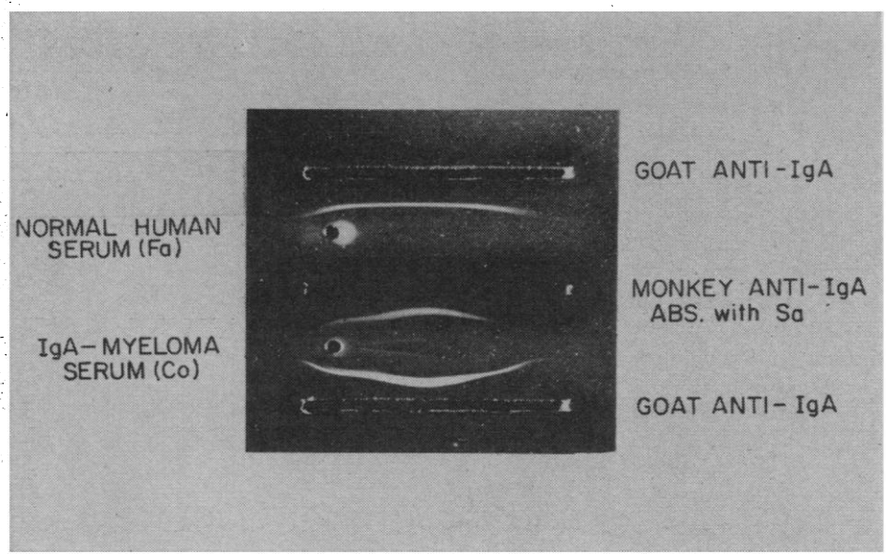

Fig. 2. Immunoelectrophoretic patterns of normal serum and IgA-myeloma serum. The IgA of the normal serum and myeloma serum are precipitated by goat antiserum to IgA. Monkey antiserum to IgA absorbed with serum Sa reacts with the Co IgA-myeloma, but not with normal serum IgA.

arated into heavy and light polypeptide chains by gel filtration through Sephadex G-200 in 1*M* propionic acid. The isolated heavy and light chains were reacted with the absorbed antiserum and neither chain gave a precipitin band in gel diffusion studies. In a separate experiment, protein Fu was reduced and alkylated as described above. The reduced, alkylated protein was not acidified. This protein preparation was tested with the absorbed antiserum and again, no precipitin band was found. Additional experiments showed that Fu-type precipitating activity could not be removed from the antiserum by absorption with Fu light chains or heavy chains. The Fu antigenic determinant, therefore, could not be localized to either the light or heavy polypeptide chain.

Analysis of the light chain types of the 51 IgA-myeloma proteins revealed that the seven Fu type proteins all had λ-type light chains, while the 44 Ma type proteins consisted of 29 with κ-chains and 15 with λ-chains (Table 1). The antiserum was not only detecting the differences between κ and λ antigens, since it reacted with some, but not all, of the λ-type IgA-proteins. The findings that all of the Fu type proteins have λ-type light chains may be due to the small number of serums studied. Additional Fu type proteins should be identified, and their light-chain type should be determined.

An attempt was made to identify Fu type IgA in normal human serum. The absorbed monkey antiserum did not give any visible precipitin bands with a human serum known to contain a normal quantity of IgA (Fig. 2). This normal serum (Fa) was the same one from which the fraction used for immunization was originally obtained. Failure of the antiserum to precipitate with normal serum IgA was confirmed by Ouchterlony studies of several other normal human serums known to contain IgA. However, molecules containing the Fu antigenic determinants must be present in normal serum since (i) the antiserum was prepared by immunization with a normal serum fraction and (ii) precipitin activity can be removed by absorption with normal serum. These facts are interpreted to mean either that only a small number of normal serum IgA molecules contain the appropriate antigenic determinant (Fu type) and that this amount of IgA is not directly demonstrable by the methods employed, or that the reaction of the antiserum with normal serum Fu type molecules leads to a soluble product.

We do not now have a specific antiserum to Ma. Therefore, neither Fu nor Ma type IgA can positively be identified in normal human serum, although both are assumed to be present.

The above findings indicate the existence of at least two antigenically distinct kinds of IgA-myeloma molecules. The chemical nature and molecular localization of these antigenic differences are not known. The Fu antigenic site detected with this monkey antiserum may include part of both the heavy and the light polypeptide chains in a region of the molecule where the chains are in close apposition because of an interchain disulfide bridge. Alternatively, the antigenic site might be present on only one of the chains (presumably the heavy chain) but require stabilization by the other polypeptide chain through a disulfide bond.

