Biosynthesis of Hyaluronic Acid in Tissue Cultures of Human Synovial Membrane.* (23947)

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It has been demonstrated(1,2) that hyaluronic acid in synovial fluid from cases of rheumatoid arthritis had a lower intrinsic viscosity than that from normal persons. Similar changes in the quality of synovial hyaluronic acid have been shown by Ragan and Meyer(3), and were ascribed by them to synthesis of incompletely polymerized hyaluronic acid. Synthesis of acid mucopolysaccharides has been studied in tissue cultures from synovial membrane by Vaubel(4), Kling *et al.*(5), Grossfeld *et al.*(6) and Castor(7). The results suggested that hyaluronic acid was produced.

The purpose of the present investigation was to study biosynthesis of acid mucopolysaccharides in tissue cultures of human synovial membranes from noninflammatory cases, and to try to obtain information on the intrinsic viscosity of the mucopolysaccharides produced.

Materials and methods. Human synovial and periarticular connective tissue were obtained by meniscectomi from male, noninflammatory cases. Pieces of tissue, removed at operation, were placed in Tyrode solution for ½ to 1 hour. Before cultivation the synovial membrane was, as far as possible, freed from subsynovial tissue. The cultures were generally made in flasks of the Grand type using culture media described by Kling *et al.* (5). Streptomycin and penicillin—50 units/ml of each—were added. The fluid phase (5 ml) of the culture medium was changed once a week. As controls, nutrient media were incubated without cells (control medium, one control for each new batch of media).† Viscosity of the fluids was measured (after centrifugation) at 37.0°-37.3°C according to Diczfalusy *et al.*(8). Ostwald viscometers (2 ml) with efflux times (for water) varying from 12 to 16 sec. were used. Relative viscosity (η_{rel}) = ratio between the efflux times for solution and solvent; specific viscosity (η_{spec}) = difference between (η_{rel}) for supernatants of cultures and the controls. Calculation of the error of the method from duplicate determinations showed that a difference in relative viscosity of 0.03 ($= 3 \times \epsilon$) was significant.

Determination of acid mucopolysaccharides. Samples were depolymerized by testicular hyaluronidase (100 VRU/ml) and deproteinized with trichloroacetic acid as described by Sundblad(1). Hexosamines were then determined according to Blix(9). The standard error of a single determination was calculated from double determinations to be ± 7.2%. The degree of interference by chromogens (10) (as compared with a solution of pure glucosamine –HCl) was so small that it could be neglected. In any case it was of no importance when the increase of hexosamines (= difference between culture medium and control medium) was reported. *Tests for characterization of viscosity increasing substance (s)*: Samples were tested for mucin clot formation according to Grossfeld *et al.*(6). The viscosity reducing effect of testicular (Hyalas ®, AB Leo Hälsingborg, Sweden) hyaluronidase, purified by chromatography, and staphylococcal hyaluronidase (Hyason®, N.V. Organon, Oss, Holland) was determined. Generally 78 VRU Hyason and up to 100 VRU Hyalas were used (all units expressed as units of Hyalas per 2 ml).‡ Incubation was for 1 hour at 37.0°-37.3°C. Dialysis was per-

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† Tissue cultivations were performed by Doc. B. Kallén at the Tissue Culture Laboratory, Inst. of Histology, Lund, Sweden.

‡ Presence of considerable amounts of inhibitors (determined according to Adner(11)) in the fluids examined, necessitated the use of these high amounts of enzymes.

