tion. The finding of two homozygous SS individuals is somewhat surprising, but we consider this to be a matter of chance. In no case were exceptions to the genetic theory observed.

These results might open new opportunities for the study and utilization of prealbumins—for instance, in genetic research and disputed paternity cases.

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Hurler's Syndrome: Demonstration of an Inherited Disorder of Connective Tissue in Cell Culture

Abstract. *Skin fibroblasts from three patients with Hurler's syndrome were grown in tissue culture and shown to contain metachromatic granules when stained for mucopolysaccharides with toluidine blue O. Similar inclusions were observed in cultures of fibroblasts from other members of the families, who appeared to be clinically normal but who were, judged from studies of pedigree, heterozygous or hemizygous for the abnormal gene.*

Tissue culture appears to provide an opportunity to study in vitro inborn errors of metabolism (*1*). If the expression of abnormal genes present in vivo can be detected in vitro at the cellular level, then such markers may be used to study both the genetic mode of inheritance of the trait and the nature of the metabolic defect. With present techniques for tissue culture, fibroblasts derived from biopsy of skin are the most consistent source of cells for studies in vitro. Inborn errors of metabolism involving connective tissue cells should be particularly amenable for study in cell culture.

Hurler's syndrome (gargoylism) is a rare inborn error of metabolism (*2*) that results in an accumulation of mucopolysaccharides in various tissues of the body; its clinical features are dwarfism, grotesque skeletal deformity, restriction of joint movements, deafness, hepatosplenomegaly, cardiac abnormalities, and mental retardation (*3*). Studies of families indicate that the condition may be inherited in an autosomal recessive fashion—classical Hurler's syndrome (*4*); or in an X-linked recessive form—Hunter's syndrome (*5*). Despite some clinical overlap (*6*), the X-linked type is milder and, characteristically, is not associated with corneal clouding. The autosomal recessive form of the disease has been further subdivided into three separable clinical entities (*7*).

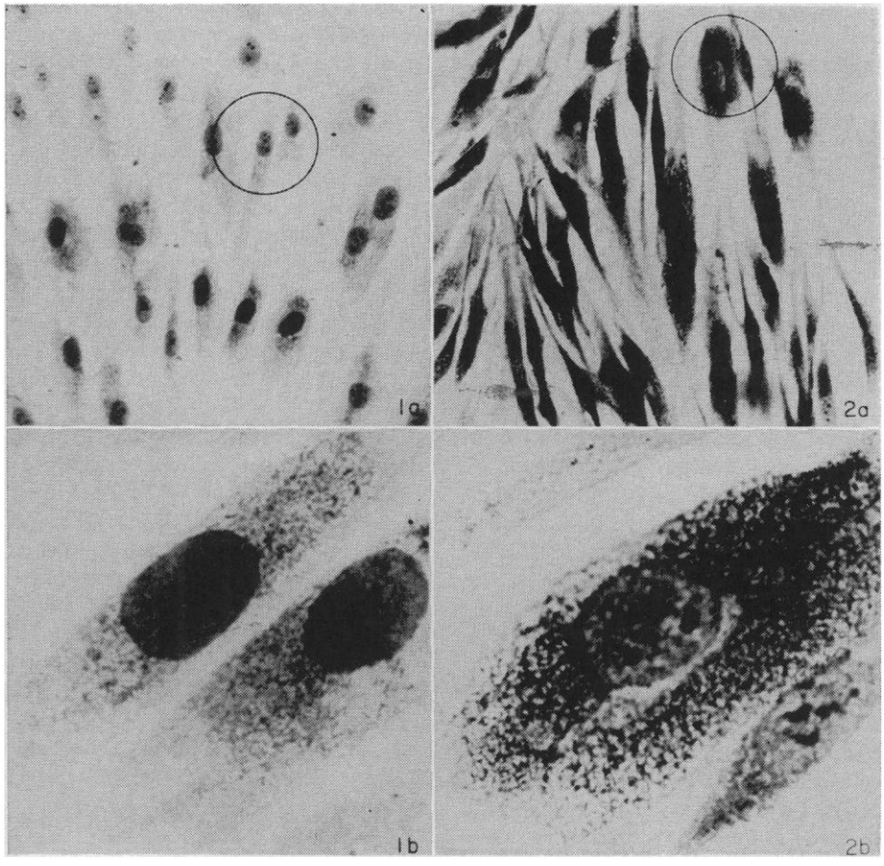
Skin was obtained by biopsy from three patients with various forms of Hurler's syndrome and from certain of their relatives. The first, a 4-year-old girl, had the clinical stigmata, including the presence of cloudy corneas, of the classical, autosomal recessively inherited type of Hurler's syndrome. The other two patients had already been investigated in this laboratory during a clinical and biochemical study of Hurler's syndrome (*8*). One was a 6-year-old boy with the clinical and biochemical signs of the X-linked, recessive form of the disease, Hunter's syndrome; he was only mildly affected, with no corneal clouding, and excreted increased amounts of chondroitin sulfate B and heparitin sulfate in the urine. The third patient, a 13-year-old boy, rather mildly affected physically but severely retarded mentally, excreted an increased quantity of heparitin sulfate in the urine. These symptoms and biochemical findings characteristically are associated with one of the autosomal-recessive forms of the disease sometimes known as the Sanfilippo syndrome (*9*). Skin was obtained by biopsy from the parents of the first two patients and, where possible, from other normal members of the families. Thirteen normal individuals (seven males and six females) served as controls. Each sample of skin (1 by 3 mm) was taken without anesthesia from the extensor surface of the upper arm. The skin was cut into 10 to 20 tiny pieces and cultured in a plasma clot in a Carrel flask by standard techniques (*10*).

