HURLER'S SYNDROME

Effect of Retinol (Vitamin A Alcohol) on Cellular Mucopolysaccharides in Cultured Human Skin Fibroblasts*

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Cell culture offers an opportunity for studying connective tissue disorders involving fibroblasts. Hurler's syndrome, an inborn error of mucopolysaccharide metabolism, is an inherited disease resulting in an accumulation of mucopolysaccharides in various tissues of the body (1–3). It has recently become evident that skin fibroblasts from affected individuals and carriers of the abnormal gene grown in cell culture contain metachromatic granules when stained for mucopolysaccharides with toluidine blue O (4). The preservation of the biochemical abnormality of Hurler's syndrome in cell culture should permit a chemical investigation of this inherited mucopolysaccharidosis at the cellular level.

Comparative studies on fibroblasts from normal subjects and patients with Hurler's syndrome provide a convenient method to amplify the present information on the environmental conditions which modify mucopolysaccharide synthesis. Fell and her colleagues (5–8), using the techniques of organ culture, found that when embryonic cartilage was grown in medium containing retinol (vitamin A alcohol), there was a marked disappearance of metachromatic material from the matrix which was associated with a 50% loss of intracellular mucopolysaccharides.

The purpose of this paper is to relate cellular mucopolysaccharides, measured quantitatively by chemical analyses, with cellular metachromasia of human skin fibroblasts and using these techniques, to study the effect of retinol on cellular mucopolysaccharides of skin fibroblasts derived from normal individuals and patients with Hurler's syndrome.

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Materials and Methods

Eight families with members affected with Hurler's syndrome have been studied. Four of the families (M. P., F. F., F. S., and P. H.) had been previously investigated in this laboratory as part of a genetic study in cell culture (4). In seven families (M. P., P. T., D. L., F. F., J. P., F. S., and P. O.) the affected child had the clinical and biochemical features compatible with the X-linked recessive form of the disease. One family (P. H.) with the autosomal recessive form of Hurler's syndrome was studied.

Skin biopsies were obtained from normal subjects, the patients with the two forms of Hurler's syndrome, and certain of their relatives. The establishment of cell lines from skin biopsies by standard culture methods has been described previously in detail (4). The cell lines studied had been grown as monolayer cultures from 1 to 12 months (4 to 29 subcultures by trypsinization) prior to this study. The initial cell innoculum per flask was 2×10^4 cells per ml. The cells were grown in Eagle's medium (9) with 10% newborn calf serum. When the cultures were established, cover slips were introduced into each flask for cytological study.

The monolayer cultures were allowed to grow for 4 to 5 wk in the same culture vessel without subculturing; the medium being changed completely twice a week. During this period cover slips were removed and stained for intracellular mucopolysaccharides with the metachromatic dye, toluidine blue O (4) and analyses of mucopolysaccharides of replicate cell cultures were performed. Four cultures were pooled for each analysis. At the end of 1 month of culture, the cells formed a compact "tissue" containing approximately 2×10^7 cells per culture.

Chemical analyses of mucopolysaccharides were performed on the cells and medium separately. The nutrient medium was decanted; the cell layer treated with trypsin (0.25%) for 10 min and then the sheet of cells shaken into a suspension. 1 ml aliquots of this suspension were taken for determination of cell protein and cell number. The sample used for the determination of cellular protein was centrifuged and washed once with balanced salt solution. The cell pellet was dispersed in a 1% aqueous solution of sodium desoxycholate and analyzed by the method of Lowry et al. (10). Cell number was determined using a Coulter counter. The remainder of the cell suspension was centrifuged and the trypsin added to the culture medium for analysis, as it had been determined that the trypsin solution contained less than 3% of the total mucopolysaccharides.

The mucopolysaccharides were isolated by the method of Wessler (11). Pronase (0.2 mg per ml, Calbiochem, Los Angeles) was added to the medium. The medium was dialyzed against 0.02 M tris/HCl buffer (pH 8.0) containing 0.1 M NaCl at 37°C for 24 hr. The medium was then concentrated by rotary vacuum evaporation at 40°C to approximately one-tenth the original volume. Pronase (0.5 mg per ml) was added to the concentrated medium and dialysis continued for at least an additional 24 hr. During the last 12 hr the sample was dialyzed against distilled water. The sample was then transferred to a centrifuge tube and the mucopolysaccharides precipitated with cetylpyridinium chloride. The precipitate was washed three times with 95% ethanol saturated with NaCl and the final precipitate dissolved in water (12). The cell pellet was suspended in 2 ml of 0.2 M tris/HCl buffer containing 40 mg pronase and incubated at 37°C for at least 24 hr. The digest was processed in the same manner as the medium with exception that concentration was not necessary. Total polysaccharide was estimated as uronic acid by the carbazole method (13).

The use of two labeled precursors enabled the study of two different steps in the biosynthesis of the mucopolysaccharide molecule (14). Acetate-H^3 (320 μc per ml medium) was used to measure synthesis of polysaccharides as this label is incorporated into the acetyl group of the hexosamine in the polysaccharide molecule. Sulfate-S^{35} (30 μc per ml medium) was used to monitor the addition of the ester sulfate group to the amino acid sugar residue of each repeating unit of the molecule. These two labels were added to the medium for the last 50 culture

hr before harvesting the cultures for analyses. The incorporation of these radioactive labels was assayed by counting their specific activities in purified samples of mucopolysaccharides prepared by a combustion method (15).

After initial analyses, the samples were reprecipitated with cetylpyridinium chloride, repurified, and the analyses repeated. A loss of less than 5% of the specific activity was noted on repeated purifications of the mucopolysaccharide samples.

The influence of retinol on mucopolysaccharide synthesis of skin fibroblasts has been studied in 3 patients (M. P., P. T., and J. P.) with the X-linked recessive form of Hurler's syndrome, and 3 normal individuals of the same age and sex. These fibroblast cultures had been grown in continuous culture for 3 months (8 subcultures by trypsinization) prior to these experiments. In the retinol studies, a commercial preparation of vitamin A alcohol (3.3×10^6 IU/gm, Mann Research Labs., New York) was dissolved in ethanol to give a final concentration of 10 IU per ml and 60 IU per ml in the culture medium. The strength of the alcoholic solution was such that the final medium contained 0.01 to 0.02% ethanol. Retinyl ester (vitamin A palmitate) in the same concentration but in aqueous solution was used in several experiments. Cultures were also grown in control medium to which neither retinol nor alcohol was added. In chemical studies the cells were grown in medium containing 60 IU retinol per ml for 5 wk prior to analyses.

