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10. Two microelectrodes were inserted into the same surface fiber 100 to 150 microns apart, one to pass a current and the other to record the action potential. The resting membrane potential was set at a value of about −75 mv by passing a constant anodal current across the membrane through the stimulating microelectrode. A first derivative resistance-capacitance circuit was used to display the rate of rise of the action potential. The time constant of the recording circuit was about 40 μsec. All experiments except those involving the use of batrachotoxin were performed at 23°C.
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13. The electrical constants of the membrane were measured by inserting two microelectrodes into a surface fiber of the muscle. For details of this technique and an explanation of the cable equations used in this study, see Albuquerque and McIsaac (*7*). For each interelectrode distance the average value of the ratio V/I was calculated from three different current pulses producing a membrane potential change of 5 to 30 mv. The $\log_{10}$ of V/I was plotted against the distance in millimeters between microelectrodes, and the data was further analyzed when a linear relationship was found. The symbol λ is the space constant and refers to the negative of the inverse of the slope of the line, R_m refers to the transverse resistance of a unit area, and ρ indicates the calculated fiber radius. The symbols τ_m and C_m refer to the time constant and capacitance of the membrane, respectively.
14. Supported by PHS grant NB-08233. J.E.W. is a recipient of a special fellowship award from the NINDS (NIH) 1 F 10 NS-02375-01 NSRA. We thank D. Timmer and M. Zelle for technical assistance.

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Correction of Abnormal Cerebroside Sulfate Metabolism in Cultured Metachromatic Leukodystrophy Fibroblasts

Abstract. *Cultured fibroblasts derived from patients with late infantile metachromatic leukodystrophy incorporated arylsulfatase A from the growth medium. Upon exposure to cerebroside sulfate, they exhibited patterns of uptake and hydrolysis indistinguishable from cells derived from control subjects. Furthermore, inclusion granules formed in the metachromatic leukodystrophy fibroblasts upon exposure to sulfatides were cleared by subsequent supplementation of the growth medium with arylsulfatase A.*

Metachromatic leukodystrophy or MLD is a hereditary neurological disorder of lipid metabolism in which there is progressive degeneration of the nervous system. Demyelination is accompanied by accumulation of cerebroside sulfates (sulfatides) in both the central and peripheral nervous systems (*1*). The biochemical lesion has been identified as a deficiency of the lysosomal enzyme arylsulfatase A (*2*). We have previously demonstrated that cultures of skin fibroblasts from patients with MLD have deficient arylsulfatase A activity and we have utilized these cultures as an in vitro model for the study of this disease (*3*).

Metachromatically staining inclusions do not normally occur in cultured MLD fibroblasts; however, when sulfatides are provided in the culture medium such inclusion granules are formed. Under similar conditions normal fibroblasts ingest the sulfatides and degrade them, as evidenced by release of inorganic sulfate into the medium (*4*). We now find that the manifestations of the genetic disorder in the tissue culture model can be corrected by an exogenous source of arylsulfatase A. When MLD cells are cultured in medium supplemented with a preparation of arylsulfatase A, the enzyme is incorporated by the cells and the functional activity of the enzyme is retained over an extended period. When these enzyme-supplemented cells are exposed to sulfatides, uptake and hydrolysis patterns characteristic of normal cells are observed. Conversely, if inclusion granules are formed by prior treatment with sulfatides, subsequent "enzyme replacement" clears the inclusions.

Procedures for initiation of primary cultures from skin biopsies, culture of outgrowths, enzyme assays, preparation of ^{35}S-labeled sulfatides, and determination of [^{35}S]sulfatide uptake and degradation have been described (*3*, *4*). Cultures for the present study were derived from two patients with late infantile MLD, an obligate heterozygous carrier (a parent of one of the patients), and a normal adult. Crude human arylsulfatase A was obtained by precipitation from pooled human urine with ammonium sulfate at 70 percent saturation as described by Austin (*5*). The precipitate was suspended in water and dialyzed against water until free of sulfate at 4°C. This material was lyophilized and stored in a vacuum at −20°C. Early studies were conducted with this material. Later studies were performed with an ammonium sulfate (40 to 50 percent saturation) fraction of the lyophilized material. Preparations of arylsulfatase A were added to culture medium to provide 2 units of enzyme per milliliter (a unit of enzyme catalyzes the hydrolysis of 1 μmole of *p*-nitrocatechol sulfate per hour) and the medium was sterilized by filtration.

