possible explanation of certain adverse physiological results occasionally experienced with the use of these emulsions.

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Production of Mucopolysaccharides by Synovial Cells in a Simplified Tissue Culture Medium. (22853)

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Acid mucopolysaccharides of connective tissue ground substance and synovial fluid "mucin" have assumed increasing importance in the light of mounting evidence that these substances are abnormal in various rheumatic disease states(1,2). Information regarding specific cell or cells of origin of the several acid mucopolysaccharides is contradictory (3,4,5) and biochemical data concerning their biosynthesis in normal tissue are fragmentary. Since most of the information concerning biosynthesis has been obtained from study of microbiologic systems capable of synthesizing mucopolysaccharides(6), it would be desirable to initiate parallel exploration at the level of the mammalian tissue cell. While circumstantial evidence of production of mucopolysaccharide by synovial cells has been obtained by tissue culture methods, previous studies are open to the criticism that the mucopolysaccharides demonstrated may well have been present in the initial culture medium. As early as 1933, Vaubel(7) reported the production of synovial "mucin" by rabbit synovial tissue grown *in vitro* in tissue culture. The production of a mucin clot on acidification of the culture medium, which contained splenic extract, was taken as evidence of "mucin" production. Vaubel regarded this phenomenon as specific for the synovial cell. Murray *et al.*(8) subsequently successfully cultured adult rat synovium *in vitro*. Their observations tended to confirm Vaubel's belief that the synovial cell in tissue culture was morphologically distinct from the "common fibroblast." Although they did not study the products of cellular metabolism in their culture media, they were unable to confirm Vaubel's finding of metachromatic staining of the cytoplasm with toluidine blue. More recently Grossfeld *et al.*(9) advanced further evidence of *in vitro* mucopolysaccharide production using cultures of rat subcutaneous tissue as well as normal and arthritic human synovium. In the culture medium which had supported synovial cell growth they measured mucin clot formation and turbidity following acidification with acetic acid. They noted that these phenomena could be prevented by

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prior treatment of the medium with either testicular or pneumococcal hyaluronidase. The culture medium used by these workers contained amniotic fluid, horse serum and, in most instances, chick embryo extract. Their observations suggested that hydrolysis of the embryo extract with hyaluronidase prior to its use as a medium component or omission of embryo extract from the nutrient medium adversely affected mucin clot production. Kling *et al.*(10) apparently were also troubled by the problem of incorporation of mucopolysaccharides into the initial culture medium and minimized this by reducing the embryo extract concentration to 2.5%. Their evidence for mucopolysaccharide production was documented by showing the presence in culture media of hyaluronidase-sensitive turbidigenic material, by mucin clot formation, by demonstrating a slight increase in viscosity of the culture medium and by showing an electrophoretic peak believed to be hyaluronic acid. It was of interest that they found no evidence of mucopolysaccharide production in cultures of para-articular tissues.

It was the purpose of the present study to detect mucopolysaccharide formation by human synovial cells using a simpler medium free of detectable preformed mucopolysaccharides and to examine the pattern of behavior of cultures from individuals of varying ages over a prolonged period of observation.

Materials and methods. Normal human synovial tissue was secured from knee joints included in amputations for peripheral vascular disease, osteogenic sarcoma, and from a 4½ month old fetus. The joints were normal by gross inspection and microscopic examination of the synovium. Less than 0.3 ml of synovial fluid was aspirated from the joints before tissue was removed for explantation. The excised tissue was rinsed with balanced salt solution(11) and cut into 1 x 1.5 mm explants. These tissue fragments were planted 6 to 10 per roller tube with a thin film of chicken plasma to improve adherence to the glass. The nutrient medium used contained 80% Eagle's basal medium(12), and 20% normal human serum. One-half ml of nutrient medium with an initial pH of approximately 7.8 was placed in each roller tube and the cultures were incubated at 37°C in a roller drum. Medium changes were carried out at 3-6 day intervals and the pH of culture media was adjusted with isotonic sodium bicarbonate when it approached pH 7.2. Frequent microscopic inspections of the roller tube cultures were made, and cover-slip cultures yielded preparations suitable for staining. The culture medium was analyzed for mucopolysaccharides by a turbidimetric method and also by a modified Sundblad‡ method for hexosamine. For turbidimetric analysis of each sample, 0.5 ml of culture medium was delivered to each of two 10 x 80 mm colorimeter tubes and adjusted to a pH of approximately 5.0 with 0.2 ml of 0.1 M acetate buffer, pH 4.91. 0.05 ml of 0.02 M phosphate buffer at pH 6.8 with 0.45% sodium chloride and 0.01% bovine serum albumin was added to the first tube and to the second tube was added 0.05 ml of the same buffer mixture containing 50 turbidity reducing units (TRU) of testicular hyaluronidase.§ Both tubes were incubated in a water bath at 37°C for 30 minutes and then turbidity was developed by addition of 0.3 ml of 1 M acetate buffer, pH 4.2. After standing for 30 minutes at room temperature, the optical transmission of the turbid samples was determined in the Coleman colorimeter, Model #9, with a 590 mu filter. The quantity of hyaluronidase-sensitive turbidigenic material in the culture medium was determined from a standard curve with purified umbilical cord hyaluronate as a reference (Fig. 1). The hyaluronic acid used as a reference standard

