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Inborn Errors of Mucopolysaccharide Metabolism

Faulty degradative mechanisms are implicated in this group of human diseases.

Elizabeth F. Neufeld and Joseph C. Fratantoni

Inherited metabolic diseases generate research activity of far greater intensity than one might expect from their relatively rare occurrence. This is because genetic disorders afford a unique opportunity to combine the concepts of genetics with the tools of biochemistry to study the metabolism of man, as has been so successfully done for the metabolism of microorganisms. The lesson of genetics is clear: genes contain the code for the structure of proteins; a mutation in a gene will result in an alteration of the specific protein to which that gene holds the code. The result may be benign or disastrous, depending on the importance of the protein to the overall metabolism and on the effect of the structural change on its function. Faced with a disease of genetic origin, the biochemist's task is to identify the altered protein which is specific to that disorder. Success may lead both to practical applications in the management of the disease and to a clearer understanding of normal metabolic processes.

Mucopolysaccharide Disorders

The best known and most severe of the inherited disorders of mucopolysaccharide metabolism is the Hurler syndrome, named after the pediatrician, Gertrud Hurler, who described it in great detail in 1919 (*1*, *2*). After several months of normal development, the infant deteriorates physically and mentally and gradually acquires an extraordinary appearance. The head is large with a flat bridge of the nose, wide-set eyes, large lips, and coarse tongue. The nasal deformity causes obstruction to breathing and may be the first abnormality noted by the parents. Growth is stunted, corneas become cloudy, and hearing deteriorates. There is widespread skeletal involvement, with stiff joints, widened ribs, and aberrant development of the vertebrae and long bones. The liver and spleen are greatly enlarged. Abnormalities are found in the walls of the major blood vessels and in the heart valves, leading to cardiovascular complications. Mental retardation is prominent, the brain suffering damage both from cellular defects and from the hydrocephalus due to impaired cerebrospinal fluid mechanics. Affected children usually do not survive through the second decade.

A closely related disorder, the Hunter syndrome, follows a milder course (*2*). Occasionally, an affected individual may live well into adulthood. The corneas remain clear, and mental retardation is variable; of the two brothers described in the original report by Charles Hunter, in 1917, one was bright (*3*).

A major difference between the Hurler and Hunter syndromes is in the mode of inheritance. The Hurler syndrome is transmitted in classical Mendelian fashion as an autosomal recessive; it can occur in children of either sex whose parents, though carriers of the Hurler gene, show no apparent abnormality. The Hunter syndrome is sex-linked, like hemophilia. Women who are carriers can transmit the disease to their sons but not to their daughters; half the daughters, however, are likely to be carriers and in turn transmit the disease to their sons.

Yet another disorder, the Sanfilippo syndrome, resembles both the Hurler and Hunter syndromes, except that the physical defects are relatively mild while mental retardation is severe. It is transmitted, like the Hurler syndrome, as an autosomal recessive. Originally thought to be a "forme fruste" (that is, an incompletely expressed form) of the Hurler syndrome, it became recognized as a separate disease entity in the early 1960's (*4*).

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The frequency of these disorders is not precisely known. We have the general impression that there are at least several hundred families in the United States who have affected children. The diseases are not peculiar to any one ethnic or racial group, and case reports from Europe suggest an incidence not unlike that in the United States. These impressions are not a valid substitute for statistics, which will hopefully become available as classification procedures become simpler.

Diagnosis of these disorders ultimately depends on the finding of elevated mucopolysaccharide excretion in the urine (*2*). About 100 mg of mucopolysaccharide, consisting primarily of chondroitin sulfate B (CSB) and heparitin sulfate (HS) (*5*) are excreted daily by Hurler and Hunter patients, compared to a normal excretion of 15 milligrams per day or less for all mucopolysaccharides combined (*6*). The ratios of CSB to HS are generally two to one in the Hurler syndrome and one to one in the Hunter syndrome, but may vary widely from this distribution. Sanfilippo patients are said to excrete HS only; however, their urine usually contains some CSB, the detection of which may be difficult by commonly used methods (*7*).

In addition, the mucopolysaccharides accumulate in many tissues. Their distribution differs from that in the urine; the liver, for instance, tends to contain mainly HS, while CSB is predominant in the spleen (*8*). It is the damage wrought by the cellular deposits of mucopolysaccharide that probably underlies most, if not all, of the clinical problems.

