MUCOPOLYSACCHARIDES PRODUCED IN TISSUE CULTURE*

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About 20 years ago, the description of the spreading reaction (1), the isolation of hyaluronic acid (2), and the description of its enzymatic hydrolysis (3) opened a new era in connective tissue research. Soon after that the enzyme-substrate nature involved in the spreading reaction and the role of hyaluronidase as a spreading factor were recognized. More recently considerable knowledge concerning the composition of the ground substance of connective tissue has been gained mainly from chemical isolation. But chemical isolation carried out directly on animal tissues failed to give information on the topology of the substances isolated, failed even to prove their origin from interfibrillar spaces, and there was no way of obtaining information about the role of the fibroblast in the formation of ground substances (4). Such information has now been obtained using the method of tissue culture.

The production of hyaluronic acid in cultures of various tissues of mesodermal derivation has been demonstrated (5) in small samples of supernatant medium from roller tube and Carrel flask cultures by the mucin clot formation test and the prevention of clot formation by hyaluronidase, thus indicating the origin of components of ground substances of connective tissues from fibroblasts. The mucin clot test, however, is only qualitative. Furthermore, it detects only highly polymerized native hyaluronic acid and hyaluronic acid produced by cells in tissue culture (this shows that hyaluronic acid produced in tissue culture, since it gives a positive mucin clot test, is, like native hyaluronic acid, highly polymerized, although smaller molecules may also be present). Neither low polymer, or partially depolymerized hyaluronic acid, nor chondroitin sulfates (molecular weight about 30 to 40,000 in contrast to about 1 to 8,000,000 for hyaluronic acid) can be detected by the mucin clot test. For this reason, and in order to characterize more completely the nature

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of the mucopolysaccharides produced by various kinds of fibroblasts, chemical isolation had to be carried out for quantitative determination of the products elaborated *in vitro*.

Methods and Materials

Tissue Culture Procedure.—Since chemical isolation requires large samples, mass tissue culture had to be used for this purpose. A method of mass tissue culture has been devised, using Petri dishes sealed with parafilm, and also Petroff flasks (6). Planting was carried out in a thin plasma layer over the entire bottom of the dish. 5 to 8 tissue fragments per square cm. were explanted, about 2 ml. of medium containing embryo extract was added, and the explants equally distributed before clotting of the medium. After about 6 hours, 12 ml. of a nutrient medium were added, consisting of 20 to 25 per cent embryo extract, 10 per cent serum, and 60 to 65 per cent amniotic fluid (7), with addition of 100 units of potassium penicillin and 100 micrograms of streptomycin per ml. Regular change of the medium was carried out twice a week and the pooled "used" medium was submitted to fractionation for mucopolysaccharides.

In cultures of human embryo skin and human and bovine embryo bone, growth began as soon as 24 hours after explantation, and after several feedings growth became luxurious. Over large areas several growth layers were superimposed upon one another forming dense mats of cells, and approximately at the 3rd to 4th week, the floor of the flask or dish was covered with thick outgrowth. Microscopic observation of cells growing in these vessels was possible up to a magnification of 400. After about 3 to 5 feedings the supernate was found to contain hyaluronic acid, as indicated by the formation of a tight mucin clot on addition of 0.2 ml. of 1 N acetic acid to 1 ml. sample containing serum protein of the culture medium. The mucin clot formation could be inhibited by hyaluronidase, both testicular and pneumococcal. In cultures of rat subcutaneous tissue, growth started much later, sometimes only at the 2nd week after explantation, and became luxurious at the 3rd to 4th week, when the mucin clot formation test in the supernate also showed production of hyaluronic acid *in vitro*. The advantage of this method of mass tissue culture is that luxurious growth can be achieved in a relatively short period of time, and samples adequate for chemical isolation of mucopolysaccharides can be obtained as soon as 6 to 8 weeks after the original explantation. Furthermore, after heavy growth in several layers has been obtained, it is possible to transform the culture in the plasma clot into a culture growing directly on glass in the same vessel by cutting out parts of the plasma clot without replacing it with new plasma. By this procedure, deterioration of growth in the plasma clot which sometimes occurred after several weeks of growth can be prevented.

