analysis showed that, on the basis of the rigorous criteria recently developed (*3*), the chromosomal complement of cells undergoing their first division after dilution of a high-density stationary culture was identical with the complement of cells kept in continuous logarithmic growth. This finding, and the fact that a culture with typical logarithmic-phase characteristics is invariably obtained by dilution of a stationary culture, indicate that the possibility of selection is remote and that most likely, logarithmic growth, starvation plateau, and stationary phase represent different functional states of the same cell population.

The plateau phase of cultures without medium renewal appears as a transient state reflecting the decrease of cellular metabolism preceding cell death. Even with glucose present, cell proliferation ceased and relative viability invariably declined below 0.90 between 48 and 72 hours (Fig. 1*C*); thus this decline indicates exhaustion of limiting nutrients other than glucose.

The average cell density of stationary cultures was 7×10^6 cells per milliliter, approximately seven times greater than the geometric-mean of the density of cells grown in culture without medium renewal from 0 to 72 hours. Exhaustion of glucose and other limiting nutrients, resulting in structural changes of the cells and loss of viability, would therefore be expected to occur within 10 hours after medium renewal. The fact that these high-density cultures were maintained for prolonged periods on a daily medium renewal schedule (Fig. 1*A*, upper curve) without changes in cell size, cellular DNA, RNA, protein (Table 1), and viability (Fig. 1*D*, upper curve), indicates that nutrient utilization rates were lower and that adaptive metabolic changes had taken place. Thus, because its availability per cell was lower, glucose was utilized at lower rates in these high-density stationary cultures. This, however, did not prevent the virtual exhaustion of glucose from the medium at 12 to 18 hours, which in turn led to extensive utilization of lactate (Fig. 1*D*). During the maintenance of these cultures, lactate utilization was a recurrent event characteristic of the second 12-hour interval between medium renewals. Figure 1, *B* and *C*, on the other hand, shows that in cultures progressing from the logarithmic to the starvation plateau phase, lactate utilization occurred only as a terminal event concomitant with loss of cell viability. This indicates that in cultures kept in the log phase of growth by periodic medium renewal and dilution, utilization of the accumulated lactate does not occur. The striking increase of the LDH activity in the high-density stationary cultures could therefore represent an adaptive response related to the recurrence of glucose exhaustion and lactate utilization in these cultures.

The finding in L cells of only one isozyme with an electrophoretic mobility similar to LDH-5 and presumably containing a majority of M (muscle)-type subunits is consistent with the shift from H (heart) to M subunit production observed in other tissues explanted in vitro (*14*). On the other hand an adaptive tenfold increase of the synthesis of this isozyme related to increased lactate utilization would seemingly be at variance with the hypothesis that the M enzyme is primarily concerned with production of lactate (*15*). The high-density stationary-cell culture system described seems well suited for further investigation of these questions and of the factors involved in the apparent shift of the emphasis of cellular metabolism from growth to maintenance. This last point is of interest in that it involves suspension cultures of a long-established heteroploid cell line where contact inhibition (*15*) would be absent.

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17 September 1965

Cellular Origin of Hyaluronateprotein in the Human Synovial Membrane

Abstract. *Bright fluorescent staining, which indicates the presence of hyaluronateprotein, was observed in the lining cells of the synovial membrane following application of rabbit antiserum to hyaluronateprotein and a fluorescein-labeled antiserum to rabbit γ-globulin. Staining was shown to be specific and due to antigenic determinants on or closely associated with the protein moiety of hyaluronateprotein.*

The anionic polysaccharides of connective tissue appear to exist as compounds with protein called proteinpolysaccharides (*1*). Most histologic methods to localize proteinpolysaccharides in tissues depend on interaction of a dye with the anionic groups of the polysaccharide (*2*). When the cationic groups of some thiazine dyes, such as toluidine blue, combine with anionic groups of the polysaccharide, the dye undergoes a metachromatic color change from blue to reddish violet. In the Hale method (*3*) a trivalent ferric cation complex becomes fixed to anionic groups of the polysaccharide, and the iron is stained by the Prussian blue technique. More recently, Alcian blue (*2*), a dye with a tetravalent cation, has been used to demonstrate the presence of polyanionic polysaccharides. To identify the polysaccharide, additional steps are needed, such as incubation of the tissue with a stain having a low *p*H (< 2), or the use of alcohol in the dye or in the wash solution to abolish metachromatic staining of hyaluronate (*2*), or removal of hyaluronate from tissue sections before staining by digestion with streptococcal hyaluroni-

dase (*4*). Although these methods have been used successfully for years, they lack specificity, and there is controversy whether hyaluronate can be identified at all by metachromatic stains (*2*).