RESULTS

Standardization of Culture Conditions for Study of Mucopolysaccharides in Cell Culture.—In preliminary experiments mucopolysaccharide synthesis was found to be influenced by the cultural conditions employed. These variables were standardized in order to obtain comparable, reproducible cellular activity. Cell growth as measured by cell number (Text-fig. 1) was essentially the same for all fibroblasts studied.

Time after Subculture.—Because the length of time after subculture by trypsinization was found to influence the amount and distribution of cellular mucopolysaccharides, cultures were analyzed without subculture for cell metachromasia and uronic acid content over a 2 month period.

Cytology: Cultures of skin fibroblasts derived from normal individuals contained cells predominantly showing no cytoplasmic metachromasia after 1 wk in culture. An occasional cell showing metachromatic granules was observed as described previously (4). During the following 5 wk without subculture, these cells grew into a dense tissue but showed essentially no metachromasia (Fig. 1).

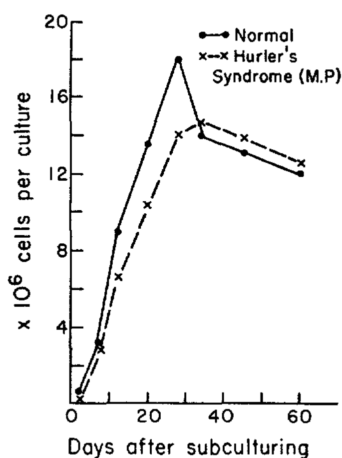
Cultures derived from the skin of patients with Hurler's syndrome (X-linked recessive, and autosomal recessive forms) contained large numbers of fibroblasts with metachromatic granules in the cytoplasm after 1 wk. During the next 5 wk culture period, the monolayer became a dense tissue. Each fibroblast became packed with metachromatic material until all the cells appeared swollen (Fig. 1). The stained culture gave the appearance of a "red rug".

Metachromatic inclusions were seen in cultures of fibroblasts derived from members of the families who were considered to be heterozygous or hemizygous for the abnormal gene. There was a marked increase in the metachromatic granules in each positive cell during the 5 wk culture period but, unlike the

findings in affected individuals, the per cent of fibroblasts containing metachromatic granules remained approximately constant under these conditions.

Chemical studies: The total mucopolysaccharide content, measured as uronic acid per cell, of fibroblasts derived from normal individuals remained relatively constant (Text-fig. 2). The cells contained approximately 14% of that found in the culture medium. The amount of uronic acid in the normal cells was little influenced by the length of time after subculture.

There was a progressive increase in the mucopolysaccharide content of fibroblasts from patients with Hurler's syndrome during the first month after



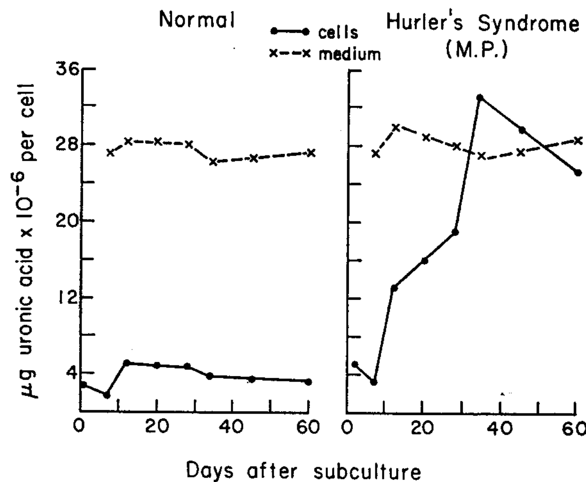
TEXT-FIG. 1. Growth of skin fibroblasts in cell culture derived from a patient, M. P., (X-linked recessive form of Hurler's syndrome), and a normal subject of the same age and sex. Cells grown in culture for 4 months prior to study.

subculture (Text-fig. 2). During the second month the amount of intracellular mucopolysaccharide remained relatively constant. The cells at this time contained 70 to more than 100% of that found in the culture medium.

Sequential analyses of the culture medium during the 2 month culture period revealed that, as in normal subjects, the amount of mucopolysaccharides in the medium measured, as uronic acid per cell, remained relatively constant (Text-fig. 2). Moreover, there was no quantitative difference in the mucopolysaccharides in the medium of cultures derived from normal individuals, and those from patients with Hurler's syndrome. The amount in the medium appeared to be independent of cellular content.

Time after Establishment of Primary Cell Line.—Since preliminary experiments indicated that the length of time the cells had been grown in culture after the establishment of a primary cell line influenced the total content of cellular mucopolysaccharides, this aspect of the problem was studied in more detail.

Cytology: The establishment of a primary cell line from a skin biopsy required approximately 4 to 8 wk, irrespective of the source of tissue. The degree of cellular metachromasia was followed from the establishment of a cell line to senescence, which usually occurred 10 to 12 months after the biopsy was taken (25 to 30 subcultures by trypsinization). Skin fibroblasts derived from a normal individual did not show any change in morphology or growth during the first 35 wk. Then the cells started to enlarge and cell multiplication decreased. By



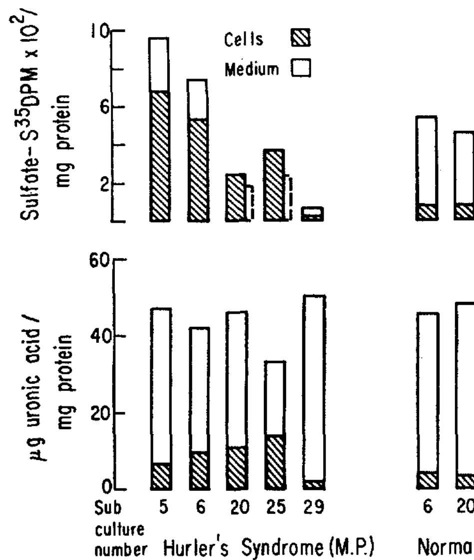
TEXT-FIG. 2. Mucopolysaccharide content of cells and medium measured as uronic acid of skin fibroblast cultures derived from a patient, M. P., (X-linked recessive form of Hurler's syndrome) and a normal subject of the same age and sex grown for 2 months without subculture. Cells were grown in culture for 4 months prior to study.

the 45th wk, the cultures showed large cells with strikingly ametachromatic cytoplasm.