Other evidence indicating multiple antigenic forms of IgA-globulin has recently been presented (*4*). It will be of interest to determine whether the subclasses of IgA described by these investigators correspond to the ones presented in this report (*5*).

WILLIAM D. TERRY
MARJORIE S. ROBERTS
Immunology Branch, National Cancer Institute, Bethesda, Maryland 20014

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3 June 1966

Vitamin C–Induced Increase of Dermatan Sulfate in Cultured Hurler's Fibroblasts

Abstract. *In fibroblasts taken from patients with Hurler's syndrome and grown in culture, dermatan sulfate constituted a larger percentage of the total sulfated glycosaminoglycans than it did in cultured fibroblasts from unaffected individuals. Moreover, the addition of ascorbic acid (vitamin C) to the culture medium markedly increased the concentration of dermatan sulfate in the Hurler's fibroblasts but not in the normal fibroblasts. The biochemical phenotype of the Hurler's cells persisted during 28 weeks of serial culture.*

Hurler's syndrome (gargoylism) is an inhibited disorder in the metabolism of acidic glycosaminoglycans (mucopolysaccharides), characterized by a massive deposition in tissues and an increased excretion in the urine of dermatan sulfate with or without heparan sulfate. Accumulation of glycosaminoglycans in the tissues is associated with dwarf-

ism, skeletal deformities, stiffness of the joints, hepatosplenomegaly, corneal clouding, deafness, mental retardation, and cardiac abnormalities. Family studies have identified both X-linked and autosomal recessive forms of inheritance. Both inherited forms of the syndrome appear to be biochemically indistinguishable. As judged by clinical observations, the X-linked form is usually milder and is not associated with corneal clouding (*1*).

The metabolic defect causing this disorder is not defined. Cell cultures of fibroblasts derived from human skin might provide a suitable model for the study of sulfated glycosaminoglycan metabolism in this disorder, if the biochemical phenotype were shown to persist in culture. Recent histochemical studies of cultured fibroblasts derived from the skin of patients with Hurler's syndrome have demonstrated the presence of distinctive, metachromatic cytoplasmic granules after the cells have been stained with toluidine blue O (*2*). Results of earlier work with short-term cultures of lymphocytes were similar (*3*). In neither study was the chemical character of the granules defined. Increased incorporation of sulfate into the pool of sulfated glycosaminoglycans of Hurler's fibroblasts in culture has been reported (*4*). We present evidence that Hurler's cells in culture contain dermatan sulfate, and that ascorbic acid stimulates the synthesis of this sulfated glycosaminoglycan.

Cells for culture were obtained by skin biopsies from the forearms of 4 children with Hurler's syndrome who have the classical symptoms of the disease and who excrete increased quantities of dermatan sulfate in the urine. Three of these patients presumably have the form caused by the autosomal recessive gene(s), while one boy has been proven to have the X-linked recessive gene. Fibroblasts derived from these patients were compared to those obtained from six unaffected individuals. Two specimens were from foreskin; one sample of each of the following: forearm, back, thigh, and a supernumerary finger made up the other four specimens.

Pieces of minced tissue were placed directly in plastic petri dishes in Eagle's minimal essential medium (MEM) (*5*) supplemented with 10 percent fetal calf serum, glutamine, serine, penicillin, and streptomycin, and were incubated at 37°C in 5 percent CO_2. A sufficient number of cells was available for experiments 6 weeks after making the initial explant. Studies were conducted on cells serially propagated for from 6 to 62 weeks. Fibroblasts, removed from the petri dish by treatment with 0.025 percent pronase in a balanced salt solution free of calcium and magnesium were centrifuged, then resuspended in Eagle's MEM. The cells (2.5×10^5 cells in 10 ml of medium) were then transferred to each plate. Five days after subculture of the cells, one half of the cultures received medium to which 50 μg of ascorbic acid per milliliter had been added; the remaining cultures received identical medium but without added ascorbic acid. The fetal calf serum used in these studies contributed 0.6 μg of ascorbic acid per milliliter of medium (*6*). The medium was replaced three times weekly. Fourteen days after the addition of ascorbic acid, the experiments were terminated, medium was removed, and the cell layer was rinsed briefly with Earle's balanced salt solution; the cells were scraped from the plate into distilled water and lyophilized. Each ten petri dishes yielded 60 to 120 mg of dried material. Total sulfated glycosaminoglycans were determined on the glycosaminoglycans isolated from the cells (*7*). The crude glycosaminoglycans were then fractionated on cellulose microcolumns by the technique of Antonopoulos *et al.*, as modified by Svejcar *et al.* (*8*).