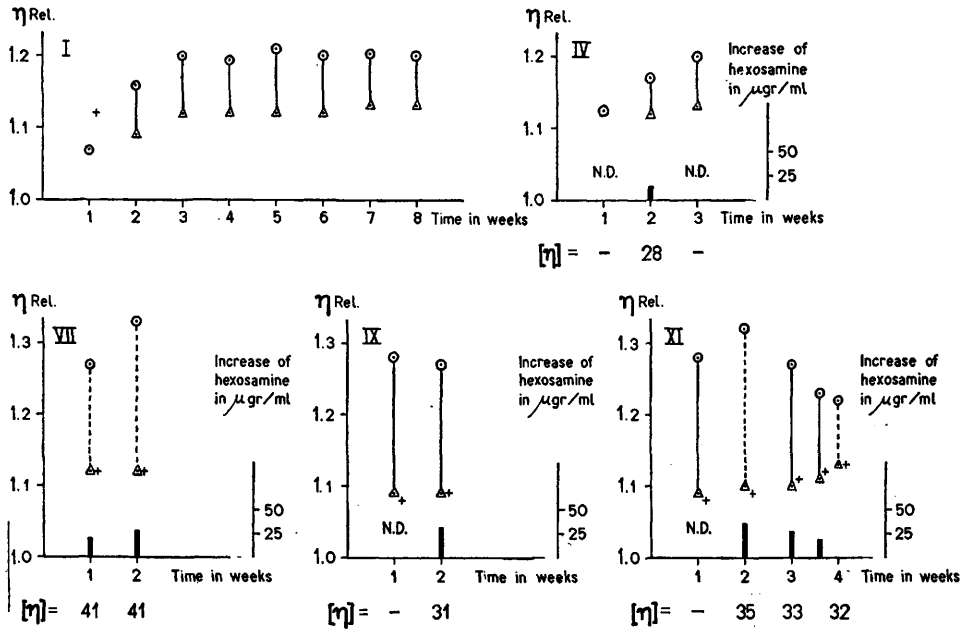

FIG. 1. Increase of viscosity and mucopolysaccharide–hexosamines in supernatants of tissue cultures from human synovial membrane. η Rel. = relative viscosity; ○ = relative viscosity before incubation with hyaluronidase; △ = relative viscosity after incubation with hyaluronidase; | = viscosity reduction after incubation with testis hyaluronidase; ¦ = viscosity reduction after incubation with staphyloc. hyaluronidase; + = relative viscosity of control medium; ▮ = increase of mucopolysaccharide–hexosamines; $[\eta]$ = intrinsic viscosity.

formed (on several samples selected at random) either as rapid dialysis according to Mies(12) against isotonic buffer saline mixture (pH = 7.0) at room temperature, or as extensive dialysis at + 4°C for about 48 hours against large volumes buffer saline (changed 4 times) with cellophane sausage tubing (Visking Corp., Chicago). The intrinsic viscosity $[\eta]$ was calculated as the ratio between specific viscosity and hexosamine concentration (in g/100 ml) x 2.12 (1:2.12 = the molar ratio between hexosamine and the repeating unit of hyaluronic acid).§

Results. In Table I are reported the results of tissue cultures, clinical material and production of acid mucopolysaccharides. The cultures were checked for cell growth and in one case a time-lapse movie was taken for about 20 hours. No signs of mitosis in the synovial cells could be observed. The liquid medium from cultures of human synovial membrane was more viscous than the liquid medium incubated without cells (= control medium (Fig. 1)). In Fig. 2 the results from

TABLE I. Clinical Material (Non-inflammatory Cases with Lesions of the Meniscus) and Tissue Cultures.

Case No.	Age, yr	Duration of disease, yr	Tissue cultures *	Production of mucopolysacch.
I	32	11–12	+	+
II	49	1/3	Fibrinolysis	—
III	59	1/4	"	—
IV	21	1/3	+	+
V	?	?	Fibrinolysis	—
VI	?	?	"	—
VII	30	<1/12	+	+
VIII	72	2	Necrosis	—
IX	53	2	+	+
X	15	1/4	+	+
XI	31	1	+	+
XII	52	1/2	+	+

* + = cell growth.

§ As shown in previous investigations a linear ratio existed between viscosity and concentration of hyaluronate preparations for values of the latter below 0.05%(1,13,14). In the present investigation this relationship was valid even for the increase of viscosity in supernatants from tissue cultures. Because of this linear ratio the intrinsic viscosity(1), defined as $\lim_{c \to o} \frac{\eta \text{ spec}}{c}$ (c = concentration) could be calculated as the ratio $\frac{\eta \text{ spec}}{c}$.

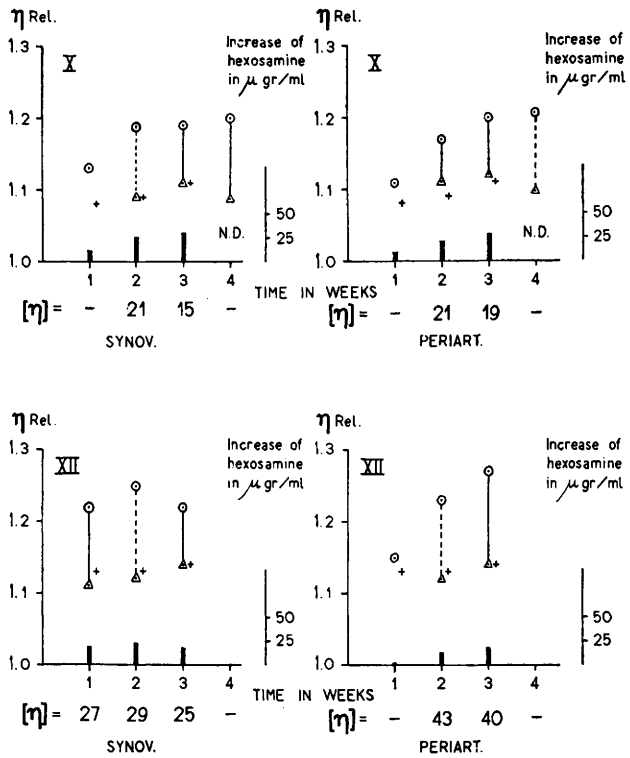

FIG. 2. Comparison between tissue cultures from human synovial membrane and from periarticular connective tissue. Symbols as in Fig. 1.