After several weeks the explants were surrounded by dense halos of fibroblastic growth; the cultures were then treated with trypsin and transferred to flasks where they became established as monolayer strains. These cultures were grown in reinforced Eagle's medium (*10*) with 10-percent newborn calf serum. No significant differences in cellular activity between the cultures from normal and affected individuals were observed. Karyotypic analysis revealed a normal chromosomal constitution.

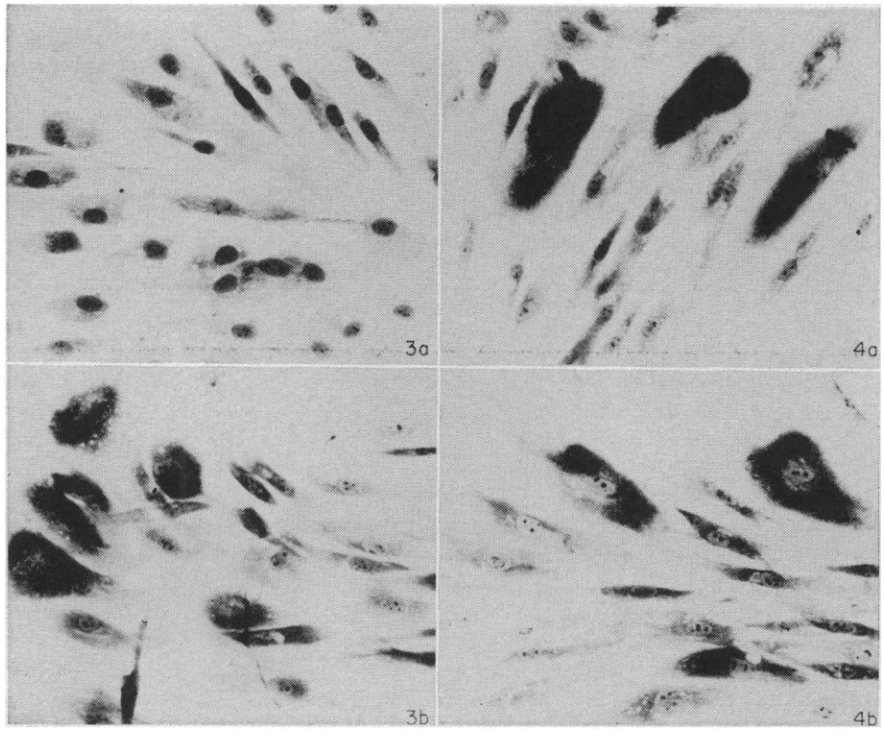
At the time of subculturing, coverslips were introduced into the flasks; from 1 to 7 days later the coverslips were removed and the cells were stained. Each coverslip was washed twice in warm balanced-salt solution, fixed in methanol for 5 minutes, and air dried. The preparation was then stained with the metachromatic dye, toluidine blue O (*11*) (0.1 percent toluidine blue O in 30-percent methanol), for 5 minutes; cleared with acetone,

Table 1. Metachromasia in skin fibroblasts 24 hours after subculturing.

