Metachromatic Leukodystrophy (Sulfatide Lipidoses) Cultured in vitro

Abstract. *Cerebral white matter from a patient with metachromatic leukodystrophy was successfully cultured in vitro. A luxuriant growth in Rose chambers and in roller tubes was apparent by the 14th day. The outgrown cells contained large numbers of granules which stained dark brown with cresyl violet–acetic acid. The growth and metachromasia were maintained up to 80 days in vitro.*

Metachromatic leukodystrophy(MLD) is a hereditary disorder of lipid metabolism; it results in excessive deposition of sulfuric acid esters of cerebroside in various organs of the human body, especially in the central and peripheral nervous system. Diagnosis of the disease is routinely made by the demonstration of metachromatic granules in the urine (Austin test) (*1*) and the finding of brown metachromasia (*2*) in a peripheral nerve biopsy. Austin *et al.* (*3*) and Mehl and Jatzkewitz (*4*) have presented biochemical evidence indicating that the basic pathologic defect in lipid metabolism is the result of an enzyme defect, namely, the lack or depletion of cerebroside sulfatase, an enzyme which splits cerebroside sulfate into cerebrosides and sulfate. Although extensive histological, histochemical, and biochemical studies of MLD have been undertaken, the cerebral and peripheral nerve tissues of this disease have not yet been cultured in vitro. We now report successful cultures of cells obtained by biopsy from the cerebrum and peripheral nerve of a 4-year-old boy afflicted with metachromatic leukodystrophy.

Under sterile conditions the surgically removed tissues (frontal cerebral cortex, subjacent white matter, and supraorbital nerve) were washed in Gey's balanced salt solution for about 2 hours, then changed to Eagle's tissue culture medium, and cut into small fragments of about 1 to 2 mm in maximum diameter. The tissues were explanted in avian-plasma clots on cover glasses in roller-tube preparations. To each tube we added 2 ml of nutrient fluid consisting of Eagle's culture medium supplemented with 20 percent fetal bovine serum and 600 mg of dextrose per 100 ml of medium. In addition, cell suspensions of white matter were prepared by mechanical aspiration through a No. 18 needle and injected into four Rose chambers filled with 2 ml of the same nutrient fluid. The culture medium was changed twice weekly. In addition, explants from both brain and supraorbital nerve were grown in Rose chambers on collagen-coated coverslips or under dialysis membranes (*5*). Cultures in these groups were maintained on Eagle's medium containing 600 mg of glucose per 100 ml of medium, 10 percent fetal bovine serum, and 10 percent whole egg ultrafiltrate. Cells were observed by phase-contrast microscopy daily for the first 2 weeks and twice a week thereafter. In some cultures, phase-contrast, time-lapse cinematography was used at different stages of culture development. After various periods in culture, some preparations were fixed with methanol and stained with May-Grünwald-Giemsa, toluidine blue, and cresyl violet-acetic acid for the detection of specific metachromasia (*2*). Other cultures were fixed in 1 percent osmium tetroxide and prepared for electron microscopy. Fragments of cerebral white matter from a 14-year-old patient who had surgery because of a cyst of the third ventricle and portions of the apparently normal cerebral cortex of an 8-year-old girl with a glioma of the thalamus were similarly prepared for studies in vitro and used as controls. Light and electron microscopy and histochemical studies for metachromasia were also done on these tissues.