Skin fibroblasts derived from patients with Hurler's syndrome showed a progressive increase in intracellular metachromasia throughout repeated subcultures. Although by the 45th wk of growth, cell division was greatly reduced, the cells were large and packed with metachromatic material.

Chemical studies: Throughout 8 months of continuous growth the fibroblasts from normal individuals showed a relatively constant mucopolysaccharide content measured as total uronic acid and by measurement of the incorporation of radioactive sulfate into the mucopolysaccharide molecule (Text-fig. 3). Moreover, mucopolysaccharide content of the cultures did not depend on length of time in culture or number of subcultures. As found with normal individuals, there was no increase in the total mucopolysaccharide content of fibroblast cultures derived from patients with Hurler's syndrome, although the intracellu-

lar mucopolysaccharide content was markedly increased (Text-fig. 3). This increase was gradual and became most apparent between the 2nd and 6th month of culture when the intracellular mucopolysaccharides increased 2 to 9 times. This increase was followed by a marked decrease as the cultures showed signs of senescence. Whereas synthesis of polysaccharides seemed relatively constant until senescence, sulfation decreased as time in culture increased (Text-fig. 3). The skin fibroblasts derived from hemizygotes showed a similar but less marked



TEXT-FIG. 3. Mucopolysaccharide content (measured as uronic acid) and synthesis (measured as incorporation of sulfate-S^{35}) of skin fibroblast cultures derived from a patient, M. P., (X-linked recessive form of Hurler's syndrome), and a normal subject of the same age and sex over a 12 month period of continuous growth.

increase in intracellular mucopolysaccharides the longer the fibroblasts were grown in culture.

Effect of Cell Density.—Since preliminary cytological observations had suggested that cells which formed dense monolayers showed increased cellular metachromasia, the specific effect of cell density was studied.

Cytology: The cells from normal individuals remained ametachromatic irrespective of cell density. In contrast, fibroblasts from affected individuals showed a decreased cytoplasmic metachromasia when the cell density was low and a continuous monolayer was not formed. If however, such cells formed a dense monolayer after subculture, the cells again contained metachromatic material.

Chemical studies: Cell density did not influence the mucopolysaccharide

synthesis of fibroblasts from normal individuals; whereas, the fibroblasts derived from an affected individual showed an increased mucopolysaccharide content with increased cell density. This increase in mucopolysaccharide was apparent in the cells and in the culture medium (Table I). At low cell densities

TABLE I

Influence of Cell Density on Uronic Acid Content of Skin fibroblasts in Culture

Normal individual					X-Linked recessive form of Hurler's syndrome (M.P.)				
Cell No. × 10^5 per flask		μg uronic acid × 10^{-6} per cell			Cell No. × 10^5 per flask		μg uronic acid × 10^{-6} per cell		
Initial	Final	Cells	Medium	Total	Initial	Final	Cells	Medium	Total
Low									
1	20	6.5	27.0	33.5	1	41.0	8.2	54.0	62.2
Medium									
5	58	4.8	29.0	34.8	5	100.0	15.9	43.0	58.9
High									
10	80	4.6	45.0	49.6	10	63.0	18.0	116.0	134.0
15	100	4.4	32.0	36.4	15	130.0	19.2	125.0	144.2

TABLE II

Cellular Uronic Acid of Skin Fibroblasts Cultures from 7 Families with X-Linked Recessive Form of Hurler's Syndrome and 1 Family with Autosomal Recessive Form of Hurler's Syndrome: All Analyses Done on Cells between 6 to 10 Subcultures

Type of Hurler's syndrome	No. of individuals studied	Total No. of determinations	μg uronic acid/mg cellular protein
X-linked recessive:			
Propositus	7	12	18.2 ± 5.4
Hemizygote	7	8	16.5 ± 9.5
Normal Relatives	9	12	5.3 ± 0.9
Autosomal recessive:			
Propositus	2	4	11.2 ± 2.1
Heterozygote	3	6	9.1 ± 3.4
Normal Relatives	3	8	5.5 ± 1.1

fibroblasts from both normal subjects and patients with Hurler's syndrome multiplied more rapidly.

Mucopolysaccharides in Cell Cultures.—After standardization of the cell density and the environmental conditions of growth, the mucopolysaccharide synthesis of cells in culture from normal subjects, affected individuals and relatives considered to be hemizygous and heterozygous were compared (Table II).

Prior to this study all cells had been grown in continuous culture for 2 to 3 months (7 to 11 subcultures by trypsinization).

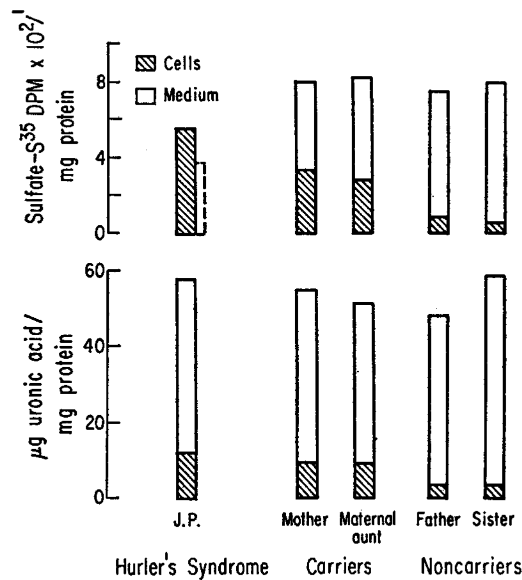
In the fibroblast cultures derived from 12 normal individuals, the total intracellular mucopolysaccharides remained relatively constant, 5.2 ± 1.0 μg per mg cellular protein. The uronic acid content of the culture medium, 58.0 ± 5.6 μg uronic acid per mg cellular protein, was 10 times greater than that in the cells and showed little fluctuation. In cultures derived from 6 patients with the X-linked recessive form of Hurler's syndrome, the cells contained approximately 3 times more uronic acid (18.2 ± 5.4 μg per mg cellular protein) than normal fibroblasts. However, the uronic acid in the medium averaged 60.0 ± 34.7 μg per mg cellular protein which was essentially the same as the uronic acid content of the medium of normal fibroblast cultures. In the cultures from the two individuals with the autosomal recessive form of Hurler's syndrome, the cells from the affected individuals contained at least 2 times more uronic acid (11.2 ± 2.1 μg per mg cellular protein) than cells from their normal relatives. The uronic acid content of the medium (46.9 ± 42.3 μg per mg cellular protein) showed great variation and was similar to the variation found in cultures from normal cells. Thus in both forms of Hurler's syndrome studied, although the total uronic acid content of the fibroblast cultures derived from affected individuals was not increased, the intracellular uronic acid was increased compared to that found in cells from normal individuals.