Source of fibroblasts	Incidence in 1000 cells (%)	Extent (%)		
		Granules		Gargoyle cells
		Few (<50 per cell)	Many	
	Hurler, autosomal recessive			
Proband	73	90	8	2
Father	60	67	29	4
Mother	30	90	5	5
	Hurler (Sanfilippo), autosomal recessive			
Proband, biopsy 1	60	65	30	5
biopsy 2	69	87	11	2
biopsy 3	76	70	24	6
	Hurler (Hunter), X-linked			
Proband, biopsy 1	99	42	50	8
biopsy 2	93	68	24	8
Father	0	0	0	0
Mother	49	74	22	4
Paternal grandmother	0	0	0	0
Maternal grandmother	22	80	14	6
Paternal aunt	0	0	0	0
Maternal uncle	3	100	0	0
	Normal			
Controls, averages of 13	0.7	100	0	0



Figs. 1 and 2. Monolayers of skin fibroblasts grown in tissue culture; preparations stained with toluidine blue O to demonstrate intracellular acid mucopolysaccharides, which stain metachromatically (pink). Fig. 1. Fibroblasts from a control. *a*, General field; nuclei and cytoplasm stained light blue; × 160. *b*, Fibroblasts circled in *a*; granules seen in cytoplasm stained light blue; no metachromasia; × 1000. Fig. 2. Fibroblasts grown from a patient with the X-linked type of Hurler's syndrome (Hunter's syndrome). *a*, General field; discrete metachromatic granules in the cytoplasm of each cell; note clear juxtanuclear areas; × 160. *b*, Fibroblasts circled in *a*; cytoplasm densely loaded with metachromatic (pink) granules.



Figs. 3 and 4 (left). Monolayers of skin fibroblasts grown in tissue culture; preparations stained with toluidine blue O. Fig. 3. Fibroblasts from parents of patient with the X-linked type of Hurler's syndrome (Hunter's syndrome). *a*, Father; nuclei and cytoplasm stained light blue; no metachromasia. *b*, Mother; cells with cytoplasm loaded with metachromatic (pink) granules and cells with cytoplasm containing only a few light-blue granules; × 140. Fig. 4. Fibroblasts from parents of patient with the classical, autosomal recessive Hurler's syndrome: *a*, Father; *b*, Mother. In both cultures are cells loaded with metachromatic (pink) granules and cells with a few light-blue granules; × 140.

acetone-xylene, and xylene; and mounted in Permount (*12*).

Cytological evaluation was based on the examination of 100 fields, each containing approximately 100 cells. Intracellular metachromatic granules were graded as follows: (i) absent, (ii) present but few, (iii) many, and (iv) "gargoyle" cells. Thannhauser (*13*) described cells loaded with metachromatic granules, present in histological sections of skin from patients with Hurler's syndrome, as gargoyle cells.

Cultures originating from all 13 controls contained an occasional cell showing metachromatic granules (Fig. 1, Table 1). Approximately 0.1 percent of cultured fibroblasts from ten of the controls had metachromatic granules; cultures derived from the remaining three controls contained 2 to 6 percent fibroblasts with metachromatic granules. These cells, which remained constant in number, could not be distinguished from cells containing metachromatic granules in cultures derived from affected individuals. Gargoyle cells were not found in cultures from controls.

Metachromatic granules occurred in 60 to 90 percent of the fibroblasts in cultures derived from patients with Hurler's syndrome (Fig. 2 and Table 1). Although serial cultures derived from one skin sample showed similar degree of cellular metachromasia, cell cultures derived by biopsies from the same individual at different times showed some slight variation (Table 1). The metachromatic granules appeared to be of uniform size; they were evenly distributed throughout the cytoplasm except for the juxtanuclear area which appeared to lack demonstrable metachromatic material (Fig. 2). Occasionally the granules were clustered in vacuoles.