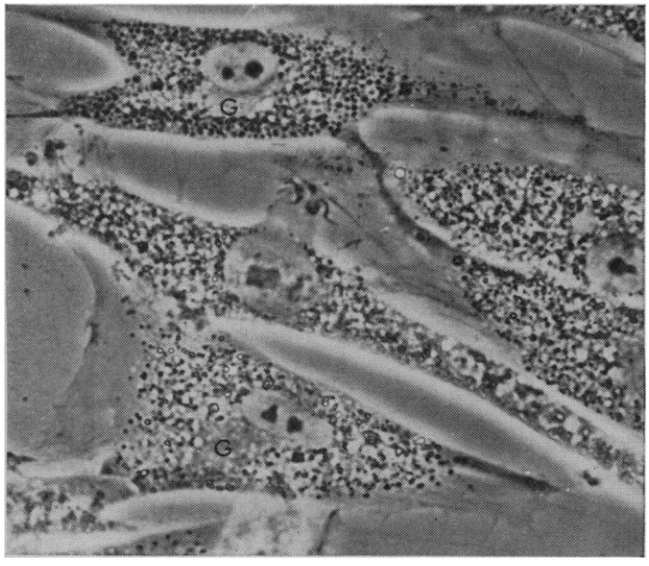
Growth of the cerebral white matter derived from cell suspensions in Rose chambers was apparent by the 6th day in culture and was vigorous by the 14th day (Fig. 1); mitotic figures, however, were only infrequently observed. Although there was some variation in the size and shape of the cells, a large majority of them appeared elongated and showed two or more processes (bipolar or multipolar cells). The nucleus was usually centrally located, occupied about one-fourth of the total cell, and contained one or two distinct nucleoli. The cytoplasm was relatively abundant and contained large numbers of dense dark granules about 1 μ in diameter. A less abundant, doubly refractile type of granule with a clear central area, but of essentially similar dimensions, was also seen in the cytoplasm, interspersed among the denser granules. Granules of both types occupied most of the cytoplasm and were immediately adjacent to the nuclear membrane. Only the Golgi zone appeared conspicuously devoid of these granules. A characteristic brown metachromasia was readily demonstrated in the granules of early cultures (6 to 14 days in vitro) with the cresyl violet–acetic acid technique (*2*) and persisted in cultures stained at 30, 60, and 80 days in vitro (Fig. 2). A moderate amount of clear, flattened membranous ectoplasm was usually present on both poles of the cells. Sometimes, great numbers of large, doubly refractile vacuoles were accumulated in both poles of the cells. The growth of cells was maintained in vitro with essentially similar characteristics up to the 70th day when the cultures began to deteriorate. By the 90th day large numbers of cells had died or were degenerated, but a few appeared quite healthy. Although there were some apparently astrocytic cells, no conclusions as to the true histological identity of most cells could be reached. Further analysis of phase-contrast photographs and of the ultrastructure may shed more light on this subject (*6*). In the Rose chamber (as observed by phase-contrast cinematography) many mobile phagocytic microglia were observed in the medium near the explant by 4 days. These were spherical elements which displayed small characteristic processes in active motion. Accompanying the microglia were a few spindle elements, each with a thin cell body tapering on each side to extremely long processes (approximately 150 μ in length in some cases), which were often multiple on each side of the cell body and arose as bifurcations at or near the cell body. Film sequences revealed frequent collisions of wandering phagocytes with such processes and subsequent temporary adhesions at the points of contact, leading to frequent tugging movements. The thin, elongated element displayed a refractile cell body which prevented detailed cytologic observations by phase microscopy. Another type of cell which emigrated from the explant in early cultures was a large stellate or bipolar cell replete with multiple refractile granules. At about 2 weeks in vitro, time-lapse sequences revealed great numbers of another type of cell adhering to the margin of the explant in

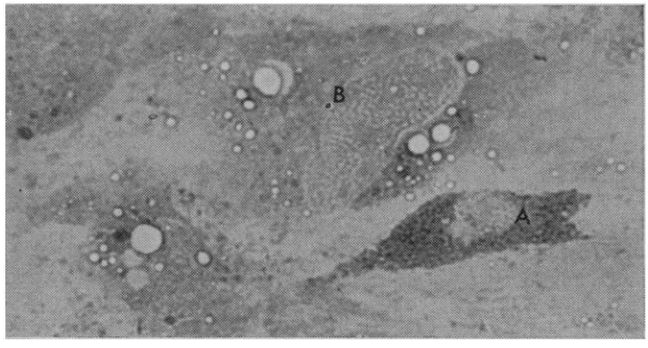
palisade fashion. These cells contained large quantities of refractile granules that stained positively for metachromatic substances with cresyl violet–acetic acid. These cells were apparently slightly mobile and occasionally became detached from each other and the free surface. The simultaneous presence of three of these cell types is illustrated in Fig. 3. Viable cultures were maintained in roller tubes and in Rose chambers for 3 months. The principal surviving cell type in long-term primary cultures of explants of white matter was the broad multipolar granular type, which also survived to this period in dissociated cultures.

The control tissues were normal in all respects. Tissue sections were negative for metachromasia. The pattern and rate of growth in vitro of normal white matter was different from that of MLD. Growth in vitro was exceedingly slow even after 14 days. Whereas in MLD 90 percent of the cultures grew rapidly, only 50 percent of the normal tissues were more or less successful; typical astrocytes were frequently identified in the latter.

Some of these cells contained a moderate amount of granules, although they were distinctly smaller and considerably fewer than in MLD. In one case, satin with cresyl violet–acetic acid gave negative results; in the other, a few cells showed a purplish-red coloration of the cytoplasm or red-brown staining in an occasional cell.