Early workers in the field believed the disorders were due to lipid storage, primarily because of the vacuolated appearance of stained sections of the liver after death, such as might be seen if stored lipid had been removed during processing of the tissues. This was an error, however; the material removed was mucopolysaccharide, which is soluble in the formalin then routinely used as fixative (*9*). Ironically, there is a problem of lipid metabolism, mainly in the brain, which has been largely ignored since the mucopolysaccharides were found to be involved. An abnormal distribution of lipids in neurons gives rise to pathological intracellular structures, called Zebra bodies because of their distinctive striated appearance in electron microscopy (*10*).

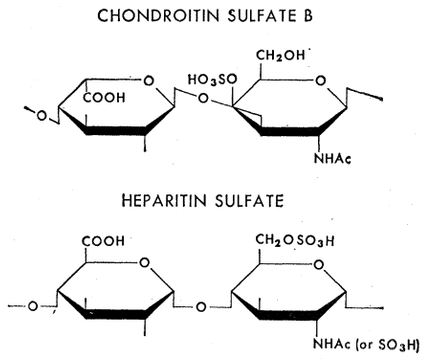

Fig. 1. Repeating units of the carbohydrate chains of chondroitin sulfate B and heparitin sulfate. *Ac*, acetate.

As most biochemical work on the mucopolysaccharide disorders concerns the Hurler, Hunter, and Sanfilippo syndromes, our discussion will be restricted to them. There are, however, several others. The very rare Scheie and Maroteaux-Lamy syndromes involve CSB; most striking is the Morquio syndrome, in which skeletal deformities but normal mental development are associated with the excretion of keratosulfate, a mucopolysaccharide unrelated to either CSB or HS (*2*, *11*). One can expect that, with the current interest in these diseases, other genetically distinct forms will be uncovered.

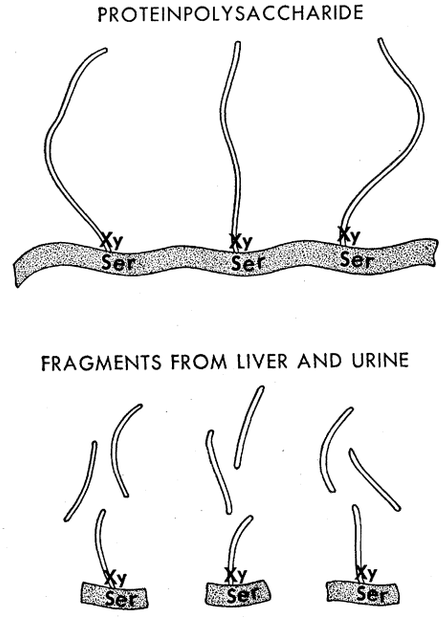

Fig. 2. Relation of fragments found in tissues and urine of patients with the mucopolysaccharide disorders to the native proteinpolysaccharide of connective tissue. Shaded areas represent protein, clear areas, carbohydrate. *Xy*, xylose; *Ser*, serine.

Biochemistry of the Mucopolysaccharides

The two compounds involved in the disorders, chondroitin sulfate B and heparitin sulfate, are normal though minor constituents of most connective tissue; however, both are quantitatively important in the blood vessels and heart valves, while CSB is a major component of skin. They occur as large polymers, consisting of a protein core with extensive carbohydrate branches—that is, as "proteinpolysaccharides" (*12*).

It is generally difficult to extract the whole proteinpolysaccharides out of connective tissue, because they are bonded to various proteins, such as collagen, that make up the intercellular matrix. Thus, there has been only one successful isolation of the protein-bound CSB (*13*), and none as yet of HS. Generally, the carbohydrate portions have been isolated and analyzed only after extensive enzymatic removal of the protein core.