Cell lines derived from patients with Hurler's syndrome had many cells with granules which were stained metachromatically with toluidine blue O; they had, in addition, the large "gargoyle" cells described by other investigators (*2*). Ascorbic acid added to the cultures of Hurler's cells increased both the number of metachromatic granules and the intensity of the staining reaction, but these observations have not been quantitated. A few cells derived from unaffected individuals also had metachromatic cytoplasmic granules, but "gargoyle" cells were never seen.

Table 1 presents pooled data on the concentration of total sulfated glycosaminoglycans in cells from six separate experiments. Ascorbic acid had no significant effect on the concentration of sulfated glycosaminoglycan in the normal cells, but produced a significant increase in that of the cultured Hurler's cells. Cell lines derived from two patients with Hurler's syndrome showed the same response to ascorbic acid after both 6 and 28 weeks of serial culture; this finding indicates that the phenotype persisted throughout this period.

Table 1. Effect of ascorbic acid on sulfated glycosaminoglycans in fibroblasts derived from four patients with Hurler's syndrome and from six unaffected individuals.

Medium	Experiments (No.)	Sulfated glycosaminoglycans (μg/mg dry cells)
Normal cells		
Control	8	2.06 ± 0.349*
Control + ascorbic acid (50μg/ml)	8	3.63 ± 1.313†
Hurler's cells		
Control	6	2.57 ± 0.670‡
Control + ascorbic acid (50μg/ml)	6	8.44 ± 1.145§

The results are expressed as the mean ± the standard error. Significance of the differences between † and §, $P < .02$; between ‡ and §, $P < .01$. No significant difference between * and †, and between * and ‡.

The crude glycosaminoglycans in cells from the six unaffected individuals and from the four patients with Hurler's syndrome were fractionated, and the concentration of the fraction containing dermatan sulfate was determined. The identity of dermatan sulfate was confirmed by its resistance to digestion by hyaluronidase and by the fact that a carbazole-naphthoresorcinol ratio of 0.95 was obtained (*9*). Ascorbic acid did not significantly increase the concentration of dermatan sulfate in normal cells, but in the Hurler's cells a distinct increase was found (Table 2). In the presence of added ascorbic acid, a clear difference in the biochemical phenotype is demonstrable between the Hurler's and the normal cells.

In cultures not supplemented with ascorbic acid, the difference in the concentration of dermatan sulfate between the normal and Hurler's cell was not

Table 2. Effect of ascorbic acid on the dermatan sulfate in cells of four patients with Hurler's syndrome and those of six unaffected individuals.

Medium	Experiments (No.)	Dermatan sulfate (μg/mg dry cells)
Normal cells		
Control	6	0.47 ± 0.205*
Control + ascorbic acid (50μg/ml)	6	1.43 ± .453†
Hurler's cells		
Control	4	1.00 ± 0.381‡
Control + ascorbic acid (50μg/ml)	4	5.03 ± 1.285§

The results are expressed as the mean ± the standard error. Significance of the differences between ‡ and §, $P < .02$; between † and §, $P < .05 > .02$. No significant difference between * and †, and between * and ‡.

significant because of the variability between experiments. However, if one considers the proportion of dermatan sulfate to the total sulfated glycosaminoglycans, a pattern characteristic of Hurler's cells became apparent in every experiment. Forty percent of the total sulfated glycosaminoglycans in the Hurler's cells appeared in the fraction containing dermatan sulfate; only 23 percent of the glycosaminoglycans from normal cells appeared in this fraction. This proportional increase in dermatan sulfate in Hurler's fibroblasts would not have become evident without fractionation of the glycosaminoglycans.

We conclude that the biochemical phenotype for Hurler's syndrome, that is, an increased concentration of dermatan sulfate, persists in cells in culture, and its phenotypic expression is reinforced by inclusion of ascorbic acid in the culture medium.

