the rRNA extracted from cells of an untreated, control culture. It was found that there was little or no decrease in the amount of 18*s* rRNA in the vinblastine-treated cells but there was a 40 to 50 percent reduction in the specific activity of the 28*s* rRNA. The nature of the vinblastine-sensititve step in the processing of the nucleolar precursor into cytoplasmic 28*s* rRNA is not evident, but preliminary experiments indicate that there is no accumulation of 28*s* rRNA in the nucleus. This tends to exclude the possibility that vinblastine sulfate acts by inhibiting transport of the mature 28*s* rRNA into the cytoplasm.

It is of interest to note that incubation of untreated cells with radioactive uridine followed by immediate phenol extraction and ethanol precipitation of the cytoplasmic RNA results in the coprecipitation of a large amount of radioactive material of very low molecular weight. This material co-sediments with 4*s* tRNA in sucrose gradient centrifugation, but runs considerably faster than 4*s* tRNA upon acrylamide gel electrophoresis. As much as 30 percent of the amount of radioactivity found in the 4*s* tRNA peak in sucrose can be seen to migrate faster than 4*s* tRNA in acrylamide gel electrophoresis (*9*). In the presence of vinblastine sulfate the amount of this material of very low molecular weight is reduced by as much as 70 percent. The difficulty in separating 4*s* tRNA from smaller material in sucrose gradient may account for the earlier reports of the high inhibition of tRNA synthesis by vinblastine sulfate (*2*).

The inhibition patterns reported here for vinblastine sulfate are also seen with vincrystine sulfate. Vincrystine sulfate is only about 50 percent as effective as vinblastine sulfate at the same concentration in inhibiting the incorporation of radioactivity into cytoplasmic RNA of HEp-2 cells; this result is in agreement with those reported for Ehrlich ascites cells in vitro (*2*).

EDWARD K. WAGNER
BERNARD ROIZMAN
Department of Microbiology, University of Chicago, Chicago, Illinois 60637

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6. The use of such a double gel allows the rRNA of high molecular weight to enter and band in the 2.7 percent gel, whereas the tRNA is concentrated at the interface between the gels resulting in a much sharper band in the 5.5 percent gel than that which would be found in a gel consisting entirely of 2.7 percent acrylamide.
7. The composition of the toluene scintillation fluid is 7.4 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene in 1 liter of toluene.
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10. Supported by grants from the American Cancer Society (E 314D and PRA-36), NSF (GB 4555), and PHS (CA 08494-03). One of us (E.K.W.) is a Helen Hay Whitney Foundation postdoctoral fellow.

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Hurler and Hunter Syndromes: Mutual Correction of the Defect in Cultured Fibroblasts

Abstract. *The biochemical defect of cultured skin fibroblasts from Hurler or Hunter patients (faulty degradation of sulfated mucopolysaccharide, resulting in excessive intracellular accumulation) may be corrected if cells of these two genotypes are mixed with each other or with normal cells. The effect is mediated by substances released into the medium.*

The Hurler and Hunter syndromes are genetically transmitted disorders, characterized by retarded growth and mentation, skeletal abnormalities, excretion of chondroitin sulfate B and heparitin sulfate in urine, and deposition of these two mucopolysaccharides (MPS) in various tissues (*1*). The biochemical abnormality, accumulation of mucopolysaccharide, is preserved in fibroblasts cultured from the skin of affected individuals (*2*). We have previously shown this accumulation to be the result of inefficient degradation of intracellular MPS, rather than of excessive synthesis or reduced secretion (*3*).

Table 1. Effect of preincubated medium on accumulation of labeled intracellular mucopolysaccharide. Hurler or Hunter fibroblasts were incubated with unlabeled medium for 2 days, followed by 2 days in medium supplemented with $^{35}SO_4$, 20 × 10^6 count/min per milliliter. In each case, 7 ml of medium was used, of which 2 ml was fresh while 5 ml had been exposed to cells of the genotype indicated.

Cells	Medium previously exposed to	MPS accumulation (count/min per mg of protein)
Hurler	Hurler	258,000
	Hunter	132,000
Hunter	Hunter	238,000
	Hurler	110,000

Although the two diseases have similar clinical and biochemical manifestations, they are genetically distinct; Hurler's is transmitted as an autosomal recessive, while Hunter's, also recessive, is X-linked (*1*). The biochemical lesion must therefore occur at different points in the pathway of MPS degradation. One would expect that hybrid cells, containing a normal allele for the Hunter gene (derived from the Hurler parent) and a normal allele for the Hurler gene (derived from the Hunter parent), might be able to degrade MPS adequately. Surprisingly, we found that Hurler and Hunter cells can compensate for each other's defect when simply mixed in culture, or even when cells of one genotype are exposed to medium preincubated with the other.