These carbohydrates consist of alternating units of uronic acid and sulfated hexosamine (Fig. 1). In CSB, the repeating dimer is L-iduronic acid linked to *N*-acetylgalactosamine-4-sulfate, whereas in HS it is D-glucuronic acid linked to *N*-acetylglucosamine, which is either sulfated in the 6-position or has an *N*-sulfate replacing the *N*-acetyl. The chains are not absolutely regular. For instance, a few of the L-iduronic acid groups in CSB may be replaced by D-glucuronic (*14*), or they may occasionally be sulfated (*15*). Likewise, there are areas in heparitin sulfate in which the glucosamine is not sulfated at all, and others in which it is doubly sulfated (*16*). A particularly interesting feature of both polymers is the junction of the carbohydrate and protein (*17*). In both cases, the chains are linked to serine by the sugar xylose. Two residues of galactose are linked to the xylose, followed by a residue of glucuronic acid and one of *N*-acetylhexosamine. Only then does the alternating sequence represented in Fig. 1 begin.

Considering the inadequacy of our knowledge of the structure of the CSB and HS proteinpolysaccharides, it is not surprising that our information concerning their metabolism is sparse. It is generally thought that the protein core is first synthesized in conventional manner, and that the carbohydrate chains are subsequently added, one sugar at a time, starting with the attachment of

xylose to serine (*18*). Sulfate is added after the appropriate sugar has been linked to the growing chain.

The sugars and the sulfate are derived from "activated" precursors—sugar nucleotides and phosphoadenosine 5′-phosphosulfate, respectively. The processes of chain elongation and sulfation occur on the membranes of the endoplasmic reticulum, as the protein progresses from the polysomes, where it was synthesized, to the exterior of the cell. Compounds which inhibit protein synthesis, such as puromycin and cycloheximide, likewise inhibit the incorporation of sugar and sulfate into polymer, confirming the view that sulfated carbohydrate chains are not prefabricated before attachment to protein (*19, 20*). This general concept is derived mainly from studies of the biosynthesis of chondroitin sulfate A and of heparin, two polymers chemically related to CSB and HS, respectively, but not involved in the disorders in question. The enzymatic syntheses of CSB and HS have not yet been reported; it is assumed that they will in general be of the same pattern, though the details will undoubtedly differ.

Even less is known about the degradation of CSB and HS than about their formation. This is an unusual situation, for the breakdown of most biopolymers had been studied long before methods were available to study synthesis. Hyaluronidase (E.C. 3.2.1.35), which destroys most of the major mucopolysaccharides, does not degrade CSB and HS (except for the few glucuronic acid bonds in CSB which are susceptible to hyaluronidase).

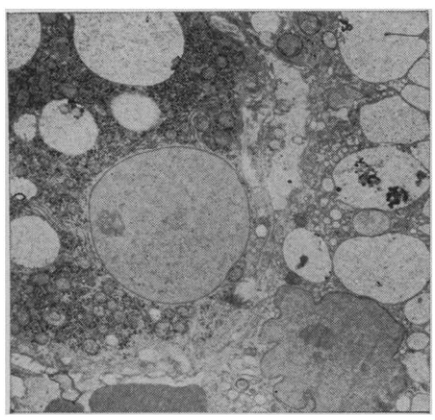

Fig. 3. Electron micrograph of liver cells in the Hurler syndrome (*22*). The large gray bodies are nuclei; the vacuoles containing finely dispersed material are presumed to be lysosomes containing mucopolysaccharide. Small, dark inclusions are also seen in some vacuoles; these probably are residues of cell fragments routinely engulfed by lysosomes. [Photograph by courtesy of Drs. F. van Hoof and H. G. Hers]

In the connective tissues of patients with the mucopolysaccharide disorders, one finds, as in normal individuals, the large proteinpolysaccharide entities of CSB and HS. But in the urine or in tissues where unusual storage takes place, such as liver, the molecules of CSB and HS are much smaller. The protein core is missing; a few amino acids are attached to some of the carbohydrate pieces, while other fragments have neither amino acids nor the region of xylose-serine linkage (*21*) (Fig. 2). It is as if the protein portion had been fully digested while the polysaccharide had been only partially cleaved. In the case of CSB, the cleavage probably occurs by the action of hyaluronidase at the occasional glucuronic acid linkages; how the breaks arise in HS is not clear.