2 cultures of periarticular connective tissue are included. After 2 or 3 weeks, the increase of viscosity attained about the same level as in corresponding cultures from synovial membranes. The increase of viscosity could be reduced to viscosity of the control medium through incubation with testicular or staphylococcal hyaluronidase (see Fig. 1 and 2). It should be mentioned that the viscosity of the control medium could not in any case be reduced through incubation for at least one hour with testicular or staphylococcal hyaluronidase. Largely parallel with the increase of viscosity, there was an increase of acid mucopolysaccharides in the liquid medium (Fig. 1, 2). In addition, the mucin clot formation test, when performed, was positive in the liquid medium after cultivation and negative in the control medium.

To find out whether hexosamine containing oligosaccharides (which are dialysable) could be demonstrated in the liquid medium, some samples were dialysed. The results indicated that small amounts of oligosaccharides were present before and after cultivation. In no case was there a significant increase of oligosaccharides after cultivation.

In tissue culture experiments, as performed, the amounts of tissue in the flasks varied, thus rendering comparisons between amounts of mucopolysaccharides produced impossible. However, an idea of their quality might be obtained from the intrinsic viscosity (see materials and methods). The numerical value of the intrinsic viscosity showed a considerable variation (15-43) from one case to another. With the exception of the synovial culture from case X, intrinsic viscosity seemed to be rather constant from week to week. Mucopolysaccharides of a synovial culture as compared with those of a periarticular origin showed about the same values of the intrinsic viscosity in case X and somewhat lower values in case XII (Fig. 2). When determining the hexosamine content of the mucopolysaccharides of the liquid medium not incubated with cells, considerable variations were found (13-34 γ mucopolysaccharide-hexosamine/

ml). In several cultures (7 out of 16) the amount of mucopolysaccharides was about 270% of that initially present in the liquid medium.

Discussion. Kling *et al.*(5) Grossfeld *et al.* (6) and Castor(7) performed quantitative determination of mucopolysaccharides (in supernatants of tissue cultures), by means of turbidimetric methods. The turbidimetric values of supernatants were related to a standard curve showing extinction values obtained from dilutions of a preparation of purified hyaluronic acid. Castor(7) compared the turbidimetric method with determinations of the hexosamine content of mucopolysaccharides (after dialysis), and found considerable differences: only 12-27% could be accounted for by turbidimetric determination of hexosamines. In the present study determination of the hexosamine content was performed for quantitative assay of acid mucopolysaccharides. Determination after dialysis of samples showed only a slight decrease of the hexosamine content in the control medium as well as in supernatants from tissue cultures. Thus production of large amounts of dialysable hexosamine containing substances, *i.e.* oligosaccharides, discussed by Castor(7) as a possible explanation of the differences found by him, could not be demonstrated in the present investigation. It should be observed that Castor(7) did not report any determinations of hexosamines *before* dialysis.

In the present study, the viscosity increasing non-dialysable substance(s), containing hexosamines and forming mucin clot could be depolymerized by testis hyaluronidase. These observations together, are in accordance with previous reports on presence of acid mucopolysaccharides in supernatants of tissue cultures from synovial membranes(4-7). As shown by Woodin(15) hyaluronic acid was depolymerized by staphylococcal hyaluronidase in contrast to chondroitin sulphate, while both these substrates were depolymerized by testis hyaluronidase. As shown in Fig. 1 and 2 the increase of viscosity could be completely eliminated through staphylococcal hyaluronidase. This observation indicates that *the increase of viscosity was caused by hyaluronic acid.*

Owing to low concentrations, errors of the methods used, and approximations implied in the calculation (all of the increase of mucopolysaccharide-hexosamines was calculated as hyaluronic acid), our values of intrinsic viscosity might be regarded as relative and semiquantitative. Still these values are of the same order of magnitude as the *in vivo* values given by Sundblad(1) for hyaluronic acid of synovial origin. Provided the increase of mucopolysaccharides (see material and methods) reflected real production, the values of intrinsic viscosity thus found are compatible with the assumption that hyaluronic acid constituted the main part of acid mucopolysaccharides produced *in vitro*. The constancy of the intrinsic viscosity from week to week observed in the present study (Fig. 1, 2) suggested that as a principle the intrinsic viscosity might be used as a measure of quality of the mucopolysaccharides produced in tissue cultures.