‡ Hexosamine was determined by extensive dialysis of the samples followed by treatment with hyaluronidase, precipitation of proteins with *hot* trichloroacetic acid, acid hydrolysis of the supernatant fluid, and analysis for hexosamine by the method of Boas (13) which involves purification by an ion exchange resin technic. The modifications of the original Sundblad method(14) indicated here were found to be necessary for accurate determination of hyaluronic acid in synovial fluid, and will be published elsewhere. The hexosamine analyses were performed through the courtesy of Mr. Donald Watson.

§ We are indebted to Wyeth Co. for the highly purified testicular hyaluronidase used.

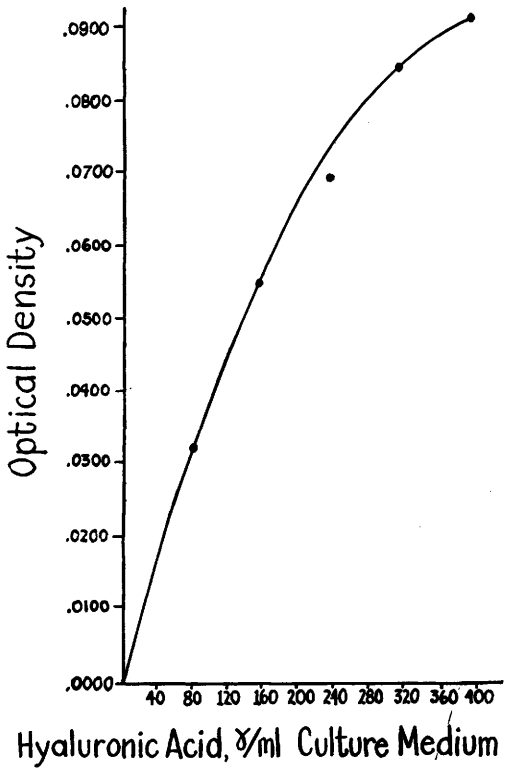

FIG. 1. Standard curve derived after adding reference hyaluronic acid to initial culture medium.

had been purified by electroconvection‖ and was quantitated on the basis of the hexosamine which it contained. The relative viscosity of the reference hyaluronic acid at a concentration of 2.8 mg/ml is approximately 32 at pH 7.0 in 0.03 M phosphate buffer.

Observations. Culture morphology and behavior. Group I cultures were derived from the suprapatellar bursa of a 67-year-old male patient with diabetes mellitus. For the first month these explants were cultured *in vitro* in a medium containing 10% embryo extract and for the succeeding 5 months they were grown in 80% Eagle's basal medium with 20% human serum. It was possible to subculture the cells mechanically five times, after which the cultures degenerated. The cellular morphology was pleomorphic, ranging from spindle and stellate shaped cells to more polygonal forms. In general, cytoplasmic granulation as demonstrated by the May-Greenwald-Giemsa stain was not marked, but a perinuclear halo of violet granules was commonly seen (Fig. 2). The pattern of growth was reticular and the rate of proliferation appeared slow. Lysis of the plasma clot was not uncommon.

Group II cultures were obtained from the knee synovium of a 14-year-old boy whose leg was amputated for osteogenic sarcoma, and were grown in the medium previously described. No subcultures were attempted. These explants showed a much more rapid initial rate of proliferation than those of Group I. This rapid outgrowth was not sustained and the cultures were lost after two months. Plasma clot lysis and decrease in pH of the medium were considerably more prominent in these cultures than in those from Group I. Cellular morphology did not differ appreciably from Group I.

Culture Group III was derived from the knee joint synovium of a 4½-month-old male fetus. The explants were removed from the area of insertion of the quadriceps complex into the patellar cartilage. Outgrowth around the fetal explants appeared at 72 hours and rapid proliferation of small granular connective tissue cells in a reticular pattern followed (Fig. 3 and 4). Concomitant with accelerated proliferation, the fetal culture produced

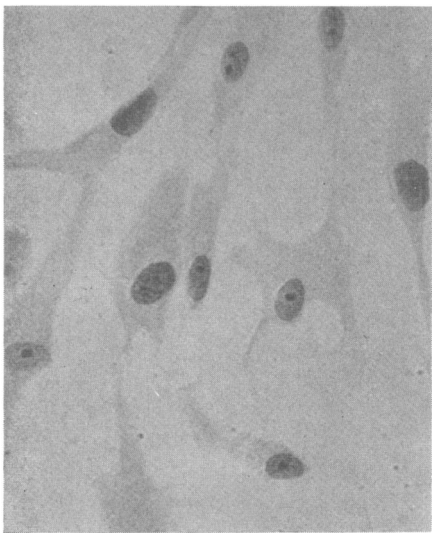

FIG. 2. Four-day cover slip culture of synovium from 67-yr-old male. May-Greenwald-Giemsa stain. × 170.