The first suggestion that a disorder of degradation might be the basis of these diseases came in 1964 from the examination of the ultrastructure of liver from Hurler patients (*22*) (Fig. 3). The cells were full of vacuoles delineated by single membranes, containing very finely dispersed material. The vacuoles were thought to be lysosomes (those cellular organelles in which breakdown of macromolecules normally takes place) pathologically engorged with undigested mucopolysaccharide; they resembled the bloated liver lysosomes of a rat that had been injected with an unmetabolizable substance such as the detergent Triton WR1339. Lysosomes filled with mucopolysaccharide have also been found in the livers of Hunter and Sanfilippo patients (*10, 23*).

Thus, the structure of the cell and the structure of the stored and excreted polysaccharide suggest that in these disorders the mucopolysaccharides are not fully degraded to small units which could be returned to the general metabolic pool. This immediately raises some obvious questions: are the mucopolysaccharides chemically faulty; does the affected individual produce them in such quantities as to overwhelm the normal disposal machinery; is there a defect in the degradative mechanism? Answers to these questions had to come from biochemical studies of isolated tissue. In the absence of animals with similar genetic disorders, such studies only became feasible in recent years with the introduction of tissue culture techniques.

Tissue Culture Studies

In 1965, Danes and Bearn (*24*) reported that fibroblasts cultured from skin of patients with the Hurler or Hunter syndromes show signs of disease in that they accumulate mucopolysaccharide. This can be determined by staining with toluidine blue, which reveals pink or "metachromatic" granules in the diseased cells (Fig. 4) or by chemical analysis (*25, 26*). Most of the mucopolysaccharide accumulated in the diseased fibroblasts is CSB; the second mucopolysaccharide (HS) found in excess in the patients is apparently not formed by skin fibroblasts and its cellular origin is presently unknown. Since skin biopsies are readily available and fibroblasts easy to grow, these observations made available lines of genetically marked cells for metabolic study of the mucopolysaccharide disorders.

Our approach was to determine, by very simple kinetics, whether accumulation of mucopolysaccharide in fibroblasts was due to an increased rate of

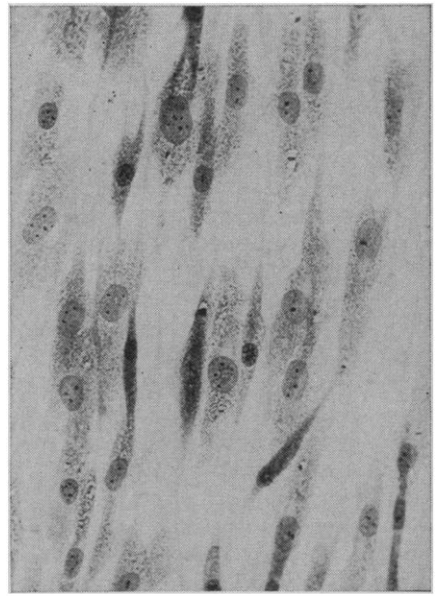

Fig. 4. Cultured fibroblasts from a Hunter patient, stained with toluidine blue. The nuclei are pale blue, while the cytoplasm is filled with intensely pink granules. Normal fibroblasts have but few granules and their cytoplasm looks bluish-purple. Hurler fibroblasts are indistinguishable from cells in this picture, whereas Sanfilippo fibroblasts generally have fewer granules and stain less intensely.

synthesis or to a decreased rate of degradation (*27*).

Cells exposed to medium containing radioactive sulfate ($^{35}SO_4$) synthesize radioactive mucopolysaccharide, the fate of which can readily be followed (Fig. 5). Incorporation of the sulfate into mucopolysaccharide is a complex process that must involve entry of the ion into the cell, activation to phosphoadenosine 5′-phosphosulfate, and transfer to the growing chains of protein-polysaccharide. Though these reactions have not been individually measured, results from inhibitor experiments (*25*) and from our kinetic experiments suggest that the disease does not affect the overall process.

Once synthesized, about three quarters of the mucopolysaccharide is secreted by the fibroblasts into the medium—a process likewise unaffected by the disease—while the remainder is diverted to a storage pool within the cell, from which the only way out is by degradation (*27*). In Hurler and Hunter fibroblasts, material is admitted into that pool at normal rates but is degraded relatively slowly. Thus in normal cells, most of the mucopolysaccharide has a half-life of about 8 hours, while a minor fraction has a half-life of about 3 days. In Hurler and Hunter cells, there is but little degradation in the first 8 hours; the half-life of the entire storage pool varies, in different cultures, from 2 to 6 days. Thus, there may be two mechanisms of degradation in normal cells, the faster of which is missing in the mucopolysaccharide disorders.