Fibroblasts were obtained from skin explants from one adult Hunter patient, four Hurler patients under the age of 6, one 5-year-old SanFilippo patient, two normal infants, and one normal adult. The cells were cultured in Eagle's minimal essential medium (with $MgCl_2$ substituted for $MgSO_4$), supplemented with nonessential amino acids, 10 percent fetal calf serum, penicillin, streptomycin, and nystatin, in an atmosphere of 5 percent CO_2. The growth rate of the cell lines was sufficiently similar to preclude overgrowth of one line by another during the experiments.

The incorporation of $^{35}SO_4$ into intracellular MPS of normal, Hurler, and Hunter cells begins at the same rate; but whereas the labeled MPS reaches a steady state in normal cells within 24 to 48 hours, it continues to increase in linear fashion in the Hurler and Hunter cells for the duration of the experiment (*3*). However, mixtures of these two genotypes with each other display almost normal kinetics, as do mixtures

of either one with normal cells (Fig. 1A). By contrast, pairing of cells from three Hurler lines results in accumulation of radioactive MPS which is precisely the calculated average.

The interaction of differing genotypes can also be detected by chase experiments (Fig. 1B). Here, Hurler and normal cells, prelabeled with $^{35}SO_4$, are replated in unlabeled medium. The normal cells lose most of their labeled MPS within 24 hours, while the Hurler cells still retain 65 percent after 2 days. The Hurler cells can be made to lose the labeled MPS if at the time of replating they are mixed with unlabeled cells of the normal, Hunter, or SanFilippo genotypes [the last is a mucopolysaccharide disorder which differs from Hurler's by some clinical manifestations and by the urinary excretion of heparitin sulfate only (*1*)]. As in the accumulation experiment, cells from another Hurler line have no corrective effect (*4*).

We had previously shown that the loss of labeled MPS from normal cells is due to degradation rather than secretion, since the radioactivity lost from the cells appears in the medium as dialyzable material. Label from MPS of mixed Hurler and Hunter cells is likewise released into the medium in dialyzable form (*5*).

Acquisition by cell mixtures of the ability to degrade MPS can also be demonstrated histologically. Whereas the cytoplasm of Hurler and Hunter fibroblasts stains strongly and metachromatically with toluidine blue (typical of MPS deposits), a mixture of these cells appears almost normal 10 days after plating, with slight or no metachromasia. The corrective effect is therefore exerted not only on subsequent MPS metabolism, but also on removal of stored material.

By what mechanism do the cells complement each other so as to be able to degrade MPS normally? We could postulate hybridization by cell fusion, so that the two genomes are combined within each cell (*6*); exchange of cytoplasmic material between cells in direct contact (*7*); or an exchange of necessary factors through the medium. The first two possibilities are ruled out and the third implicated by the corrective effect observed when cells are allowed to accumulate label in the presence of medium that had been preincubated with cells of the other genotype (Table 1). The corrective effect is greater if cells are in contact with complementary medium for 2 days prior to addition of label; presumably, the pool of preexisting MPS is reduced during this period. Depletion of the preexisting pool of MPS is observed as partial loss of metachromasia when Hurler cells are incubated for 10 days in medium previously exposed to Hunter or normal cells, and, conversely, when Hunter cells are incubated with medium that had been exposed to normal or Hurler cells.

The interaction of cells by way of secreted material may be the basis of the normal phenotype of mothers of Hunter patients (*1*). These women are heterozygotes for the mutant gene, which is located on the X chromosome. However, only one of the two X chromosomes is believed to be active in any one cell; this theory, usually referred to as the Lyon hypothesis (*8*), has been experimentally verified for Hunter heterozygotes by clonal separation of normal and abnormal fibroblasts (*9*). Were