In the families with the X-linked recessive form of Hurler's syndrome, 7 sets of parents were studied. The uronic acid content of the fibroblasts from hemizygous mothers (16.5 ± 9.5 μg per mg cellular protein) was only slightly less than that of their affected sons. The fibroblasts from the noncarrier fathers and sibs had an intracellular uronic acid content (5.3 ± 0.9 μg per mg cellular protein) similar to that of unrelated normal individuals. The uronic acid content of the media (mothers, 92.0 ± 39.4 μg per mg cellular protein; fathers, 58.7 ± 21.4 μg per mg cellular protein; sibs, 58.0 ± 5.6 μg per mg cellular protein) showed minor variations which were not considered significant.

In the family with the autosomal recessive form of Hurler's syndrome, the uronic acid content of the fibroblasts from the heterozygous mother, father, and paternal first cousin (9.1 ± 3.4 μg per mg cellular protein) were similar to that of the affected sons. Uronic acid of the medium (62. 9 ± 36.5 μg per mg cellular protein) was similar to that found in cultures from their affected sons and from normal relatives.

Measurement of mucopolysaccharide synthesis (Text-fig. 4) of cells derived from several members of a family with the X-linked recessive form of Hurler's syndrome indicated that the total amount of mucopolysaccharides synthesized, measured as incorporation of either acetate-H^3 or sulfate-S^{35}, appeared to be the same irrespective of the donor, but the distribution between the cells and medium was markedly different. In fibroblast cultures from normal individuals, in this case the noncarrier father and sister, the mucopolysaccharides synthe-

sized in the cell rapidly accumulated in the medium. Whereas in cultures derived from the affected individual (J. P.), the sulfated mucopolysaccharides tended to stay in the cell resulting in an increase in intracellular mucopolysaccharides. The distribution of sulfated mucopolysaccharides did not appear to be dependent or influenced by the concentration in the medium as there was a marked variation in the mucopolysaccharide content of the medium without any evident influence on the distribution of sulfated mucopolysaccharides. This



TEXT-FIG. 4. Mucopolysaccharide content (measured as uronic acid) and synthesis (measured as incorporation of sulfate-S^{35}) of skin fibroblast cultures derived from members of the J. P. family (X-linked recessive form of Hurler's syndrome) grown for 5 wk without subculture. (Radioactive label added to medium for last 50 culture hr.)

intracellular retention of newly synthesized sulfated mucopolysaccharides was noted in cells from hemizygotes as well (Text-fig. 4).

Preliminary studies indicated that retention of newly sulphated mucopolysaccharides also occurred in cells derived from patients with the autosomal recessive form of Hurler's syndrome.

The Effect of Retinol on Synthesis of Mucopolysaccharides in Cell Culture.—As no individual differences were noted in the influence of retinol on fibroblasts from 3 normal individuals and 3 patients with the X-linked recessive form of Hurler's syndrome, the results were pooled and considered in two groups; the effect of retinol on fibroblasts derived from normal subjects and those derived from patients with Hurler's syndrome.

Cytology: The addition of retinol to the medium did not influence the morphol-

ogy or cytological appearance of skin fibroblasts from normal individuals. 1 month after transfer of normal cells to a medium containing retinol, the cells formed a compact layer and continued to show no cellular metachromasia and little cytoplasmic vacuolization. Although, as in normal cells, retinol did not appear to influence the cellular morphology of fibroblasts from patients with the Hurler's syndrome, its influence on cellular metachromasia was conspicuous (Fig. 2, Table III). 1 month after subculture control medium, the cells derived from Hurler's syndrome were packed with metachromatic granules so that the stained culture gave the familiar appearance of a "red rug". Duplicate cultures

TABLE III

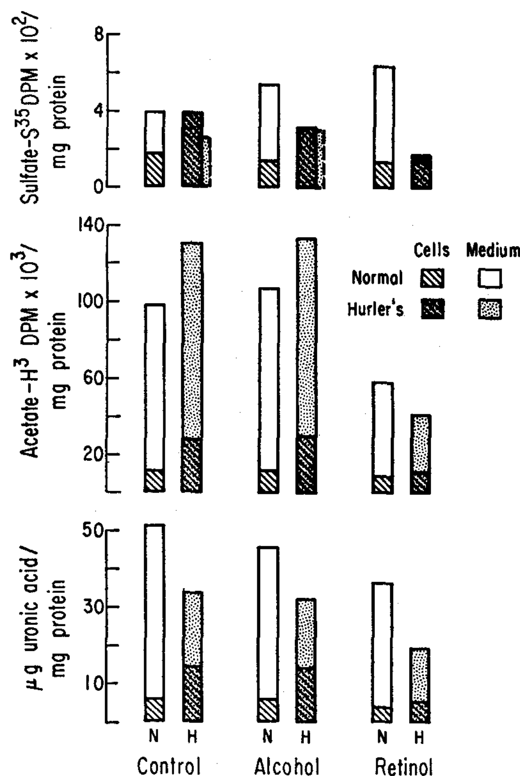
Metachromatic Granules in Skin Fibroblasts from a Patient (M.P.) with the X-Linked Recessive Form of Hurler's Syndrome

Medium		Metachromatic cells, % (1000 cells counted)							
		Days after subculture							
Retinol	Alcohol	1	5	14	21	30		37	52
IU/ml	%								
0	0	65	65	67	88	95	All cultures transferred to control medium	98	95
0	0.02	66	68	68	92	99		97	94
30	0.01	57	29	34	4	4		28	95
60	0.02	50	25	23	3	4		18	96
Retinyl ester									
60	0	70	72	72	94	99		99	97

grown in medium to which alcohol (0.02%) had been added showed a similar degree of cellular metachromasia. However, fibroblasts grown in medium containing retinol gradually lost their cellular metachromasia so that 1 month after subculture the cells appeared ametachromatic and could not be distinguished, by the degree of cellular metachromasia, from fibroblasts derived from a normal individual. The ametachromatic cells contained clear cytoplasmic vacuoles (Figs. 2 and 3) which appeared to represent regions where mucopolysaccharides had been stored. With prolonged culture in the presence of retinol, the cells gradually lost their cytoplasmic vacuoles. The influence of retinol on cellular metachromasia was not concentration dependent (Table III). Retinyl ester in the same concentration, but in aqueous solution, had no noticeable effect on cellular metachromasia. The loss of cellular metachromasia under the influence of retinol was completely reversible. 22 days after the cells had been transferred to a medium lacking retinol, the cellular

metachromasia had reverted to that seen when the cells were grown continuously in normal medium (Fig. 2, Table III).

