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Received July 1, 1965. P.S.E.B.M., 1965, v120.

Rat Epiphyseal Cartilage: II. Sulfate-35 Incorporation into Proteinpolysaccharide, *in vitro*.* (30570)

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The isolation of a crude proteinpolysaccharide (PP) from epiphyseal cartilage in relatively large yields has been reported(1). This communication reports our observations on the incorporation of media radiosulfate into the crude PP of rat epiphyseal cartilage employing standard *in vitro* technique.

Methods and materials. Animal and tissue preparation. Male weanling rats, 21 days old (40-45 g) of the Sprague-Dawley strain (Charles River Labs.) were fed on Purina chow and water *ad libitum* until experimentation. The animals were anesthetized and killed by cervical dislocation and the lower extremities rapidly dissected free. The proximal tibial and distal femoral epiphyses after exposure were separated from their shafts by a slice through the metaphyseal junction of the epiphyseal plate. The epiphyses were further cleaned of adherent tissue and 3 longitudinal slices were made producing 4 segments approximately 0.5 to 1.0 mm in thickness. The slices were kept on an iced petri dish until all dissections were completed, and then placed in tared 25 cc incubation flasks containing 3.0 cc of incubation media. The tissue was proportioned in essentially 2 designs so that the tissue from one animal was present in each flask or the tissue of one half of one animal was present in each of two paired flasks. All incubations were begun less than 30 minutes after the animals were killed. Usually 6 to 12 animals were used in each experiment. They were 22 to 25 days old.

Media and incubation. Krebs-Ringer bicarbonate (KRB) was used in all experiments containing one-half the suggested calcium concentration and fortified with glucose at a concentration of 100 mg% unless otherwise specified. The total volume in each flask was 3.0 cc and contained 3 microcuries of $Na_2S^{35}O_4$ per flask (18.2 curies/mole-specific activity New England Nuclear Co.). After the cartilage was added to the tared flasks the wet weight of the cartilage segments was determined. The flasks were then covered with serum stoppers and incubated at 37°C in a metabolic shaker (70-75 cycles/minute) following 10 minutes of aeration with 95% O_2, 5% CO_2 mixture.

Isolation of proteinpolysaccharide (PP). The cartilage segments were rapidly removed from the media, washed with 100 cc of distilled water and quickly frozen. Preliminary attempts to stop the reaction by addition of

* These studies were supported in part by grants-in-aid from Nat. Inst. of Arthritis and Metab. (AM-03967) and Funds for Research and Teaching, Dept. of Nutrition, Harvard School of Public Health.

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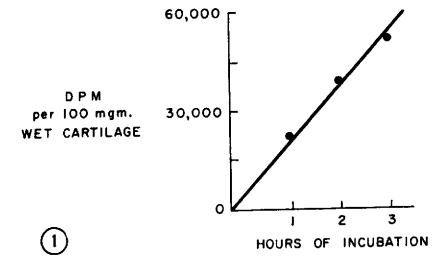
acid led to the formation of PP-collagen complexes not easily separated from the matrix. The cartilage segments were then homogenized within 48 hours or dried in acetone and stored. Proteinpolysaccharide was isolated by the method of Schubert(2) and Bollet(3) as previously described(1). The crude PP isolated was reprecipitated to constant specific activity and constant protein to polysaccharide (uronic acid) ratio (3 times) and the final material was dissolved in a suitable volume of 0.5N sodium hydroxide.

Analytic methods and radioactivity measurements. PP protein was determined by the Lowry method(4) with albumin as the standard and PP uronic acid (U.A.) by the method of Dische(5). Glucose was determined by the method of Nelson and Somogyi(6) and lactate by the method of Barker and Summerson(7). Radioactivity measurements were made in the liquid scintillation system of Bray(8) with an average total efficiency of 35%. Each sample was corrected for quenching by addition of an internal standard.