The pattern of mucopolysaccharide metabolism by Hurler and Hunter cells is so strikingly different from the normal that it can be used for prenatal diagnosis, a situation in which clinical observation is obviously impossible. The fetus is constantly shedding cells into the amniotic fluid, a sample of which can be withdrawn from the uterus as early as 14 weeks after conception. Of the many cell types originally present in the fluid, fetal fibroblasts are the only ones to multiply in culture. Like fibroblasts from skin biopsies, they show excessive accumulation of radioactive mucopolysaccharide if the fetus is affected with the Hurler or Hunter syndrome (*28*).

When one observes the pattern of mucopolysaccharide metabolism in Hurler and Hunter fibroblasts, one cannot see any significant difference between them, just as one cannot tell the two syndromes apart by examining the mucopolysaccharides in the urine of affected individuals. If, however, one mixes fibroblasts from Hurler and Hunter patients, the defects cancel out, and the mixed cells produce a normal pattern (*29*) (Fig. 6). The presence of both cell types in the same dish allows each one to metabolize mucopolysaccharide normally. The basis for this correction turns out to be very simple. Because the genes responsible for the two disorders are different, the fundamental biochemical defects—that is, the proteins whose structure is encoded in those genes—must differ, however similar the end results might be. Hurler cells are normal with respect to the Hunter defect, and vice versa. The fibroblasts of one genotype are able to supply, through the medium, some factor that cells of the second genotype lack.

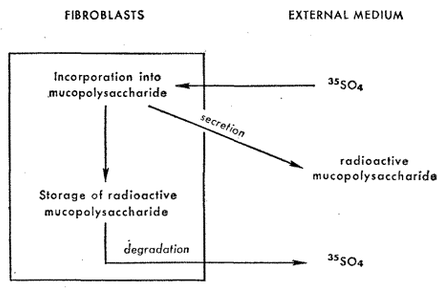

Fig. 5. Schematic outline of the metabolism of sulfated mucopolysaccharide in fibroblasts.

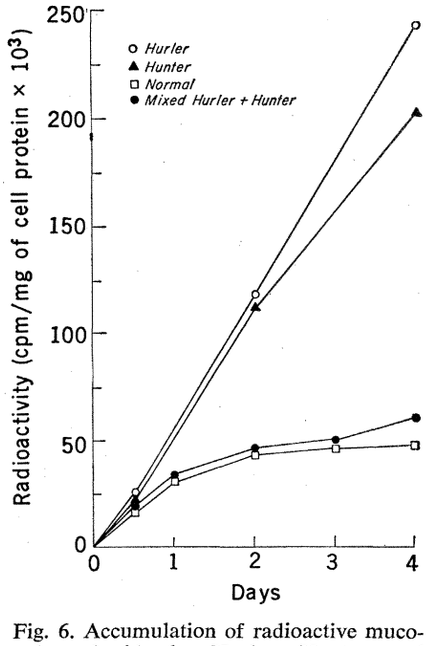

Fig. 6. Accumulation of radioactive mucopolysaccharide by Hurler, Hunter, and normal fibroblasts, as well as by a mixture of Hurler and Hunter fibroblasts.

As expected, the ability to correct the defect of Hurler cells is not limited to Hunter cells but is a property of all fibroblasts, provided they are not of the Hurler genotype (*30*). Those tested include cells derived from normal individuals, from Hurler heterozygotes, and from individuals affected with several other mucopolysaccharide disorders or with unrelated genetic disease. Similarly, the metabolism of Hunter cells is corrected by fibroblasts of all genotypes tested except those of the Hunter genotype. These determinations can be made either by mixing fibroblasts or by assaying the corrective activity of the medium in which fibroblasts had been incubated for 3 days. Preliminary results suggest that the defect in Sanfilippo fibroblasts can be similarly corrected (*31*).