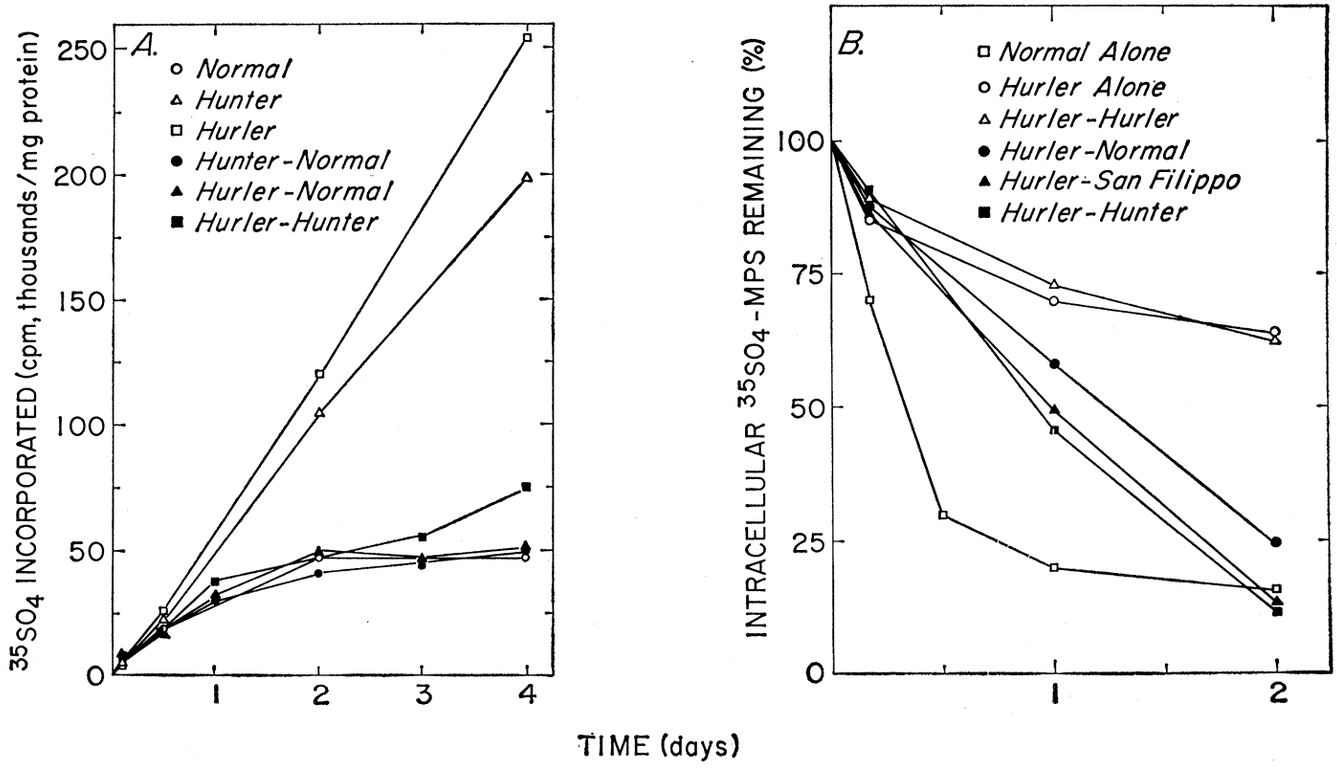

Fig. 1. Effect of mixing cells of varying genotypes on accumulation (A) and chase (B) of labeled intracellular mucopolysaccharide. (A) Hurler, Hunter, or normal cells were trypsinized and replated singly or in combination at a density of about 2×10^6 cells per plate. After 2 days, 6 ml of medium containing $^{35}SO_4$ (9×10^6 count/min per milliliter) was added. Ratio of cells in the mixed plates—Hurler : Hunter, 44 : 56; Hunter : normal, 43 : 57; Hurler : normal, 38 : 62. (B) Hurler and normal fibroblasts had been previously labeled for 3 days in medium containing $^{35}SO_4$ (8×10^6 count/min per milliliter). After trypsinization they were plated in unlabeled medium; the Hurler fibroblasts were plated either alone or with an approximately equal number of unlabeled cells of the indicated genotype. Radioactive mucopolysaccharide was determined as previously described (*3*). *cpm*, Counts per minute.

the expression of the gene entirely intracellular, we might expect the carrier, who is a mosaic of normal and Hunter cells, to display symptoms—as is the case for heterozygotes of some variants of glucose-6-phosphate dehydrogenase deficiency, likewise an X-linked trait (*10, 11*).

Isolation and identification of the corrective factors in the medium will clarify the normal mechanism of MPS degradation, and, eventually, open up possibilities of replacement therapy. An obvious immediate use of the technique of cell mixing is in the differentiation of the genetic mucopolysaccharidoses.

JOSEPH C. FRATANTONI
CLARA W. HALL
ELIZABETH F. NEUFELD
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

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4. Correction of the defect in the chase experiments (Fig. 1B) is only partial, while that in the accumulation experiments (Fig. 1A) is essentially complete. This is probably due to a difference in experimental procedure: in the accumulation experiments, cell mixtures had been plated together 2 days before labeling, while in the chase, observation was begun immediately after mixing. We speculate that time is required to decrease the large pool of preexisting MPS of Hurler and Hunter cells before normal kinetics can be achieved; indeed, cells plated together for 5 days before beginning the chase (2 days without $^{35}SO_4$ and 3 days in the presence of isotope) lose 80 percent of the label in 24 hours, as do normals.
5. In both cases, the product recovered in the medium migrates as inorganic sulfate on electrophoresis in 0.05*M* sodium tetraborate; however, no definitive identification has yet been performed.
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11. Danes and Bearn (9) have observed the presence of about 50 percent metachromatic cells in uncloned fibroblasts from Hunter heterozygotes. Superficially, this seems to be at variance with our results, in which normal cells correct the defect of Hunter cells mixed with them. However, it is obvious that the concentration of complementary factors must depend on conditions of growth, especially on cell density and medium volume, so that the degree of correction will vary in different experimental conditions. Metachromasia, which is not a quantitative test, should not be expected to reveal partial correction.
12. We thank Drs. A. S. Dekaban, R. R. Howell, C. Jacobson, F. M. Rosenbloom, H. R. Sloan, W. B. Uhlendorf, and J. H. Zelson for kindly supplying us with skin biopsies or fibroblast cultures.