Fibroblast cultures derived from both parents of one of the patients, in whom pedigree studies indicated an autosomal recessive type of inheritance (Fig. 4

and Table 1), had cells containing metachromatic granules; the cells could not be distinguished morphologically from similar cells in cultures from affected individuals. Gargoyle cells also occurred in small numbers in cultures obtained from both parents (Table 1); because the parents were presumed to be heterozygous for the abnormal gene, the presence of such cells in fibroblast cultures from both parents suggested that the abnormal trait was detectable in the heterozygous state.

Although the fibroblast cultures derived from the father of the patient with the X-linked type of the disease contained no metachromatic cells (Table 1 and Fig. 3*a*), the cultures derived from the mother contained approximately 49 percent of cells with metachromatic granules (Table 1 and Fig. 3*b*). The fibroblast culture from the maternal grandmother of this patient contained approximately 22 percent of metachromatic cells, including some gargoyle cells (Table 1), whereas the culture from the paternal grandmother contained only an occasional metachromatic cell (0.1 percent). Fibroblast cultures from a paternal aunt and a maternal uncle showed negligible metachromasia (Table 1). Proportions of cell populations showing metachromasia remained constant throughout the first 4 months in culture. Other members of this family are now being studied.

Acid mucopolysaccharides stain metachromatically with cationic dyes such as toluidine blue O probably because of the availability of consecutive, regularly spaced anionic groups along the carbohydrate chain (*14*). Although nucleic acids and some acidic lipids of high molecular weight may give a similar but weaker staining reaction, these substances stain purple to violet whereas the sulphated acid mucopolysaccharides stain a strong pink (*15*). It has been shown that fibroblasts from tissues of various origin produce acid mucopolysaccharides in vitro (*16*); and that cultured fibroblasts, regardless of origin, secrete predominantly hyaluronic acid, with only minor secretions of sulphated acid mucopolysaccharides (*17*).

Our observations indicate that, as with cultured lymphocytes (*18*), fibroblasts from patients with Hurler's syndrome have markedly greater contents of acid mucopolysaccharide than those from controls. Moreover, fibroblast cultures from both normal parents of patients having the autosomal recessive form of the disease had detectable metachromatic granules (Fig. 4, *a* and *b*). As to the family of the person having the X-linked type of the disease, the hemizygous state was not demonstrable in vivo but was clearly detectable in vitro. It will be of interest to see whether cloning of the fibroblasts from the mother and maternal grandmother discloses two populations of cells, as has been found in other X-linked genetic traits (*19*).

It is too early to assume that the abnormal gene for Hurler's syndrome can always be detected in cell culture; further studies of more families are required to investigate variation of the expression of the gene under in vitro conditions.

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Response of the Pupil to Steady-State Retinal Illumination: Contribution by Cones

Abstract. *Response of the pupil to steady-state retinal illumination was measured in an observer who lacked functioning rods. At high intensities, this response was as great as that of a normal eye. These results cannot be explained by the hypothesis that only rods are receptors for the steady-state response.*

In 1962 Alpern and Campbell (*1*) summarized the evidence for the idea that the human pupillomotor photoreceptors are both rods and cones. This idea has found general acceptance insofar as responses of the pupil to transient stimuli by light are concerned, but recently Bouma (*2*) questioned it with regard to the steady-state response. Evidence to support the original idea seems overwhelming: (i) steady-state directional sensitivity of the pupillomotor photoreceptors (*3*), (ii) relatively high efficiency of the central retinal area in evoking response by the pupil (*2*), and (iii) continued narrowing of the pupil to increases in light intensity at levels and field sizes that insure that every rod in the retina has been saturated (*2*). Bouma, however, has offered special hypothesis to overcome this evidence, adopting the view that the excitation of rods and of rods alone initiates the steady-state response.

Our experiments demonstrated directly the contribution of cones to the steady-state response; we examined an eye in which exhaustive psychophysical and electrophysiological methods had failed to reveal evidence of functioning rods. This patient (M.L.) has been studied extensively and his color vision, electroretinogram (ERG), electroculogram, and dark-adaptation have been described (*4*). These data together with the results of detailed ophthalmological examination lead to the conclusion that he represents that variety of Oguchi's disease in which no trace of activity by rods appears even after the subject has been in the dark for several hours.

Figure 1*a* shows the change in the absolute visual threshold for a 1.8-degree blue (453-nm) test flash, exposed for 1 second in the nasal field 15 de-