‖ Abstract by Roseman, Watson, Duff and Robinson in *Fed. Proc.*, 1955, 14, No. 1.

FIG. 3. Three-day cover slip culture of synovium from 4½-mo-old fetus. May-Greenwald-Giemsa stain. × 170.

more acid than previous strains. Mechanical division of the culture for one subculture was successful. Culture degeneration and death occurred after 6 weeks.

Pattern of mucopolysaccharide synthesis. The quantity of hyaluronidase-sensitive poly-

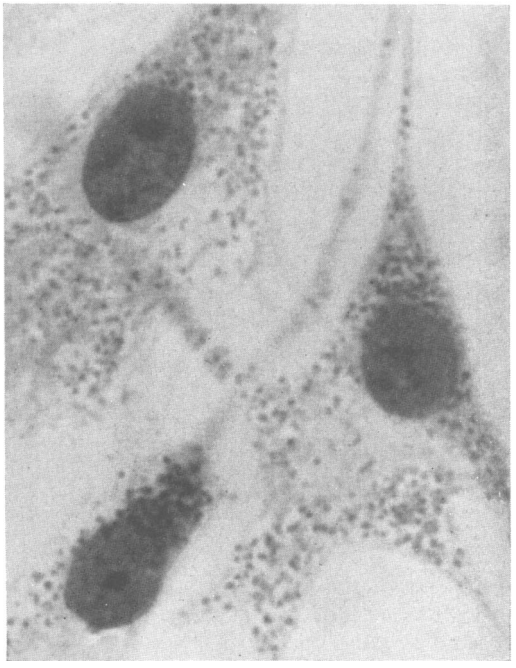

FIG. 4. Same culture as Fig. 3. × 756.

TABLE I. Hyaluronic Acid Content of Culture Media.

Culture group	Medium	% EE50*	Incubation time of medium tested, days	Time post explantation, days	Hyaluronic acid, γ/ml†
None	$ESM_{80}HS_{20}$	20	0	0	1540
	$AF_{80}HS_{20}$‡	20	0	0	1080
	$ESM_{80}HS_{20}$§	0	0	0	0
I	$858_{50}HS_{50}$‖	0	6	49	80
	$ESM_{80}HS_{20}$	0	4	97	36
		0	4	101	76
		0	4	172	84
		0	3	175	72
		0	4	179	72
		0	4	183	60
		0	4	187	60
II	$ESM_{80}HS_{20}$	0	9	15	244
	$ESM_{60}HS_{40}$	0	7	32	95
	$ESM_{80}HS_{20}$	0	5	37	132
		0	4	41	112
		0	4	45	128
		0	3	48	120
		0	4	52	104
		0	4	56	108
		0	4	60	172
III	$ESM_{80}HS_{20}$	0	8	8	519
		0	7	15	264
		0	5	20	168
		0	4	24	120
		0	4	28	76
		0	3	31	136
		0	4	35	40
		0	4	39	38
		0	4	43	28
		0	3	46	84

* % EE50 indicates % of medium represented by half diluted chick embryo extract (Difco).

† Hyaluronic acid determined by turbidimetric technic described.

‡ $AF_{80}HS_{20}$ represents amniotic fluid, 60%, human serum, 20%.

§ $ESM_{80}HS_{20}$ refers to 80% Eagle's basal medium with 20% human serum.

‖ $858_{50}HS_{50}$ indicates Parker's medium 858(16) diluted 1:1 with human serum.

saccharide found in the culture media of 3 groups of human synovial explants is tabulated (Table I). From the table it is apparent that in our hands the turbidimetric method shows no mucopolysaccharide in the simplified culture medium *per se* in contrast to large amounts found when embryo extract is a component of the medium. Turbidimetric measurement of polysaccharide synthesis by explants grown in a medium containing embryo extract would appear to be open to serious question, since small increases measured by difference would be of doubtful sig-

nificance. In all 3 culture groups mucopolysaccharide appeared to be released by the cell cultures into the nutrient medium. For each culture group the first few days *in vitro* showed the greatest polysaccharide yield with a tendency to a lower, relatively constant level of polysaccharide production in later weeks.