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Isopycnic Separation of Subcellular Components from Poliovirus-Infected and Normal HeLa Cells

Abstract. *A rapid method for determining the density of ribonucleoprotein particles and complexes has been developed. The method involves glutaraldehyde fixation of sucrose gradient fractions and immediate centrifugation for 5 hours through preformed cesium chloride gradients. There is little or no aggregation of particles, so that components which co-sediment in sucrose gradients are resolved by the cesium chloride gradient. By this method the densities of HeLa cell ribosomes, polyribosomes, and subribosomal particles have been determined. Furthermore, the poliovirus replication complex has been separated from polyribosomes and its density has been found to be unaffected by treatment with ethylenediaminetetraacetic acid.*

The separation of subcellular particles containing RNA is usually effected by rate-zonal centrifugation through gradients of sucrose. As investigation of cellular physiology has progressed, problems have arisen which cannot be solved by rate-zonal analysis because of its low resolving power and the apparent co-sedimentation of disparate elements. This is especially true in the study of virus replication. A separatory method based on isopycnic banding of formaldehyde-fixed subcellular complexes in cesium chloride has provided a second dimension of analysis and has been successfully used in a number of studies (*1, 2*). The aldehyde fixation is necessary to stabilize the complexes against the dissociative effects of CsCl (*3*). The methods which have been published, however, are lengthy and the steps of the procedure may produce selective losses.

To obviate these difficulties we have developed a rapid method of isopycnic centrifugation using glutaraldehyde fixation and preformed cesium chloride gradients. Glutaraldehyde was chosen because it is a bifunctional reagent which is known to fix tissue specimens rapidly (*4*). In this report we present the method and illustrate its utility in the investigation of certain questions (*5*).

To carry out the analysis, cytoplasmic extracts are prepared and then fractionated through 15 to 30 percent linear sucrose gradients in RSB (0.01*M* tris, 0.01*M* NaCl, 0.0015*M* $MgCl_2$ at *p*H 7.4) by standard techniques (*6*). After centrifugation, the gradients are separated into fractions while the absorbance at 260 mμ is monitored by a Gilford recording spectrophotometer. Samples of 0.8 ml from appropriate regions or individual fractions of the sucrose gradients are fixed at 4°C by the addition of 0.2 ml of 33 percent glutaraldehyde (Fisher Scientific Co., biological grade) which has been neutralized to *p*H 7.0 with 1*M* $NaHCO_3$ just prior to use. The fixed samples are immediately layered onto preformed linear gradients of CsCl of appropriate density (*7*). Sucrose need not be removed from the samples, because the density of CsCl at the top of the gradients is always greater than the density of 30 percent sucrose (1.14 g/cm^3). Comparison of samples containing sucrose with samples freed of sucrose by dialysis or centrifugation shows that the sucrose layer above the CsCl gradient does not alter the apparent densities of subcellular particles. Dialysis of materials after exposure to glutaraldehyde often leads to artifacts and should be avoided.

The linear CsCl gradients are preformed in a volume of 5 ml in 9/16 by 3¾ inch (1.4 by 9.5 cm) cellulose nitrate tubes (Spinco) with CsCl solutions of two different densities made up in RSB containing 0.8 percent BRIJ-58. Addition of the non-ionic detergent BRIJ-58 prevents loss of materials due to absorption to glass and cellulose nitrate tubes. However, the solubility of BRIJ-58 in CsCl becomes limited when the density is higher than 1.70 g/cm^3. Polyallomer tubes have been found to produce artifacts and should not be used. After layering the sample, the tube is filled with Nujol or another mineral oil. The tubes are then centrifuged for 5 hours at 4°C at a speed chosen to produce 170,000*g* at the midpoint of the gradient. These tubes can be centrifuged either in the SW41 Spinco rotor or in the SB-283 IEC rotor. After deceleration with the brake on, drops are collected from the bottom of the tubes directly into disposable glass tubes and are assayed for refractive index, absorbancy at 260 mμ, and acid-precipitable radioactivity (*6*). Densities are obtained from the refractive index measurement. Because of the sucrose and glutaraldehyde on the top of the gradient, neither density nor absorbancy in the top fractions is shown in the figures. Recovery of radioactivity in the