When MLD fibroblasts were cultured in medium containing arylsulfatase A, they incorporated the enzyme and achieved intracellular specific activity of 0.3 to 0.4 unit per milligram of protein (Fig. 1). No decrease of this activity was observed over a 6-day period. In contrast, the enzyme activity in the growth medium had a half-life of less than 24 hours and no demonstrable activity remained at day 6. A similar precipitous loss of activity was observed in growth medium incubated without cells. The confluent enzyme-containing fibroblasts were harvested by trypsinization on day 7, replated at the same density, and incubated with medium unfortified with enzyme. The specific activity of the intracellular arylsulfatase A remained essentially unchanged for four more days. On day 11 the cells were again removed by trypsinization, but this time they were replated lightly to allow replication and growth. There was gradual loss of specific activity to day 16. The loss was presumably due to dilution by cell growth.

The MLD cells cultured in medium containing an arylsulfatase A preparation that had been inactivated by prior incubation for 6 days at 37°C contained no detectable arylsulfatase A. This finding makes it unlikely that the observed intracellular activity was due to some mechanism other than incorporation of active enzyme by the MLD cells.

When MLD cells were pretreated with arylsulfatase A, trypsinized, replated, and grown in the presence of [^{35}S]sulfatide, they metabolized the exogenous sulfatide in a manner indistinguishable from that of normal or heterozygous cells. They failed to accumulate excess sulfatide and excreted significant accounts of inorganic [^{35}S]-sulfate into the medium. In contrast,

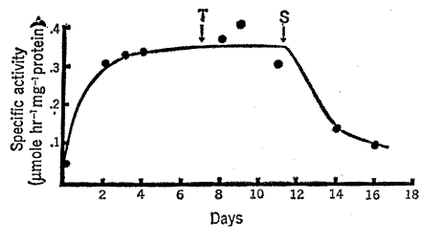

Fig. 1. Intracellular levels of arylsulfatase A after treatment of MLD fibroblasts with exogenous enzyme. A total of 145 mg of lyophilized enzyme preparation derived from the 70 percent ammonium sulfate precipitate of urine was added to growth medium to give a final concentration of 2 units of arylsulfatase A activity per milliliter of medium. The medium was then sterilized by filtration. Confluent cultures of MLD cells were incubated with this medium for 7 days, at which time the cells were washed, trypsinized with 0.25 percent trypsin, replated at the same cell density, and incubated with normal medium. After 4 days the cells were trypsinized and replated at one third the original density to permit growth. For arylsulfatase A assays, cells were harvested by trypsinization and centrifugation, suspended in an equal volume of water, and lysed by freezing and thawing six times. The lysate was centrifuged in a Spinco Microfuge and the supernatant fluid was assayed for protein and for arylsulfatase A activity; *T*, trypsinization; *S*, subculture.

cells from the same MLD strains that had not been previously treated with enzyme produced no inorganic sulfate and showed large intracellular accumulations of the sulfatide (Fig. 2).

A corollary experiment was also performed in which MLD cells were pretreated with [^{35}S]sulfatide by culturing for 10 days in medium supplemented with the radioactive lipid. At the end of this time, the cells had incorporated approximately 10 percent of the available sulfatide and had produced no detectable inorganic [^{35}S]sulfate. The treated cells were trypsinized, washed, and subcultured. After 1 day the medium was replaced with that containing arylsulfatase A and was incubated further for 6 days. About half of the radioactivity (3150 count/min) was recovered from the medium as inorganic sulfate while the remainder (3015 count/min) was associated with the cells as unchanged intracellular sulfatide. In a parallel culture, pretreated with sulfatide but not exposed to arylsulfatase A, no ^{35}S-labeled inorganic sulfate was found in the medium and all the radioactivity (6440 count/min) was recovered as intracellular sulfatide.