Hyaluronate has been isolated from normal human synovial fluid combined with about 2 percent protein, a compound called hyaluronateprotein (*5*), or HP. It is certain that hyaluronate is produced by cells in the synovial membrane, for its synthesis was demonstrated in synovial membrane slices (*6*), and it was identified in the medium in which synovial cells were grown in tissue culture (*7*). The type of cell that elaborates the hyaluronate has not been identified. In sections of synovial membrane, metachromatic staining was demonstrated in the intercellular spaces of the lining cells at the surface. Prior digestion of the sections with streptococcal hyaluronidase abolished metachromatic staining (*4*), which suggests the presence of hyaluronate. The cellular origin of hyaluronate, and so presumably of HP, would appear to be the synovial lining cells. These cells are present at the surface of the synovial membrane (Fig. 1*A*), show higher activities of oxidative and hydrolytic enzymes than the fibroblasts below the lining (*8*) (Fig. 1*B*), and possess a complex fine structure compatible with absorptive and secretory activities (*9*). These findings are no more than suggestive evidence, however.

Methods more specific than the histologic ones described above are needed to locate proteinpolysaccharides in tissues or even in cells. An immunofluorescent method would be a good approach: rabbit antiserum to the proteinpolysaccharide would be layered on the tissue section, and the sites of antibody fixation visualized with a fluorescein-labeled antiserum to rabbit γ-globulin. However, until recently, attempts to produce antibodies to proteinpolysaccharides of connective tissues have been unsuccessful. Now there is evidence that antibodies to cartilage proteinpolysaccharides have been produced in rabbits (*10*), which makes it possible to use immunofluorescent methods to demonstrate the presence of proteinpolysaccharides in cartilage (*11*). This report describes the first immunofluorescent study during which a specific antiserum to HP was used to identify those cells in the synovial membrane in which this proteinpolysaccharide is preferentially localized.

Hyaluronateprotein was isolated principally by ultrafiltration from normal human synovial fluids (*5*). Mild