Results. Initial experiments indicated not only rapid incorporation of labeled media sulfate into the sulfated glycosaminoglycans isolated as described, but very little incorporation into the glycosaminoglycans remaining in the residual matrix (isolated by papain digestion, 0.2 N extraction, and cetylpyridinium chloride precipitation). These newly synthesized or sulfated glycosaminoglycan complexes were also found to be easily extractable since homogenation periods of less than 30 minutes led to no significant changes in total amount of radioactivity recovered in the reprecipitated PP. Since almost all of the radioactivity (average 90%) incorporated was observed in this PP fraction, the data are expressed in total radioactivity recovered in the isolated PP along with its specific activity (DPM/μg PP U.A.). Since PP is composed of 2 moieties, protein and polysaccharide, the protein moiety is presented as micrograms of albumin equivalents and the polysaccharide in micrograms (μg U.A. $\times$ 3.25‡). The total of these 2 figures agreed closely with the dry weight of this material and the expression of

‡ Based on the 30.8% U.A. concentration of potassium chondroitin sulfate ($C_{14}H_{19}NSO_{14}K_2$).



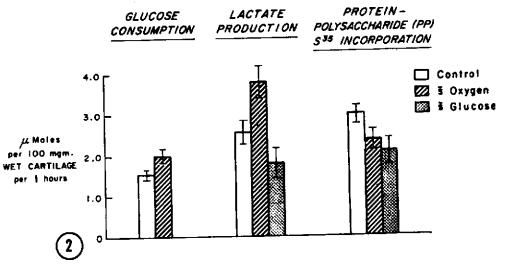


FIG. 1. Cartilage segments were incubated as described for increasing periods of time. Each point represents mean of 3 flasks.

FIG. 2. Cartilage segments from 5 animals were divided equally between the same number of paired flasks. In exp. 1, the effect of anaerobism was evaluated by substitution of 95% N_2-5% CO_2 gas during aeration. In exp. 2, glucose was omitted from incubation media in the experimental flasks. Control data are presented in a composite fashion and PP S^{35} incorporation is expressed in micromoles of media sulfate incorporated $\times$ 300 for convenience.

P values observed:

Exp. 1. Glucose consumption, $<.01$; Lactate production, $<.001$; PP S^{35} incorporation, $<.01$.

Exp. 2. Lactate production, $<.0005$; PP S^{35} incorporation, $<.001$.

the polysaccharide/PP weight ratio had a similar relation to the per cent of polysaccharide in this complex as calculated from the molar ratio of nitrogen/hexosamine(1).

As demonstrated in Fig. 1 the rate of radiosulfate incorporation was linear within the 2-hour incubation time employed. Although the results are expressed in DPM incorporated/100 mg wet cartilage, an equally linear relation was obtained when DPM/μg PP U.A./100 mg wet cartilage was employed.

The results obtained on different groups of animals of ages ranging in individual groups from 22 to 25 days old are presented in Table I. Total radioactivity incorporated into the PP fraction remained relatively constant with approximately a 5% S.D. within individual flasks in any one experiment and less than a 10% S.D. between different groups

TABLE I. Epiphyseal Segment from Individual Animals of 4 Different Ages Was Incubated as Described. The U.A. content and total radioactivity of the isolated PP are expressed per 100 mg of wet cartilage incubated.

		Proteinpolysaccharide		
Age, days	No. of flasks	Uronic acid, mg	Specific activity, DPM /μg U.A.	Total radioactivity, DPM × 10^4
22	3	.385	100.0	3.85 ± .09
23	5	.376	94.3	3.54 ± .19
24	5	.334	107.5	3.59 ± .39
25	5	.319	116.1	3.70 ± .14

and ages. While this parameter remained rather constant, analyses of the isolated PP revealed significant changes in composition (Table II). When compared with the body weight and cartilage wet weight, a progressive decrease in polysaccharide, protein and PP was associated with growth. The decrease in the polysaccharide fraction of PP was proportionally greater and resulted in a decreasing polysaccharide/PP weight ratio. Comparison of the same ratio in the PP isolated from the same group of animals on successive days led to similar changes which were also statistically significant in 2 individual experiments ($p<0.01$).

Since the specific activity of the media sulfate is known, superficial calculation allows for an estimate of the glycosaminoglycan synthesis or sulfation occurring within the period of incubation (Table III)(9,10). Also included in the latter table are the results obtained on glucose consumption and lactate production.