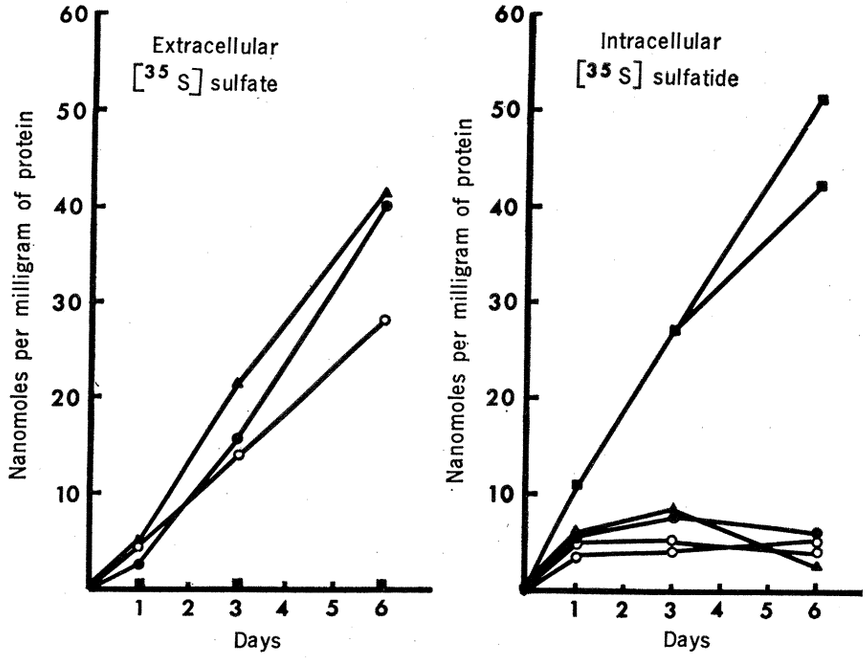

Fig. 2. Normalization of sulfatide uptake and hydrolysis by MLD cells previously treated with arylsulfatase A. Growth medium was supplemented with urinary extract (40 to 50 percent ammonium sulfate fraction) to give a final concentration of 2 units of arylsulfatase A activity per milliliter. Cells from two strains of MLD cells were incubated in this medium for 4 days, trypsinized, and subcultured into 60-mm petri dishes. At the same time 60-mm petri dishes were inoculated with normal cells, heterozygous cells, or untreated MLD cells. After 1 day, medium containing 60 μg of [^{35}S]sulfatide (1.16×10^6 count/min per milligram) was added to each dish. One dish of each set was harvested at the times indicated and assayed for protein, intracellular sulfatide, and extracellular inorganic sulfate. ●——●, Heterozygote; ▲——▲, control; ○——○, pretreated MLD cells; and ■——■, untreated MLD cells.

It has been shown by electron microscopy that sulfatides incorporated by cultured MLD cells are located in structures that appear to be lysosomal (*6*). It is presumed that exogenously provided sulfatide is ingested by pinocytosis and accumulated in lysosomes. Arylsulfatase A provided in the medium is likewise taken up into lysosomes by pinocytosis. This mechanism provides a logical explanation for the events observed in the present study. Arylsulfatase A activity is conferred upon MLD fibroblasts genetically deficient in arylsulfatase A by "enzyme replacement"; functional sulfatide sulfatase activity is present whether enzyme replacement takes place prior to or subsequent to exposure to sulfatides. The remarkable stability of the enzyme after it has been ingested, especially as compared to that remaining in the growth medium, suggests that reintroduced lysosomal enzymes are resistant to the normal lysosomal degradative processes.

The concept of enzyme replacement therapy for storage diseases is not unique. A number of studies have been reported in both in vitro and animal systems, as well as in human trials (*7*). The present studies provide confirmation that this approach is entirely feasible and that cultured fibroblasts serve as a useful model. Moreover, the ambiguity of success of clinical trials, potential immunological complications, and instability problems indicate that many refinements and modifications of the enzymes may be required before a safe and effective clinical enzyme replacement regimen can be developed. Fibroblast model systems, such as the system recorded here, should be a valuable adjunct for this development.

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Production of Hemadsorption-Negative Areas by Serums Containing Australia Antigen

Abstract. *Exposure of human Wi-38 cells to human serums containing Australia antigen, and presumably serum hepatitis virus, renders the cells refractory to infection by Newcastle disease virus as detected by the hemadsorption-negative plaque test for intrinsic interference. Induction of the Newcastle disease virus refractory state could be passed in cell culture with up to a 1 : 100,000 dilution of material obtained from cells "infected" with serums containing Australia antigen after filtration (0.45-μm pores) and heating to 60°C for 1 hour. Human antiserums to the Australia antigen prevented induction of the Newcastle disease virus refractory state.*

Australia antigen (Au) has been found specifically in the serums of patients with serum hepatitis (*1*), and serums containing this antigen have been found to transmit serum hepatitis (*2*). It appears that Au [and the SH antigen which is probably identical with it (*3*)] is either a component of the serum hepatitis virus or intimately associated with this virus. Serums containing this antigen were used in our attempt to develop a tissue culture assay system for serum hepatitis virus. Since cytopathic changes caused by the virus have not been described, a noncytopathic assay system was sought and is described herein.