Chemical studies: When fibroblasts derived from normal individuals were grown in medium containing retinol for 5 wk, the total mucopolysaccharide content was sharply reduced (Text-fig. 5). The uronic acid content of the cells



TEXT-FIG. 5. The influence of retinol on mucopolysaccharide content (measured as uronic acid) and synthesis (measured as incorporation of acetate-H^3 and sulfate-S^{35}) of skin fibroblast cultures derived from a patient, M. P., with the X-linked recessive form of Hurler's syndrome, (H), and a normal individual (N) of the same age and sex. Analyses done at end of a 5 wk culture period. (Radioactive labels added to medium for last 50 culture hr.)

and medium were both decreased by approximately 30%. The incorporation of acetate-H^3 indicated that total polysaccharide synthesis was reduced approximately 40%. Although the incorporation of sulphate-S^{35} into intracellular mucopolysaccharides was decreased by the same order of magnitude, approximately 35%, the mucopolysaccharides which accumulated in the medium showed no decrease in sulphation.

In cultures derived from 3 patients with the X-linked recessive form of

Hurler's syndrome grown in medium containing retinol for 5 wk, the uronic acid content of the cells and medium were also reduced by approximately 60% (Text-fig. 5). The radioactivity measurements indicated that both polysaccharide synthesis and sulfation of the mucopolysacharides were both decreased by approximately 60%.

When retinol was removed from the medium, the cells gradually regained their normal cellular mucopolysaccharide content. After 3 wk, these cells could no longer be distinguished from cells grown in retinol-free media either from their degree of metachromasia, uronic acid content, or ability to incorporate sulfate into the hexosamine moiety of the mucopolysaccharide molecule.

DISCUSSION

With the demonstration that fibroblasts from patients with Hurler's syndrome and individuals heterozygous or hemizygous for the abnormal gene showed increased cellular metachromasia (4), the possibility arose that this increased intracellular mucopolysaccharide should be detectable using quantitative chemical methods. As both hyaluronic acid and chondroitin sulfate have been reported to be synthesized by fibroblasts in culture (16, 17), the uronic acid levels are a reflection of the total polysaccharide present in the cultures. In order to study the sulphated mucopolysaccharides, chondroitin sulfate B and heparitin sulphate, which are increased in the urine of patients with both forms of Hurler's syndrome studied (18), the incorporation of sulphate-S^{35} into the hexosamine moiety of the mucopolysaccharide molecule was followed (14). However, as chondroitin sulfate produced by fibroblasts in cell culture has been found not to be fully sulphated, the results in the present study were considered to be qualitative. Further studies are in progress to evaluate the degree of sulphation of the mucopolysaccharide molecule synthesized under the present standardized culture conditions. As sulphation of acid mucopolysaccharides has been considered to proceed independently of synthesis (16) and at a different rate from polysaccharide synthesis (20), acetate-H^3 was also added to the medium to monitor polysaccharide synthesis.

Preliminary chemical analyses of cells and medium from routine fibroblast cultures failed to reveal any appreciable quantitative difference between normal and Hurler's cells. It was assumed that the amount of mucopolysaccharide in the cells was detectable by the more sensitive staining techniques and that the culture conditions would have to be modified to increase mucopolysaccharide content before chemical analyses would be possible.

Many factors must play a role in modifying mucopolysaccharide production in cell culture (19, 20). In this study only those factors which became evident during the routine culture of fibroblasts have been investigated and standardized. It was our goal to establish the conditions under which fibroblasts would synthesize mucopolysaccharides at a constant and measurable rate.

The early cytochemical studies had shown that cellular metachromasia of Hurler's cells, increased markedly when the cells were allowed to grow without repeated subculture or trypsinization. When such cultures were studied over a 2 month period without subculture, it was found that the amount of intracellular mucopolysaccharides increased, correlating well with the increase in cellular metachromasia (Text-fig. 2). In contrast, the mucopolysaccharide content of the medium remained constant and contained approximately the same amount as that found in the medium from normal fibroblast cultures and was thus not directly related to the intracellular content. Maximum mucopolysaccharide synthesis has been demonstrated in normal embryonic fibroblast cultures during periods of rapid growth (20, 21). However, Morris (20) found that sequential analyses of media from extremely dense, nongrowing stock cultures showed that mucopolysaccharides were produced at a moderately high and constant level. The conditions established in the present study lead to long periods of slow growth, very comparable to those reported by Morris (20). As long as the cultures were actively growing, up to approximately 8 to 10 months of continuous culture, mucopolysaccharide synthesis was relatively constant although there was evidence of decreased incorporation of sulfate in cultures from patients with Hurler's syndrome grown for several months in culture (Text-fig. 3). By the time the culture showed signs of senescense, mucopolysaccharide synthesis had decreased. These observations are consistent with the notation that growth and differentiation are not reciprocal cellular functions (22).

Cell crowding has been reported to decrease mucopolysaccharide synthesis by normal fibroblasts in culture (20). Increasing the initial cell density 15-fold did not influence the mucopolysaccharide synthesis of fibroblasts from normal individuals (Tables I). The increase of intracellular mucopolysaccharide reported (20) to occur in fibroblasts from normal rats when growth rate is low or cell density high was not noted in this study. Increased cell density in fibroblast cultures from Hurler's patients caused a marked increase in the mucopolysaccharide content of both the cells and medium (Table I).

Under standard conditions, the distribution of mucopolysaccharides in cultures from patients with Hurler's syndrome was quite different from that found in normal cells. The cells contained three times as much mucopolysaccharide as the normal fibroblast cultures (Table II). Radioactive labeling experiments which measured polysaccharide synthesis and sulfation substantiated the observation that newly synthesized sulfated mucopolysaccharides tended to remain in the cell (Text-fig. 4). A 3- to 4-fold increase in cellular mucopolysaccharides in fibroblasts cultured from Hurler's syndrome has also been recently reported by Matalon and Dorfman (23). Brante (24) has suggested that the accumulation of intracellular granules may occur because of some molecular abnormality which results in failure of extrusion into the extracellular space.