In general, the use of a rich nutrient solution containing embryo extract has been found to promote production of mucopolysaccharides (possibly because the culture medium has to provide material for resynthesis of enzyme systems not only for growth, but also for the elaboration of mucopolysaccharides), although it is unknown which substances specifically promote mucopolysaccharide production.

General Chemical Procedure.—The samples of culture fluid, which were stored at −15° to −20°C. until analysis, were precipitated with acetone and the precipitate was washed

with acetone and ether and dried. The dried powders were suspended in water and digested with pepsin at pH 1.5 followed by digestion with trypsin at pH 7.5 at 37°C. in the presence of toluene. The fractions obtained by differential precipitation with ethanol as calcium salts (8) were analyzed for nitrogen, hexosamine, uronic acid, and sulfate, and the optical rotation was determined. The hydrolysis of the substances was determined by measuring the increase of reducing sugar on incubation with testicular hyaluronidase. The amino sugars of each class of mucopolysaccharides, after acid hydrolysis, were determined by paper chromatography according to Stoffyn and Jeanloz (9). Other analytical methods used have been described previously (8). Embryo extract was processed in a similar manner. The content of mucopolysaccharide in the embryo extract of the medium could in no case account for more than $\frac{1}{10}$ of that recovered, and was considered negligible.

RESULTS

1. Human skin, planted in 28 culture dishes, gave luxurious growth in several layers. The cells had the appearance of typical fibroblasts, with good

TABLE I

Human Skin (Fractionated as Ca Salts)

Preparation No.	EtOH fraction	Yield‡	N	Hexosamine	Uronic acid	SO_4	$[\alpha]_D$	Hyaluronidase digestion
MV 81 I*	25–33 per cent	63 mg.	3.90	32.0 per cent	36.4 per cent	0.5 per cent	not done	testicular+ pneumococcal+
MV 81 II	33–40 per cent	25 mg.	—	29.0 per cent	29.2 per cent	9.1 per cent	−30	testicular+ pneumococcal+

* 81 I very viscous.

‡ Yield signifies the total weight of material recovered from all dishes.

transparency, and only few granules were visible at low magnification. No epithelial cells were seen. Chemical isolation gave two fractions: In fraction 1, 63 mg. hyaluronic acid was isolated; in fraction 2, 25 mg. chondroitin sulfate (probably C, judged from its solubility in ethanol), which was not fully sulfated (Table I).

From analytical data and enzymatic digestion, as well as viscosity measurements, the major fraction is hyaluronate, the minor fraction is apparently chondroitin sulfate C. No chondroitin sulfate B was found.

2. Embryonic human bone (calvarium), planted in 15 culture dishes, showed very good and abundant growth of elongated spindle-shaped cells in several layers, with quite clear cytoplasm and not many granules. Results from chemical isolation are shown in Table II and indicate that about equal quantities of hyaluronic acid and chondroitin sulfate were elaborated by this sample of human bone. Further characterization of the material was not feasible.

3. Embryonic beef bone (membraneous and limb bone) was planted in 40 culture dishes. Luxuriant growth of fibroblasts, morphologically not distinguishable from other fibroblasts, was observed, and 1500 ml. of pooled supernate were harvested and submitted to fractionation and chemical isolation.

Nineteen grams of acetone-dried material were recovered and found to contain appreciable amounts of acid mucopolysaccharide, part of which was sulfated. No further characterization was carried out.

4. Rat subcutaneous tissue was planted in 18 dishes and gave very good growth of fibroblasts in several growth layers. Results of chemical isolation are shown in Table III.

TABLE II

Human Membrane Bone (Calvarium)

Total mucopolysaccharide (unfractionated)

Preparation No. MV 73	Yield*	Hexosamine	Uronic acid	SO_4
Cell mass	114 mg.	23.6 per cent	25.6 per cent	4.4 per cent
Culture fluid	72 mg.	23.2 per cent	23.7 per cent	3.8 per cent

Combined solid and liquid phase fractionated as Ca salts

	EtOH fraction	Yield*	Hexosamine	Uronic acid	SO_4	$[\alpha]_D$
A	33 per cent	26 mg.	31.5 per cent	37.3 per cent	2.4 per cent	−66
B	40 per cent	25 mg.	23.8 per cent	33.6 per cent	9.6 per cent	−25
C	50 per cent	7.2 mg.	—	30.1 per cent		

* Yield signifies the total weight of material recovered from all dishes.