Absence of both media glucose and atmospheric oxygen (nitrogen-CO_2 aeration mixture) produced significant changes in PP sulfate incorporation (Fig. 2). Both conditions were associated with depressed sulfate incorporation into the isolated PP of 30% and 15% respectively. Analysis of the PP revealed no significant qualitative changes. Anaerobism increased lactate production (90%) and increased glucose consumption (28%) which would appear to indicate the presence of the Pasteur effect. Lactate production in the absence of added glucose averaged 1.21 μmoles/100 mg wet cartilage and suggests an endogenous source of glucose.

That varying ionic concentration modifies metabolism *in vitro* is well known, and an attempt to evaluate the effects of altered calcium and magnesium on these parameters was conducted, especially since these two ions were observed to alter the calcification process in rachitic rat cartilage(11). As Table IV demonstrates, an increased calcium concentration was associated with a decrease in sulfation while lowering the magnesium concentration increased the same process. A calcium concentration of 2.5 mmoles also depressed glucose consumption (13%) and lactate production (9%). Decreasing the ionic concentration of magnesium in comparison

TABLE III. Data on Metabolism of Rat Epiphyseal Cartilage Incubated *in vitro*. Results are derived from 5 experiments containing 3 to 5 flasks and values are expressed in terms of 100 mg wet cartilage incubated.

Parameter	
Glucose consumption, μM/hr	1.50 ± .14
Lactate production, μM/hr	2.50 ± .40
Sulfate incorporation	
Media specific activity, DPM/μM	1.85 × 10^6
PP total radioactivity, DPM	1.92 × 10^4
PP sulfate incorporation, μM/hr	1.04 ± .09 × 10^{-2}

TABLE II. The PP Isolated from the Incubated Cartilage of Different Animals Was Analyzed for U.A. and Protein. Data are expressed in terms of total polysaccharide (U.A. × 3.25) and protein isolated per 100 mg wet cartilage and the polysaccharide to PP (mg polysaccharide + mg protein) ratio. The results of 23 such analyses have been grouped into 3 on the basis of age (A = 21-22, B = 23-24, and C = 25 days old).

			Proteinpolysaccharide		
Group	Body wt, g	Cartilage wet wt, mg	Polysaccharide, mg	Protein, mg	Polysaccharide/PP ratio
A	46.5 ± .8	186 ± 16	1.626 ± .187	1.086 ± .105	.602
B	51.7 ± 2.3	220 ± 20	1.155 ± .119*	.835 ± .111*	.583
C	56.1 ± 4.1	263 ± 20	1.045 ± .079†	.811 ± .034‡	.561

* A *vs* B, $P < .01$. † A *vs* C, $P < .01$; B *vs* C, $P < .10$. ‡ A *vs* C, $P < .01$; B *vs* C, $P > .10$.

TABLE IV. Cartilage Segments from Each of 5 Animals Were Divided Equally Between the Same Number of Paired Flasks. Control flasks contained KRB prepared as described. Experimental flasks contained KRB with altered concentrations of calcium or magnesium. Data are derived from 2 experiments testing each alteration individually.

		KRB ion concentration, mM	Glucose consumption, μM/hr	Lactate production, μM/hr	Sulfate incorporation, μM/hr $\times 10^{-2}$
Calcium	Control	1.25	1.46 ± .15	2.41 ± .13	.96 ± .05
	Experimental	2.50	1.27 ± .22	2.20 ± .13	.79 ± .04*
Magnesium	Control	1.30	1.63 ± .50	2.66 ± .22	1.21 ± .05
	Experimental	.65	2.23 ± .37	3.03 ± .37	1.36 ± .06†

* Statistically significant, $P < .05$.

† Statistically significant, $P < .02$.

was associated with a 37% increase in glucose consumption and a 14% increase in lactate produetion.