These procedures provide a relatively simple diagnostic test. The laboratory must carry lines of fibroblasts from Hurler and Hunter patients whose clinical diagnosis is unambiguous. Classification of unknown fibroblasts depends on their ability to effect correction in these lines. Those which correct the defect of Hurler cells cannot be of the Hurler genotype, and those which correct the defect in Hunter cells cannot be of the Hunter genotype. The results are often of more than abstract interest: accurate classification is obviously essential for genetic counseling.

Considerable effort is now being devoted to the purification and characterization of the corrective factors. They are heat-labile macromolecules which seem to behave as proteins (*30, 31*). They are found inside the fibroblasts as well as in the medium. Because the factors are genotype-specific, they must be closely related to the basic defect. Conceivably, they may turn out to be those proteins whose structure is determined by the normal counterparts (alleles) of the Hurler and Hunter genes, respectively. Perhaps the factors are enzymes catalyzing different steps of a multistep sequence in the degradation of mucopolysaccharides—for example, cleavage of the protein core or of the carbohydrate branches, or removal of the sulfate groups. Discovering a function, on a molecular level, for the corrective factors, will no doubt require elucidation of the normal pathways of CSB and HS catabolism.

Some Physiological Implications

It is now clear why the mucopolysaccharide disorders are inherited as recessive traits; that is, why the carrier parents of affected children are normal in every respect. The father and mother of Hurler patients carry in each of their cells a Hurler gene and its normal counterpart, which code for a defective protein and a functional one, respectively. The amount of functional protein made by these individuals under the direction of the single normal gene is apparently sufficient to insure adequate degradation of CSB and HS. It may not be sufficient, however, in some conditions of tissue culture; cultured fibroblasts and lymphocytes from Hurler heterozygotes have been shown to display metachromasia (*24, 32*), a finding which should prove useful for the screening of carriers.

The situation is different in the case of the Hunter mothers (since this is an X-linked disorder, the fathers, who have only one X chromosome, cannot be carriers). The X chromosomes behave differently from the other 22 pairs in that only one is functional in any one cell, the other having been inactivated in early embryonic life (*33*). Since the inactivation is a random one, mothers of Hunter patients are mosaics, half of whose cells are normal and half of whose cells might have been expected to store mucopolysaccharide and cause some manifestations of disease. The presence of the two types of cells in fibroblast cultures of Hunter heterozygotes has been demonstrated by Danes and Bearn (*34*). Since the women are not clinically affected in any way, one must assume that their cells of normal genotype are able to supply the defective ones with the requisite factor, as fibroblasts were found to do in culture. This kind of autotherapy gives reason to hope that the factors may eventually be useful in the treatment of the disorders.

A corollary to the hypothesis of degradative failure is that the amount of mucopolysaccharide excreted in the urine of Hurler children [sometimes as high as 200 milligrams per 24 hours (*7*)] is roughly comparable to that catabolized by normal children. Unfortunately, there are no direct studies of mucopolysaccharide turnover in humans or animals against which this figure can be checked. There is, however, an indirect measurement. In the liver and kidney, there is a metabolic pathway called the glucuronic acid cycle, the major function of which is probably to salvage the glucuronic and iduronic acids that would be formed during the breakdown of mucopolysaccharides and to return their carbon skeletons to the general metabolic pool (*35*). The amount of uronic acid that passes through this cycle is known from studies of pentosuria—a rare genetic disorder, totally benign, in which the glucuronic acid cycle is interrupted by an enzyme deficiency so that affected individuals excrete in their urine an intermediate metabolite of this cycle (*36*). About 1 to 4 grams per day of uronic acid flows through the cycle in an adult, corresponding to 3 to 12 grams of mucopolysaccharide broken down. This value would include all the mucopolysaccharides—hyaluronic acid, chondroitin sulfates A and C, as well as CSB and HS. In that context, it seems highly reasonable to postulate that normal children can catabolize as much as 200 milligrams per day of CSB and HS.