TABLE III

Rat Subcutaneum

705 ml. culture fluid. Yield: 101 mg. (0.58 per cent of dry weight) isolated as unfractionated total mucopolysaccharide.

Preparation No. 93 A.	Hexosamine	Uronic acid	SO_4	$[\alpha]_D$
	28 per cent	30.1 per cent	7.3 per cent	−44

Paper chromatography of hexosamines showed almost equal quantities of glucosamine and chondrosamine.

This sample represents a mixture of equal parts of hyaluronic acid and chondroitin sulfate. The sulfate content was one-half the amount expected of chondroitin sulfate.

DISCUSSION

In the organism only synovial fluid and vitreous humor (and some malignant tumors of mesodermal origin) contain a single mucopolysaccharide, hyaluronic acid; other tissues produce one or two chondroitin sulfates and also, except hyaline cartilage and cornea, hyaluronic acid. Cultured cells of human and bovine bone, human skin, and rat subcutaneous tissue behave

in vitro as they would in the organism (10), producing both chondroitin sulfate and hyaluronic acid. In some regards, however, certain differences have been found in cultured cells as compared with the findings in the organism. In tissue culture, no chondroitin sulfate B was detected which, at least in bovine and porcine adult skin, is the predominant component. Embryonic pigskin, however, contains very little chondroitin sulfate B. Furthermore, the chondroitin sulfates were markedly undersulfated. Whether this means that sulfur metabolism is impaired in tissue culture to a greater extent than is the synthesis of the rest of the chondroitin sulfuric acid molecule is yet unknown. How the conditions prevailing in tissue culture might modify the synthesis of chondroitin sulfate is a question of great interest, and further work is necessary to show whether any change in the composition of the culture medium will increase the sulfate uptake by the molecule of chondroitin sulfate, and whether increase or decrease of sulfate uptake might occur spontaneously after much longer periods of time of cultivation. In the animal as well as in tissue slices, the influence of a number of factors on the sulfate exchange in chondroitin sulfate has been demonstrated with radioactive sulfate. Cortisone and salicylic acid, for instance, strongly inhibit, whereas thyroxin, liver homogenate, and glucose increase the fixation of labelled sulfate in mucopolysaccharides (11).

On the other hand, incomplete sulfation of mucopolysaccharides produced in tissue culture might be related to a tendency of cultured cells to revert to an early stage of cell metabolism, since it has been assumed that unsulfated chondroitin might be the precursor of chondroitin sulfates (12). It is unlikely that the undersulfated chondroitin sulfate fractions result from desulfation because glycosulfatases have not been detected in tissues. Failure of sulfation should thus be regarded as the probable reason for the production of undersulfated chondroitin sulfates in tissue culture.

It is possible that in tissue culture rather than mixtures of hyaluronate, chondroitin, and chondroitin sulfate, hybrid molecules are produced which contain both sulfated components containing galactosamine, and nonsulfated components containing glucosamine. Hybrid molecules have not been found in nature, but have been produced *in vitro* by transglycosylation (by testicular hyaluronidase) (13). The production of hybrids in tissue culture might partly explain the present results. This cannot be proven at the present time, but if hybrids were produced in tissue culture, it would follow that polymerization takes place rather extracellularly, if we assume that different cells produce different mucopolysaccharides.

It is not quite clear in what form chondroitin sulfates occur in tissues. It is known, however, that they are bound to protein much more firmly than is hyaluronic acid (14). In contrast to hyaluronate, which occurs both in fluids and in structural elements, chondroitin sulfates in the organism as a rule do

not occur in fluids. In tissue culture, it has been shown that chondroitin sulfates produced by fibroblasts are in solution in the culture medium.

SUMMARY

1. A method of mass tissue culture has been devised by which, in a relatively short period of time, samples large enough for chemical isolation of mucopolysaccharides can be obtained.

2. Chemical isolation of acid mucopolysaccharides from mass cultures of human fetal skin, human fetal bone, bovine fetal skin, and rat subcutaneous tissue has been carried out. It has been found that the fibroblasts of each of these tissues produce in tissue culture more than one mucopolysaccharide, namely, hyaluronic acid, and a chondroitin sulfate.

3. The chondroitin sulfate produced by fibroblasts of the above tissues in tissue culture was not fully sulfated. The possible significance of this finding is discussed.

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