Discussion. Previous publications have demonstrated that incorporation of radiosulfate into organic matrix is synonomous with glycosaminoglycan synthesis or sulfation(12) and, as revealed by radioautography, the majority of this radioactivity appears in proximity to the zone of provisional calcification in the epiphyseal plate and secondary ossification center(13,14). Previous evidence also suggests that this label is rapidly incorporated into newly synthesized complexes and extruded into the matrix within 2 hours, if not before(15,16). The mechanisms suggested previously may be assumed to occur *in vitro* and it would appear that media sulfate is being incorporated into these newly synthesized structures. Also suggested from this data is a relatively constant rate of production within this age group. The ability to recover the labeled material with this homogenation technique may be indicative of a more soluble form of newly synthesized material which has not become bound to the collagenous matrix. The high rate of incorporation of radiosulfate into glycosaminoglycans of epiphyseal cartilage in comparison to similar estimates of incorporation in other tissue is probably due to the rapid growth of epiphyseal cartilage and is consistent with the embryonic character of this tissue. The changes observed in the polysaccharide/PP ratio with age and growth of this tissue are consistent with our previous observations(1). No further conclusions can be drawn from these confirmatory findings although most recent evidence suggests that the difference observed in this ratio may be attributed to a decrease in the glycosaminoglycans extracted by this method.

The depression of PP synthesis under anaerobic conditions and by the omission of glucose is in agreement with the known oxidative requirements necessary for the generation of energy sources and nucleotide precursors as well as the incorporation of hexoses into carbohydrate moieties. The lack of a more marked inhibition may be due to a low oxygen requirement of cartilage and the presence of an endogenous source of glucose. Although a Pasteur effect was not demonstrated in this type of cartilage previously (17), the tissue employed in these experiments is from older animals which have formed rather large ossification centers. Also, the Pasteur effect has recently been demonstrated in bone(18). Others have shown that alterations in the ionic environment during the incubation of isolated tissues have resulted in the modification of tissue metabolism and the changes observed in these experiments only suggest that similar operative mechanisms occur in epiphyseal cartilage.

Summary. 1. The incorporation of radiosulfate into PP of rat epiphyseal cartilage was studied in association with glucose consumption and lactate production. 2. Calculations based on the specific activity of media sulfate suggest rapid sulfation of PP within the age group of the animals employed. 3. Chemical analysis of the isolated PP revealed significant changes in the ratio of its two major constituents with age. 4. The effect of oxygen lack and omission of media glucose on these parameters was evaluated along with changes in divalent cationic concentra-

tions. 5. These observations are discussed in terms of present knowledge of the metabolism of epiphyseal cartilage.

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Received July 1, 1965. P.S.E.B.M., 1965, v120.

Inhibition of Plaque Formation by Myxoma and Fibroma Viruses with Pyrimidine Analogues.* (30571)

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Several pyrimidine analogues have been reported to interfere with the synthesis of infective viral DNA(1). Our studies reveal that infection of tissue culture with myxoma or fibroma viruses is a good model system for detection of such viral DNA antimetabolites.

Materials and methods. A. Tissue cultures. 1. Rabbit testicular continuous cell line (RT_2). RT_2 cells, a continuous cell line initiated in our laboratories, were grown with Eagle's medium in Hanks' BSS + 10% fetal bovine serum in 16-oz prescription bottles. Roller tube cultures were prepared from these cultures.

2. Primary rabbit kidney cultures (PRK). Kidneys from 3-week-old rabbits were trypsinized and planted in 2-liter Povitsky bottles, using Eagle's medium in Hanks' BSS supplemented with 5% calf serum. The Povitsky cultures were expanded into 3-oz prescription bottles employing 10 ml Eagle's + 5% calf serum.

3. Rabbit kidney continuous cell line (RK_{13}) -glaxo. The growth medium consisted of medium 199 + 5% fetal bovine serum which was reduced to 2% in the maintenance medium. The same procedure was employed as with the PRK cells.

B. Test systems. In RT_2 cells the Sanarelli myxoma virus was added to the roller tubes in 0.2 ml amounts, and the cultures immediately fed with 2 ml of maintenance medium containing the desired concentration of compound. The cultures were examined daily for CPE.

In PRK and RK_{13} cultures the myxoma virus was added to the monolayer for 2 hours to allow adsorption, the cultures washed twice, then 10 ml of the maintenance medium containing compounds was added. After incubation at 37°C for 4 days the fluids were removed and plaques counted under a stereoscopic microscope after staining with carbol fuchsin. The Kilham fibroma virus did not

* This research has been supported by the CCNSC, Nat. Cancer Inst., Nat. Inst. Health, under Contract No. PH 43-65-42.