Cultures of normal cells also showed consistent, albeit small, increases in dermatan sulfate concentration when grown in medium supplemented with ascorbic acid. Because a significant quantity of ascorbic acid was present in the fetal calf serum incorporated into the medium, further study is required to determine whether ascorbic acid is necessary for dermatan sulfate synthesis. In studies of whole animals, the concentration of dermatan sulfate has been reported to be lower in the aortas of scorbutic guinea pigs than in those of well-nourished animals, and a decrease in its synthesis has been observed in granulomas produced with polyvinyl sponge in guinea pigs deprived of ascorbic acid (*10*).

Since iduronic acid is a characteristic constituent of both dermatan sulfate and heparan sulfate, the glycosaminoglycans affected in Hurler's syndrome, the basic defect in Hurler's syndrome may be related to the epimerization of glucuronic acid to iduronic acid, and ascorbic acid may participate in this reaction.

IRWIN A. SCHAFER
JULIA C. SULLIVAN
JIRI SVEJCAR
JORGE KOFOED
WILLIAM VAN B. ROBERTSON
Department of Pediatrics,
Stanford University School of Medicine,
Palo Alto, California 94304

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11. Supported in part by grants from the National Foundation, the John A. Hartford Foundation, and from NIH, AM-05223 and HD-00560.

2 May 1966

Surface Area of Human Erythrocyte Lipids: Reinvestigation of Experiments on Plasma Membrane

Abstract. *Ratios of the lipid monolayer area to the erythrocyte surface area are 2:1 at low surface pressures and approach 1:1 at collapse pressures. Unsaturated phospholipids in cholesterol-phospholipid complexes of membrane extracts resemble their saturated derivatives at collapse pressures. Area ratio and phospholipid area data are related by an equation that tests hypothetical values for molecular areas used in membrane models.*

The Davson-Danielli concept of the cell membrane as a lipid bilayer covered with protein is a generally accepted working hypothesis for membrane structure (*1*), and it is supported by the classic experiments of Gorter and Grendel (*2*), who extracted lipids from erythrocytes, spread the extract as a monomolecular film, and determined the ratio of film area to erythrocyte area. This ratio would be 2 : 1 for a bimolecular layer of lipids at the erythrocyte surface. Since a 2 : 1 ratio was found with erythrocytes from a number of mammalian species, Gorter and Grendel suggested that sufficient lipid was present for a bimolecular layer. A different structure was later proposed by Dervichian and Macheboeuf (*3*) who performed similar experiments and found 1:1 ratios which indicated a monomolecular layer at the cell surface. This discrepancy is readily explained by the fact that Gorter and Grendel measured the film area at the first detected surface pressure, whereas Dervichian and Macheboeuf assumed that the lipid film at collapse pressure more nearly resembled a membrane structure. These studies are cited in discussions of membrane structure even though they both have serious limitations in experimental design (*4*). Gorter and Grendel used a selective lipid solvent, acetone, to extract erythrocytes and presented little data on lipid recovery. Dervichian and Macheboeuf extracted an arbitrarily defined "loosely bound" lipid fraction with 10 percent ethanol in ether at room temperature. This lipid, a fraction of unknown composition, represented only 70 to 80 percent of the erythrocyte lipid. Furthermore, new surface area data are now available for erythrocytes. Gorter and Grendel, for example, assumed that the surface area for human erythrocytes was 99 μ^2 whereas recent measurements give 145 ± 8 μ^2 as the surface area (*5*).

In our study, we measured the surface pressure–area isotherms for total lipid extracts from human erythrocytes. Total lipid extracts were used because almost all red cell lipids are localized in the cell membrane (*4*). Force-area curves were then used to calculate the ratio of lipid to erythrocyte area in a reexamination of the Gorter-Grendel and Dervichian-Macheboeuf experiments. Average molecular areas for erythrocyte phospholipids at different film pressures were also calculated from lipid composition and force-area data. Experiments yield data on the molecular area which may be used in developing membrane models. We suggest that a molecular area obtained for membrane lipid at the film pressure corresponding to a bimolecular layer is undoubtedly more acceptable for the calculations than molecular areas extrapolated from synthetic lipid mixtures at arbitrary film pressures (*6*).

Experiments were performed with human blood which was characterized by red cell count, hematocrit, and hemoglobin content. The disodium salt of ethylenediaminetetraacetic acid was the anticoagulant. Red cells were centrifuged and washed several times by resuspension in isotonic saline; the buffy layer was removed by suction. Lipids were extracted from a suspension of known cell count by adding the suspension to six volumes of a mixture of isopropyl alcohol and chloroform