Lysosomal Enzyme Studies

While our approach has been to isolate factors which differentiate the mucopolysaccharide metabolism of normal fibroblasts from that of cells of the Hurler and Hunter genotypes, even in the absence of information regarding the function of these factors, other investigators have examined known lysosomal enzymes regardless of whether they have any apparent relation to the metabolism of CSB and HS (it must be remembered that enzymes degrading these two polymers remain to be discovered). This approach has yielded unexpected results. A pattern emerges, in which livers of Hurler, Hunter, and Sanfilippo patients are much richer in several hydrolytic enzymes, particularly in *N*-acetyl-β-glucosaminidase (E.C. 3.2.1.29) and β-glucuronidase (E.C. 3.2.1.30), but poorer in hyaluronidase, sulfatase, and especially β-galactosidase (E.C. 3.2.1.23) (*37–39*). The deficiency of the last enzyme can also be observed in brain and skin (*38, 40*); in some cases it is mild (50 percent depression of the normal values), in other cases pronounced (over 90 percent depression). Of the several forms of β-galactosidase that can be separated by starch-gel electrophoresis, only one is decreased (*41*). The deficiency in β-galactosidase activity is so reproducible that the question has been raised as to whether it could be the fundamental defect in the three mucopolysaccharide disorders. We consider this unlikely, primarily because patients with generalized gangliosidosis, a severe disorder in which β-galactosidase is almost totally lacking, do not store or excrete excessive amounts of CSB and HS (*42*); in addition, the aberrant mucopolysaccharide metabolism of Hurler and Hunter fibroblasts is corrected by fibroblasts of the generalized gangliosidosis genotype, which are devoid of β-galactosidase (*43*).

The deficiency of β-galactosidase may explain, however, the slightly increased amount of lipids containing terminal galactose residues in the brain of patients with the three mucopolysaccharide disorders. These (and other) lipids aggregate in the Zebra bodies alluded to earlier. In this one respect, there is some overlap with the manifestations of generalized gangliosidosis, in which massive deposits of galactose-containing lipids occur.

The finding of a redistribution of lysosomal enzyme activity points up the fact that metabolic pathways do not operate in a vacuum, that malfunction in one area may have far-reaching consequences, and that at least some of the clinical manifestations may not be the result of the primary defect, but of some metabolic perturbation far removed.

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of McKusick (*2*), who also established a numerical system that is widely used: Hurler, I; Hunter, II; Sanfilippo, III; Morquio, IV; Scheie, V; Maroteaux-Lamy, VI. A plethora of other designations can be found in the literature.
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Flaking Stone with Wooden Implements

Flaked stone artifacts from Palliaike, Chile, suggest that wooden flaking tools were used in the New World.

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In recent years the study of prehistoric flaked stone tools has been rejuvenated by the development of modern flintworking. Efforts to replicate the techniques of manufacture and the form of prehistoric stone tools have increased the value of the archeological objects as an instrument for interpreting human history. François Bordes, Jacques Tixier, and I have worked independently and together at the manufacture of flaked stone tools in order to understand the alternate ways by which any tool type might have been made. The use of wooden flaking implements has rarely been considered, because such percussion or pressure-flaking tools do not usually survive in archeological deposits. There must have been times and places in which wooden percussors were used by prehistoric man. This article reports an effort with wooden flaking implements to reproduce stone tools from one of those times and places.

At Palliaike Cave in southern Chile, stemless pressure-flaked points made of basalt and varieties of siliceous stone (Period 3) were found (Fig. 1, a–c), but no bone compressors or percussors were discovered in association with the points. Since well-preserved bone was found in the cave, Junius Bird became curious to determine what implements and techniques were used in their manufacture. He wondered if, like certain Australian aborigines, these people could have used wooden implements; therefore, at his suggestion I decided to try replication with wooden flakers.

Bird generously provided seven examples of the points, as well as a variety of Calafate hardwood (*Berberis buxifolia*) and a small supply of native coarse-grained basalt. The pressure technique used on the points could not be called "classic" or "extremely refined"; nevertheless, planning and control were evident in the flake detachment, and thus I was eager to accept the challenge of replication with wooden implements. Since lithic materials are harder than steel, it may be difficult to visualize shaping and forming them with a wooden tool; but it can be done. The use of a wooden flaker is seldom considered because at New World sites it is common to find compressors of antler, bone, or ivory, and other less perishable tools, which are more resistant to decay than wood. It is entirely possible, however, that wood, because of its perishable nature, has gone unrecorded as material used for flaking stone. Wooden pressure tools are used in the Kimberley region of western Australia (see *1*), and there is a possibility that wood was used as a pressure tip in Mexico for making blades (*2*). As this article will demonstrate, archeologists must on some

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