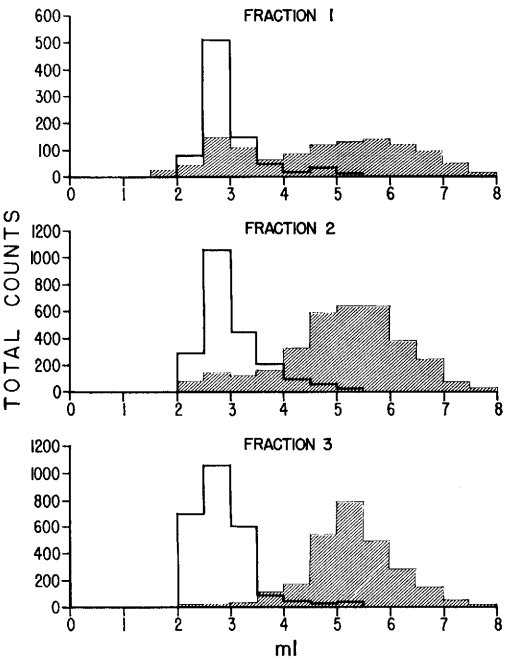

FIG. 2. Total radioactivity of each 0.5 ml fraction from a Sephadex G-50 column. Fraction 1 is the combined 0-25% ethanol fraction; fraction 2, the combined 25-40% ethanol fraction; fraction 3, the combined 40-50% ethanol fraction. Clear area was obtained prior to hyaluronidase digestion. Hatched area was obtained after hyaluronidase digestion.

Discussion. Franco-Browder, De Rydt and Dorfman have isolated a material from the limb buds of stage 22 to 23 chick embryos which has the same electrophoretic mobility as chondroitin sulfate(8). In the experiments reported here, a material has been isolated from the same source which is recognized by the radioactive sulfate bound to it. This material can be obtained in a soluble form only after incubation in 0.5 M sodium hydroxide and digestion with papain. It is excluded by Sephadex G-50, indicating that it has a molecular weight greater than 8,000. Over 90% of this material is sensitive to hyaluronidase. As it was extracted here, it is quite diverse in its properties. If the table of properties compiled by Meyer, Davidson, Linder and Hoffman is used(11), the presence of chondroitin sulfates A, C, and a small amount of B is indicated. It would be desirable to investigate this result through chemical analysis, but sufficient material has not yet been collected for this to be possible. If chondroitin sulfate is being rapidly synthesized in the limbs, one would expect to find low sulfate chondroitin sulfate as well as the fully sulfated forms(12). The properties of the material could also be due to residual material bound to the polymer, since digestion with papain would probably leave polypeptide chains remaining attached to the polysaccharide(13). However, it is clear that the material is some form or forms of chondroitin sulfate.

Franco-Browder, De Rydt, and Dorfman have suggested that induction of cartilage in the somite of the early chick embryo may be concerned with "the relationship of polysaccharide and protein synthesis"(8). They suggest that the synthesis of polysaccharide may be more rapid than the synthesis of protein, and that matrix formation must wait upon the synthesis of sufficient protein. This proposal would not seem to apply to the limb bud. When limb buds are extracted by a procedure similar to that of Malawista and Schubert, the material obtained will not go into solution readily. It is not made easily soluble by incubation at alkaline pH. A soluble material is obtained only after treatment with papain. It is possible, of course, that the protein found in matrix is different

from the protein found bound to the sulfated polymer prior to matrix formation.

The only metachromatic material which has been reported in the limb bud of the 3- to 3½-day chick was found in tiny granules in the golgi of vitally stained tissue(14). At 4½ days, this material had moved into the cytoplasm as a few large purple granules. Fixed material does not seem to show any metachromatic material at this time. This sequence of events conforms with that reported by Godman and Porter for differentiation of cartilage cells in the mouse(15). It is possible that polysaccharide-protein complex production commences very early in embryogenesis and that the product is stored in the golgi in packets so well covered that after fixation dyes cannot penetrate. This material may be released into the extracellular spaces at a much later time, either due to some stimulus or to the presence of some additional component.

Summary. Limb buds which had been allowed to take up radioactive sulfate were extracted and fractionated in the presence of crude non-radioactive polysaccharide-protein complex from 12-day chick embryos. The radioactive material was not soluble and did not become soluble after incubation for 18 hours at 37° in sodium hydroxide but did become readily soluble after digestion with papain. After treatment with sodium hydroxide and papain, the radioactive material was excluded by Sephadex G-50, but after treatment with hyaluronidase, the material was held up on the same column. Fractionation of the calcium salts of the radioactive material suggested the presence of chondroitin sulfates A, C, and a small amount of B.

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Absence of Antibody Plaque Forming Cells in Spleens of Thymectomized Mice Immunized with Sheep Erythrocytes.* (30073)

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Recent investigations in several laboratories have demonstrated that the thymus gland plays an important role in development of immunologic mechanisms(1-9). Removal of the thymus in early life, prior to maturation of immunologic competence, is associated with major disturbances in immune functions in experimental animals. Thymectomy at or near birth generally results in impairment of the ability to reject skin or tumor homografts(1-3). Early postnatal thymectomy of experimental animals also impedes or prevents development of delayed skin reactivity to serum protein antigens or

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