Criteria for identification of turbidigenic substance as mucopolysaccharide. Culture media which have supported the *in vitro* maintenance and proliferation of synovial connective tissue cells contain one or more substances which develop turbidity on acidification. Treatment of the sample with testicular hyaluronidase will prevent or markedly reduce such turbidity formation. Positive identification of the turbidigenic substance as a mucopolysaccharide would follow only if it were possible to use pure hyaluronidase. Testicular hyaluronidase with 1250 TRU/mg obviously may contain other enzymes capable of hydrolyzing turbidigenic materials. We have noted that 50 TRU of testicular hyaluronidase is very active in preventing turbidity formation by several hyaluronate and chondroitin sulfate preparations. The testicular hyaluronidase proved to be contaminated with a significant amount of ribonuclease since it was shown to be active against ribonucleic acid. No activity against desoxyribonucleic acid could be demonstrated. The testicular hyaluronidase used in this study has equivalent activity against added hyaluronic acid in both initial culture medium and that which had supported cell growth. Fifty gamma of crystalline ribonuclease was shown to have equivalent turbidity-preventing activity against ribonucleic acid added to both initial medium and medium which had been incubated with metabolizing synovial cells. Crystalline ribonuclease had, however, no capacity to inhibit the turbidity formation found in culture media which had nourished synovial cells. These observations indicate that the test culture media do not contain ribonucleic acid in a significant quantity and suggest that the turbidigenic material is a mucopolysaccharide. To further substantiate the belief that the turbidigenic material in synovial culture media is a mucopolysaccharide, selected samples were analyzed for hexosamine. Table II compares the quantity of hyaluronic acid in samples of media as measured by the turbidimetric and hexosamine methods.

TABLE II. Comparison of Hyaluronic Acid Values Measured by Hexosamine and Turbidimetric Methods.

Culture group	γ hyaluronic acid/ml by hexosamine method	γ hyaluronic acid/ml by turbidimetric method	% of turbidimetric value accounted for by hexosamine
$ESM_{80}HS_{20}$	.0	.0	
I	8.7	72	12
II	28.9	108	27
	28.9	132	22
III	48.1	264	18
	25.5	136	18

The turbidimetric and chemical data support the thesis that synovial cells will release a mucopolysaccharide into the culture medium, but do not allow one to distinguish between hyaluronic acid and chondroitin sulfate as the substance produced. To aid in this distinction we prepared bacterial hyaluronidase from the filtrate of cultures of *Clostridium perfringens*¶ using the technic described by Baker *et al.*(15). This bacterial hyaluronidase completely prevented turbidity formation of synovial fluid hyaluronate added to initial culture medium but was not detectably active against chondroitin sulfate added to initial culture medium. *Clostridium perfringens* hyaluronidase proved capable of preventing turbidity formation in the dialyzed culture medium which had supported synovial cell growth, thus supporting the likelihood that the mucopolysaccharide present is hyaluronic acid.

Discussion. From the turbidimetric and chemical data it appears that *in vitro* cultures of normal human synovial tissue can produce acid mucopolysaccharide from the constituents of a semi-synthetic medium. The medium found satisfactory here was Eagle's basal medium (containing 12 amino acids,

¶ We are indebted to Dr. Donald J. Merchant of our Medical School for the strain of *Clostridium perfringens* used.

vitamins, l-glutamine, electrolytes and glucose) supplemented with 20% human serum. Although isolation and final chemical characterization of the mucopolysaccharide is not practicable with the small quantity of medium available here, the data are consistent with the belief that hyaluronic acid is the substance found in the culture media. The substantially greater quantities of mucopolysaccharide found by turbidimetry than by the Sundblad hexosamine method might be explained in at least three ways: (1) the hyaluronate used as a reference standard may have been sufficiently denatured in the process of isolation so as to yield less turbidity per unit of hexosamine than the material produced by the synovial cell culture; (2) a substantial proportion of the turbidigenic material may not be hexosamine-containing mucopolysaccharide; (3) the dialyzable mucopolysaccharide fraction of the culture medium, which is not measured by the Sundblad hexosamine method, may actually contribute to the higher turbidimetric values.

It must be remembered that the "synovial" cultures described here and elsewhere are imperfectly characterized both qualitatively and quantitatively. Hence, the possible mixture of cell types within impure strains, as well as unmeasured variations in number of cells acting on a substrate, subjects the critical comparison of different cell strains to many uncertainties. It is apparent that the similarities in mucopolysaccharide concentration among the three groups of cultures related to time post-explantation may just as easily be an artifact arising from differences in number of metabolizing cells as evidence of similar biosynthetic capacities of synovial cells from individuals of different ages.

Summary. Synovial tissue has been shown to be capable of *in vitro* production of a mucopolysaccharide believed to be hyaluronic acid. Cultures from donors ranging from a fetus to an elderly male shared this capacity for periods of 6 weeks to 6 months. This process is supported by a nutrient medium that is 80% defined and excludes embryo extract. Culture behavior and cellular morphology are described.

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