The present experiments indicated that irrespective of the amount of mucopolysaccharide in the medium, there was an increase in the mucopolysaccharide content of Hurler's cells as compared to normal fibroblasts. Further studies are in progress to determine to what extent this retention occurs in cells from hemizygotes and heterozygotes.

Cellular metachromasia has proved to be a useful genetic marker for studying the mode of inheritance of the different mucopolysaccharidoses (4). In all seven families with the X-linked recessive form of Hurler's syndrome (Table II), cellular mucopolysaccharide content could be used to distinguish the affected individual and the hemizygote from the normal individual (Table IV), although the total mucopolysaccharide content of fibroblast cultures or the amount in the culture medium did not permit this distinction. These results were in accord

TABLE IV

Statistical Analyses: Cellular Uronic Acid Content of Skin Fibroblasts from 7 Families with the X-Linked Recessive Form and 1 Family with the Autosomal Recessive Form of Hurler's Syndrome

Subjects	X-Linked recessive			Autosomal recessive		
	No. of observations	*t* test	*P*	No. of observations	*t* test	*P*
Propositus: normal	24	9.0345	<0.01	12	4.8214	<0.01
Heterozygote: normal	20	3.6451	<0.01	14	2.3750	>0.02, <0.05
Propositus: heterozygote	20	0.4474	>0.5	10	0.6667	>0.50

with the findings on cellular metachromasia (4), where the amount of cellular mucopolysaccharides also could not be used to distinguish the affected individual from the hemizygous state.

In the family with the autosomal recessive form of Hurler's syndrome (Table II), the amount of cellular mucopolysaccharides agreed well with the degree of cellular metachromasia. The affected individual could be distinguished from the normal individual by the amount of cellular mucopolysaccharides (Table IV) but not by the amount in the medium or total mucopolysaccharides of the culture. The heterozygous state, which by cellular metachromasia and chemical analyses appeared to be intermediary in mucopolysaccharide content between the normal fibroblast and the Hurler's cells, could only be distinguished chemically from the normal and not from the affected individual.

Retinol is not regularly added to culture medium, since no nutritional requirement for retinol has been demonstrated in vitro (25). If retinol had been added to the normal culture medium, the increased intracellular mucopolysaccharides in fibroblasts from individuals affected with Hurler's syndrome and carriers (4) might not have been detected.

In studying the influence of excess retinol on embryonic cartilage, Fell and her associates (6) found that loss of cellular metachromasia was preceded by an inability of the cartilage to fix sulfate and was accompanied by a loss of sulfate already present. Our experiments were undertaken to see whether retinol in concentrations similar to those encountered in man in hypervitaminosis A would influence the cellular production of mucopolysaccharides. Since Gribetz et al. (26) have found that patients with hypervitaminosis A had blood levels in the range of 20 to 30 IU per ml, of which 70% was present as the free alcohol, a comparable concentration range (10 and 60 IU per ml) was chosen for this study. The results of these experiments have indicated that retinol decreases mucopolysaccharide synthesis and that this affects the production of polysaccharides and their degree of sulfation (Text-fig. 5). On the basis of changes in cell metachromasia, mucopolysaccharide content and incorporation of radioactive material into newly synthesized mucopolysaccharide, there appeared to be a gradual decrease in the synthesis of cellular mucopolysaccharides rather than a sudden liberation of cellular mucopolysaccharides from the Hurler's cells as might be expected if the action of retinol was primarily on the lipoprotein membranes of the cell (27). In agreement with previous reports (7), vitamin A had to be in a lipid soluble form to have any influence on cellular mucopolysaccharides as retinyl ester (water soluble) had no detectable influence on cellular metachromasia. Alcohol, in the concentration used in the retinol experiments, affected neither cellular metachromasia nor mucopolysaccharide content.

Many aspects of the role of retinol in the control of normal tissue matrix are not fully understood. Its role in the synthesis and liberation of mucopolysaccharides from cells loaded with mucopolysaccharides, as in the case of Hurler's syndrome, remains obscure. Several possibilities should be considered in addition to its influence on cellular membranes. It has been reported that in vitamin A deficiency there may be an inhibition of the release of synthesized proteins from cells (28). As the suggestion has been made (24) that the defect in Hurler's syndrome lies in the protein moiety of the chondroitin sulfate–protein complex, the influence of retinol on this protein portion should be investigated. Further studies are in progress to clarify the site of action of retinol on mucopolysaccharide biosynthesis and storage.

These studies raise the possibility that the administration of retinol to patients with Hurler's syndrome might favorably influence the synthesis and storage of cellular mucopolysaccharide in this condition. This possibility is being investigated.

SUMMARY

Skin fibroblasts from eight families with Hurler's syndrome (X-linked recessive and autosomal recessive) and normal individuals have been studied in cell culture. A good correlation between cellular metachromasia and the quanti-

tative estimation of intracellular mucopolysaccharides was observed provided the culture conditions were standardized.

In both forms of Hurler's syndrome intracellular mucopolysaccharide content could be used to distinguish the affected individual and the carrier from the normal subject, although the total mucopolysaccharide content of fibroblast cultures or the amount in the culture medium did not permit such a distinction.

Retinol, in concentrations similar to those encountered in man in hypervitaminosis A, caused a reduction in total mucopolysaccharide content of fibroblast cultures from normal and affected individuals. Cultures from three patients with the X-linked recessive form of Hurler's syndrome showed a gradual but marked decrease in cellular metachromasia and approximately 60% decrease in mucopolysaccharide content. Synthesis of polysaccharides and sulfation appeared to be equally affected. On removal of retinol from the medium the content of intracellular mucopolysaccharides returned to pretreatment levels. The possible relevance of these findings to the treatment of Hurler's syndrome is discussed.