In contrast to results published by Kling *et al.*(5), production of hyaluronic acid could be demonstrated even in cultures from periarticular connective tissue. The intrinsic viscosity of supernatants from these cultures seemed to show the same or a somewhat higher value if compared with the intrinsic viscosity in the corresponding synovial cultures.

Of interest is the question from what sources the acid mucopolysaccharides are synthesized. The increase of mucopolysaccharides in the supernatants of tissue cultures (main part of increase is believed to be produced by living cells) indicates that other sources than mucopolysaccharides of the nutrient medium are used in the synthesis. It has been shown by Yielding *et al.*(16), in their investigation on human synovial slices, that both the hexosamine and glucuronic acid parts of hyaluronic acid can be synthesized from glucose.

Summary. Production of acid mucopolysaccharides in tissue cultures from human non-inflammatory synovial membranes was studied. The results indicate that hyaluronic acid was the chief acid mucopolysaccharide produced. Hyaluronic acid was synthesized by the synovial as well as by the periarticular tissues. The results did not indicate an ap-

preciable production of (dialysable) hexosamine containing oligosaccharides. Intrinsic viscosity of the acid mucopolysaccharides produced varied from one case to another but kept rather constant from week to week.

1. Sundblad, L., *Acta Soc. Med. Upsal.*, 1953, v58, 113.
2. Blix, G., *Acta Orthoped. Scand.*, 1949, v18, 18.
3. Ragan, Ch., Meyer, K., *J. Clin. Invest.*, 1949, v28, 56.
4. Vaubel, E., *J. Exp. Med.*, 1933, v58, 63.
5. Kling, D. H., Levine, M. G., Wise, S., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 261.
6. Grossfeld, H., Meyer, K., Gedman, G., *ibid.*, 1955, v88, 31.
7. Castor, W., *ibid.*, 1957, v94, 51.
8. Diczfalusy, E., Fernoö, O., Fex, H., Högberg, B., Linderot, T., Rosenberg, Th., *Acta Chem. Scand.*, 1953, v7, 913.
9. Blix, G., *ibid.*, 1948, v2, 467.
10. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 533.
11. Adner, P. L., *Acta Soc. Med. Upsal.*, 1948, v53, 39.
12. Mies, H. J., *Klin. Wschr.*, 1953, v31, 159.
13. McClean, D., Hale, C. W., *Biochem. J.*, 1941, v35, 159.
14. Swyer, G. J. M., Emmens, W., *ibid.*, 1947, v41, 29.
15. Bárány, E. H., Woodin, A. M., *Acta Physiol. Scand.*, 1955, v33, 257.
16. Yielding, L., Gordon, T., Bunim, J., *Ann. Rheum. Dis.*, 1957, v16, 128.

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Relationship of Steroid and Pituitary Hormones to Myocardial Calcification in the Mouse.* (23948)

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Calcification of the myocardium can readily be induced in the mouse with hydrocortisone(1), and increases in blood calcium to toxic levels have been demonstrated in the dog following administration of the steroid (2). Furthermore, it is recognized that, in the human, hyperfunction of the adrenal glands is often accompanied by a negative calcium balance; if excessive production of corticoids is maintained for a prolonged period of time, as occurs in Cushing's syndrome, myocardial damage, calcific deposition, and osteoporosis may result(3). In an earlier study with intact C3H mice, it was established that the mineral deposit associated with the cardiac lesion is a calcium salt and that the degree of calcification is related to the dosage of hydrocortisone administered(1). The participation of other steroid and pituitary hormones in this degenerative process is described here.

Procedure. Female mice of the C3H strain were used throughout the study, with the exception of a few males that were included in order to ascertain whether or not endogenous androgen would afford some protection to the heart. The mice were injected subcutaneously once daily with the hormones for 15 days, beginning at 50 days of age. Hypophysectomies were performed at 35 days of age, ovariectomies and adrenalectomies at 42 days of age. The adrenalectomized animals were given free access to 1% sodium chloride water and to tap water. The animals were fed a modified McCollum's Diet I, *ad libitum*(4), except for those whose sodium or potassium intake was restricted. At autopsy, the heart was inspected for grossly visible calcific deposits and the intensity of the calcification was estimated on an arbitrary scale of 0 to 5. In addition, the weights of the adrenals, heart and right kidney were recorded. The hydrocortisone, corticosterone, desoxycorticosterone (DOCA), testosterone, and estradiol were administered as

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