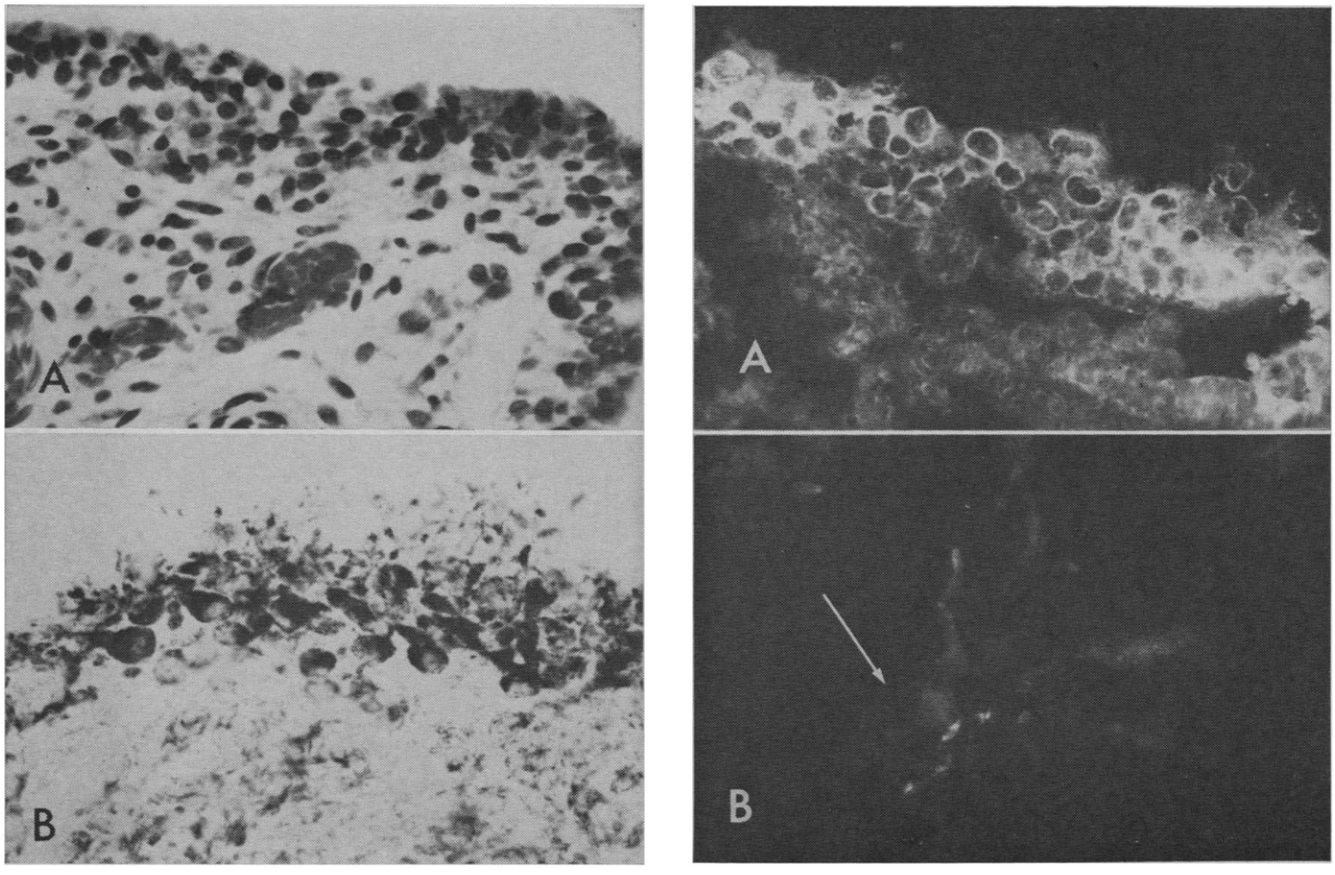

Fig. 1 (left). *A*, Section of synovial membrane from the knee of a patient with early osteoarthritis. Note the layers of lining cells at the surface. Hematoxylin and eosin stain (× 410). *B*, Same tissue, stained for diphosphopyridine nucleotide–diaphorase activity by the tetrazolium method (*8*). Black deposits of formazan outline cytoplasm of lining cells and their processes. Delicate tips of some processes extend into the joint cavity. Note relative lack of staining of cells deeper in the synovial membrane (× 410). Fig. 2 (right). *A*, Section of synovial membrane from a normal knee. The globulin fraction of rabbit antiserum to hyaluronateprotein (HP) (absorbed with human serum) was applied, followed by the fluorescein-labeled globulin fraction of horse antiserum to rabbit γ-globulin. Note bright fluorescence in the cytoplasm of the lining cells and in the intercellular spaces of some of these cells. Many of the large nuclei of the lining cells are unstained, but some show fluorescent stippling. Cells other than the synovial lining cells show relative absence of fluorescent staining (× approximately 550). *B*, Section of synovial membrane treated exactly as described in Fig. 2*A* except for prior absorption of the rabbit antiserum to HP with the protein moiety of HP (see text). Essentially no fluorescence is observed in the location of the lining cells (arrow) (× approximately 550).

isolation procedures were used to preserve the antigenic properties of HP since earlier failures to produce antibodies to hyaluronate (*12*) might have been due, in part, to impairment of antigenicity during purification. The isolated HP mixed with Freund's complete adjuvant was used to immunize rabbits. The globulin fraction of rabbit antiserum was precipitated by adding an equal volume of saturated $(NH_4)_2SO_4$; the precipitate was dissolved in a phosphate-saline buffer (*p*H 7.1) and dialyzed for 2 days at 5°C against 500 ml of this buffer, which was changed twice daily. Prior to use, the globulin fraction was absorbed with excess normal human serum. This absorbed globulin fraction did not agglutinate sheep red blood cells that had been treated with tannic acid and coated with human serum. Antibody to HP was demonstrated in the absorbed globulin fraction by its ability to specifically retard the electrophoretic mobility of HP labeled with I^{131} (*13*). The details of these methods and results will be reported elsewhere (*14*).