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EXPLANATION OF PLATES

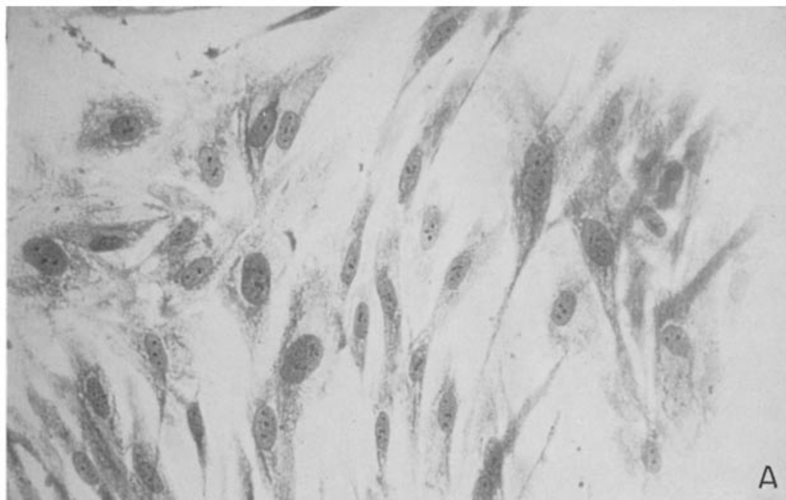
PLATE 130

FIG. 1. Monolayers of skin fibroblasts grown for prolonged periods in culture without subculture. Preparations stained with toluidine blue O.

A. Fibroblasts from a normal individual grown for 1 month in culture without subculture. No metachromasia. × 1000.

B. Fibroblasts from a patient, M.P., with the X-linked recessive type of Hurler's syndrome, grown in culture for 1 month without subculture. Every cell contains metachromatic material. × 1000.

C. Fibroblasts from a patient, M.P., with the X-linked recessive type of Hurler's syndrome, grown in culture for 2 months without subculture. Cytoplasm packed with metachromatic material which gives a swollen appearance. × 1000.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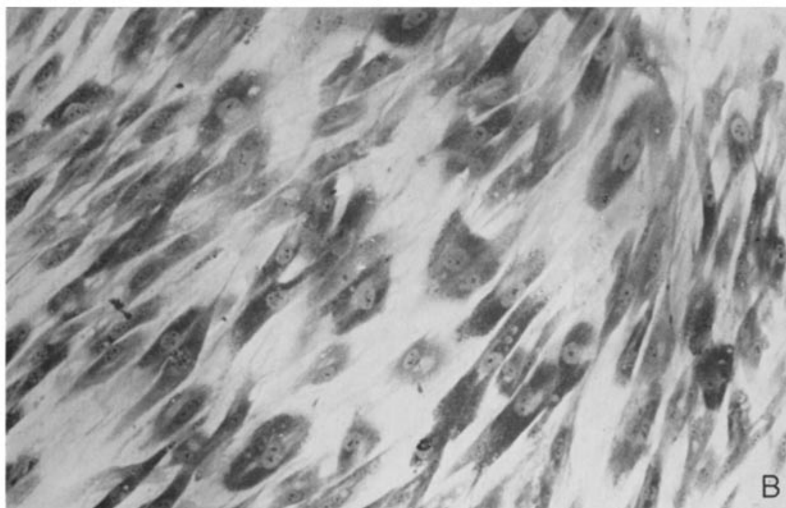
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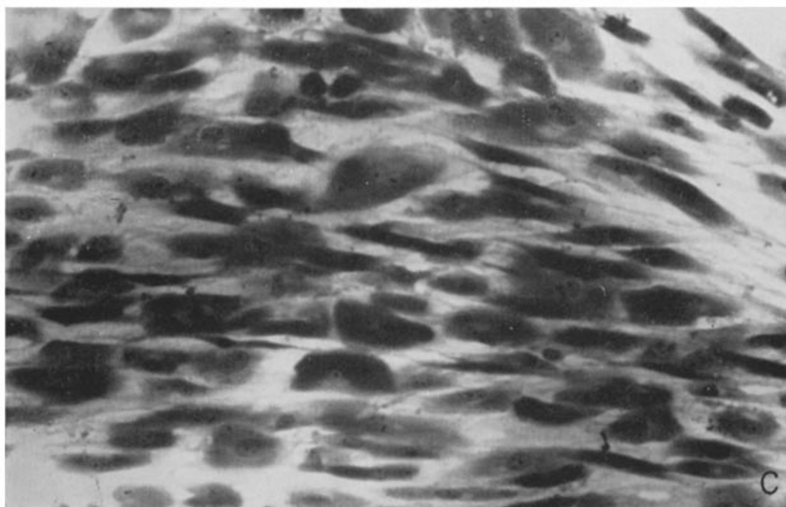
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PLATE 131

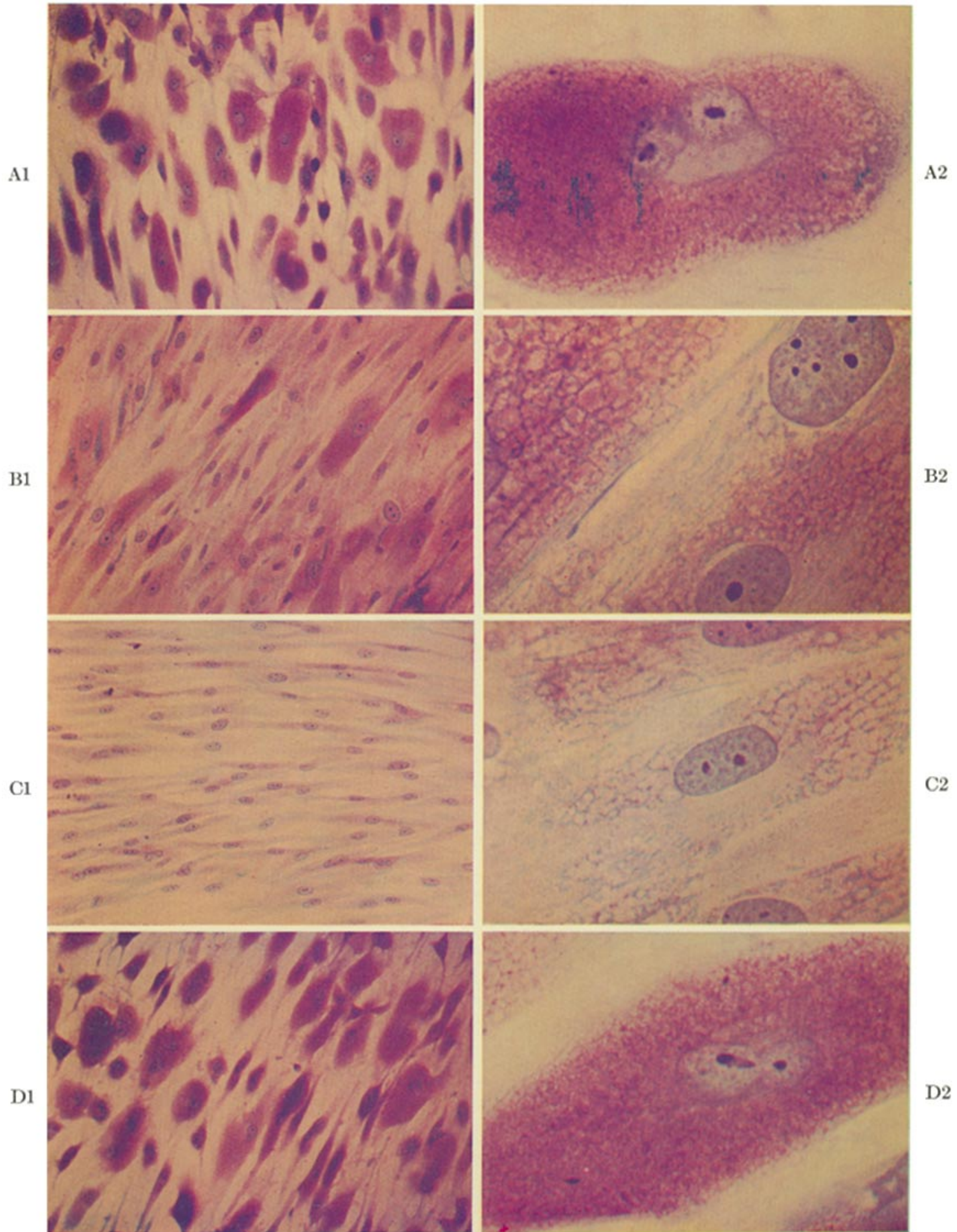
FIG. 2. Monolayers of skin fibroblasts from a patient, M.P., with the X-linked recessive form of Hurler's syndrome, grown in tissue culture. Preparations stained with toluidine blue O.