Inhibition of the replication of Newcastle disease virus (NDV) has been described as an assay system for rubella virus growing under noncytopathic conditions (*4*). Bovine erythrocytes are absorbed by tissue culture cells in which NDV replicates, whereas the rubella-infected cells are refractory to NDV superinfection and therefore do not absorb the erythrocytes. This noninterferon-mediated interference against superinfection with NDV has been called intrinsic interference (*5*). Subsequently, Sindbis and West Nile viruses, poliovirus (*5*), lymphocytic choriomeningitis virus (*6*), infectious bronchitis virus (*7*), cytomegalovirus (*8*), and reovirus (*9*) have also been shown to induce intrinsic interference when growing under noncytopathic conditions. We have sought interference with the development of NDV hemadsorption as an assay system for serum hepatitis virus.

When Au was found in the plasma contributed by a 42-year-old blood donor to the Children's Hospital Medical Center in Boston, Dr. Sherwin Kevy arranged for the donor to be bled again and we were sent 200 ml of Au-positive serum. This donor developed a severe case of hepatitis 4 weeks after the collection of blood. His serum (Newhall specimen) was used as a virus source while optimal conditions for the assay system were developed.

At present, the test is done as a modification of that previously described (*10*). Wi-38 cells in screw-top tubes are treated with phosphate-buffered saline (PBS) containing 25 μg of diethylaminoethyl (DEAE) dextran per milliliter. The tubes are kept at 37°C for 1 hour while on a roller wheel. After aspiration of the dextran, 0.3 ml of specimen fluid (containing Au and presumably containing serum hepatitis virus) is placed in each test tube and the tubes are kept at 37°C on a roller wheel for another hour. Each tube then receives 1.5 ml of minimal essential medium (MEM) with 3 percent calf serum and the tubes are incubated at the optimal temperature of 35°C. All tubes are aspirated after 24 hours and receive fresh medium. The cells remain at 35°C with medium changed every 4 days until the time of challenge with NDV. Medium is removed from the cells and NDV (California) is added at a multiplicity of 10 plaque-forming units (assayed on chick embryo fibroblasts) per cell in 0.3 ml of medium containing 25 μg of DEAE dextran per milliliter. The tubes are placed at 37°C for 1 hour on a roller wheel, and the unattached NDV is removed by aspiration. The monolayers are rinsed once with 3 ml of PBS. To preclude spurious binding of red cells due to the presence of residual input NDV, the rinsed monolayer is exposed to 0.3 ml of NDV antiserum for 30 minutes at 37°C. Antiserum is aspirated and the monolayers are washed twice with 3 ml of PBS, flooded with 1.5 ml of MEM + 3 percent calf serum, and incubated at 37°C for 15 hours. The medium is removed and replaced with 3 ml of a suspension of washed bovine erythrocytes in cold PBS at a concentration of 6×10^7 cells per milliliter. Red cell adsorption is carried out for 30 minutes at 4°C. The tubes are then gently rinsed with cold PBS to remove unattached erythrocytes and 1.5 ml of cold PBS is added to each tube after the last rinsing. The monolayers are then examined under the microscope.

Serial tenfold dilutions of the specimen were each inoculated into replicate tubes for each experiment. One tube for each dilution was then challenged with NDV each day. Experiment with the Newhall specimen revealed that areas of nonhemadsorbing cells first appeared on the 8th to 12th days after infection. These areas were surrounded by hemadsorption positive cells. The hemadsorption negative areas were first seen in tubes inoculated with higher dilutions of the specimen (at a 100,000-fold dilution) and 2 to 3 days later were seen in tubes inoculated with more concentrated specimens. The effect was produced with serum diluted as great as 10,000,000-fold. The hemadsorption negative cells were normal in appearance in two-thirds of the experiments and appeared nonviable in one-third of the experiments. In all cases control cells which had not been inoculated with hepatitis specimens but were otherwise treated in the same manner as the infected cells showed complete hemadsorption of red blood cells following challenge with NDV. Monolayers infected with a given dilution of specimen have hemadsorption negative areas after three to five consecutive daily challenges with NDV, and then subsequent challenges of further monolayers in-