Synovial membrane was obtained either by needle aspiration or at arthrotomy from patients with osteoarthritis, torn menisci, or rheumatoid arthritis. The specimens of synovial membrane were placed in chilled tissue-culture medium (*15*) for transportation to the laboratory. After quick-freezing on a Freon plate, serial sections 4 to 8 μ thick were prepared, and on one of these the layer of lining cells was identified by a polychrome stain. The sections were air dried, fixed in 10 percent buffered formalin for 20 minutes, and then washed briefly in buffered saline. The serum-absorbed globulin fraction of rabbit antiserum to HP was layered over the partially dried slides, and the sections were kept in a moist chamber for 30 minutes. After the sections were washed four times in buffered saline, the fluorescein-labeled globulin fraction of horse antiserum to rabbit γ-globulin (*16*) was layered on the slides and left for 30 minutes. Then the slides were washed as before, mounted in glycerin, and viewed under a fluorescence microscope (*17*). Photographs were taken with high speed Ektachrome film at exposures of 4 minutes, and from these, black and white prints were reproduced.

Bright yellow-green fluorescence was observed (Fig. 2*A*) in the lining cells, their intercellular spaces, and, to a lesser extent, in the endothelial cells of blood vessels. Cellular detail was sufficiently well preserved to enable one to appreciate localization of fluorescence in the cytoplasm of the lining cells. Nuclei were unstained or showed occasional fluorescent stippling. A number of experiments were performed to insure that staining was specific for HP. When the serum-absorbed globulin fraction of normal rabbit serum was used as a control, no fluorescent staining was observed; nor did the fluorescein-labeled horse globulin produce fluorescence when applied directly to the sections. When rabbit antiserum was absorbed with whole HP that had been digested with testicular hyaluronidase (300 units at 37°C for 30 minutes) (*18*) to reduce its viscosity, and then applied to synovial membrane sections as above, fluorescent staining was almost completely abolished. When the rabbit antiserum was absorbed with hyaluronidase-digested cartilage proteinpolysaccharide (*19*), or with heparin, no diminution of fluorescence was observed. These results confirm the specific localization of HP in the lining cells and provide an additional reason to believe that HP is synthesized by these cells and secreted into the synovial fluid.

Experiments showed that the antigenic sites of HP that react with the rabbit antiserum are closely associated with the protein moiety of HP. The HP was digested with trypsin, and the intact polysaccharide part was separated from the peptide fragments by ultrafiltration on a Millipore filter (pore size, 0.1 μ). Absorption of the rabbit antiserum with the polysaccharide part produced no diminution of fluorescent staining. In another study, HP was digested with testicular hyaluronidase, and the intact protein moiety was separated from the polysaccharide fragments by means of a column of Sephadex (G-100). Absorption of the rabbit antiserum with the protein part abolished fluorescent staining of the lining cells of the synovial membrane (Fig. 2*B*); absorption with the polysaccharide fragments did not diminish fluorescence. Thus, the antiserum reacts with antigenic sites on or closely associated with the protein moiety of HP. Amino acid analysis of this protein showed a high content of serine, glycine, and glutamic and aspartic acids; hexuronic acid, glucosamine, galactosamine, and galactose were also present (*20*). Agar-diffusion studies showed no precipitin lines between the protein moiety of HP and three potent rabbit antiserums to human serum; the protein was clearly not the serum α-globulin which is bound to HP in pathological synovial fluids (*21*); and although the antiserum to HP was absorbed with an excess of human serum prior to use, intense fluorescence of the synovial membrane surface was still observed. From all these observations it can be concluded that the protein moiety of HP is not a serum protein, and that it is presumably synthesized with the hyaluronate in the synovial lining cells.

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17. Zeiss Standard Universal with Osram HB 200 mercury lamp, exciter filters UG 2 and BG 12, and dark-field condenser and barrier filter 41-65.
18. Generously supplied by Wyeth Inc., Philadelphia, Pa.
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3 August 1965