A. Grown for 5 wk in medium with no retinol added. A 1, general field, × 320; A 2, fibroblast with cytoplasm filled with metachromatic granules, × 2000.

B. Grown for 3 wk in medium with retinol added. B 1, general field, × 320; B 2, fibroblasts showing decreased metachromasia and vacuoles in the cytoplasm, × 2000.

C. Grown for 6 wk in medium with retinol added. C 1, general field, × 320; C 2, fibroblasts showing ametachromatic cytoplasm and a decrease but not absence of cytoplasmic vacuoles, × 2000.

D. Grown for 4 wk in medium with retinol added and then grown for an additional 3 wk without subculture in medium with no retinol added. D 1, general field, × 320; D 2, fibroblast with cytoplasm filled with metachromatic granules, × 2000.



(Danes and Bearn: Hurler's syndrome)

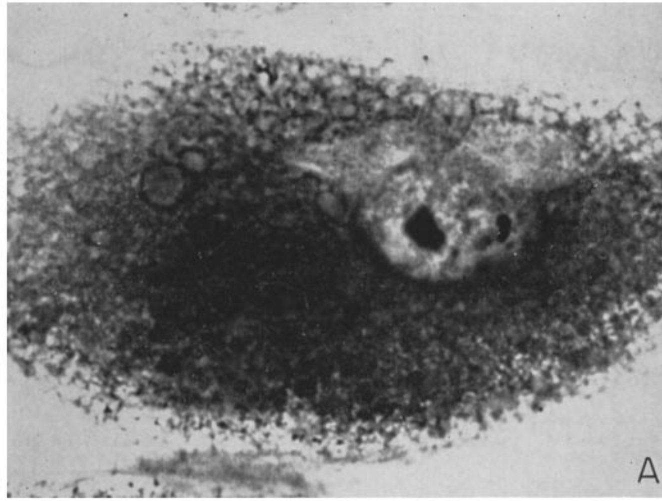
PLATE 132

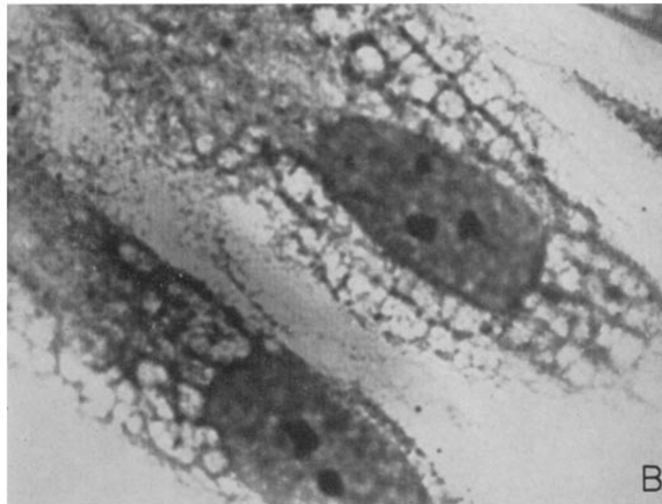
FIG. 3. Cells from monolayer cultures of fibroblasts from a patient, M.P., with the X-linked recessive form of Hurler's syndrome, grown in tissue culture. Preparations stained with toluidine blue O.

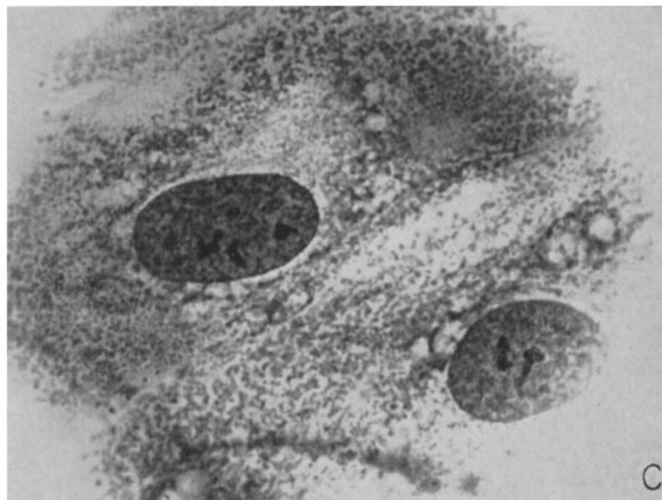
A. Grown in medium with no retinol added for 1 month without subculture. Cytoplasm filled with metachromatic material. × 4000.

B. Grown in medium with retinol added for 1 month without subculture. Cytoplasm contains little metachromatic material but vacuoles are present. × 4000.

C. Grown in medium with retinol added for 2 months without subculture. Cytoplasm contains very little metachromatic material and vacuoles are seen infrequently. × 4000.







(Danes and Bearn: Hurler's syndrome)