This is apparently the first time that cerebral or peripheral nerve tissues (or both) from patients with metachromatic leukodystrophy have been cultured in vitro. These techniques can be of value in the study of the biology and behavior of human neural tissues of this disease. The characteristic feature of the cultured cells appeared to be the perinuclear accumulation of large numbers of granules in the cytoplasm, except for the Golgi area. The brown metachromasia demonstrated in these granules is histochemical evidence for the presence of abnormally large amounts of cerebroside sulfate in the cultured cells. The luxuriant growth after 14 days in culture and the evidence of cell division by mitosis (as observed by phase-contrast cinematography) suggest that the observed cells were not only migrating cells from the original explant but





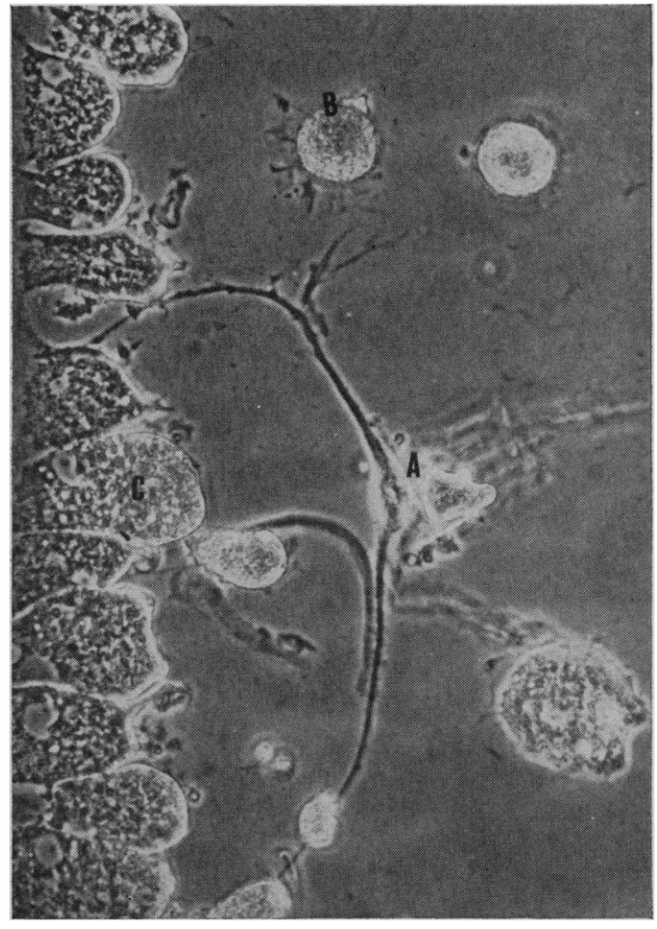


Fig. 1 (upper left). Cerebral white matter, 14 days in vitro, showing accumulation of large numbers of dense granules and smaller number of refractile granules in most of the cytoplasm but not in the Golgi zone (*G*). Variation in cell shape also shown. Phase-contrast photomicrograph of living cells (× 675). Fig. 2 (lower left). Cerebral white matter, 60 days in vitro. Cytoplasm of cell (*A*) is almost completely filled with dense granules which appear metachromatically dark brown. Another cell (*B*) shows a very large nucleus, large refractile cytoplasmic vacuoles, and only a few metachromatically stained small granules. Cresyl violet–acetic acid stain of Von Hirsch and Peiffer (× 450). Fig. 3 (right). Cerebral white matter, 12 days in vitro in Rose chamber. Edge of explant is at left. Three cell types are visible. (*A*) Spindle element with long processes; (*B*) phagocyte of the microglial type; and (*C*) a large element appearing at the margin of the explant in great numbers and containing refractile granules that stain metachromatically with cresyl violet–acetic acid. Cell *C* occasionally emigrated from the margin and assumed a globoid shape. Abstract from a phase-contrast time-lapse film at one frame per minute (20 × objective and 6 × eyepiece).

actually were new cells derived from the orginally explanted cells through cell division. The brown metachromasia consistently observed in vitro in MLD was not seen to any significant degree in the normal cultured white matter and cerebral cortex.

The value of tissue culture for the study of hereditary metabolic disorders was demonstrated by Danes and Bearn (*7*), who were able to maintain (for periods up to 8 months) viable cultures of skin fibroblasts from patients with Hurler's disease (an inborn error of mucopolysaccharide metabolism). When stained for mucopolysaccharides with toluidine blue O, 60 to 100 percent of the cultured cells contained pink or red metachromatic granules. On the other hand, similar cultures of normal controls showed that only 0.1 percent of the cells contained metachromatic granules.

Although more definite proof is needed, our study suggests that the biochemical alterations of metachromatic leukodystrophy (intracellular accumulation of cerebroside sulfate) may be transmitted from cell to cell, at least under our conditions in vitro. If this hypothesis is substantiated by other techniques, such as thymidine incorporation, subcultures, and development of a cell line, the use of cultured cells from patients with metachromatic leukodystrophy for the further study of this disease or even for therapeutic trials will be valuable.

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Thyroxine Interaction with Actinomycin D and Possible Biological Implications

Abstract. *Actinomycin D and thyroxine interact in solution* (p*H 8 to 10*), *as revealed by changes in the absorbance of actinomycin D. Thyroxine can prevent the growth-inhibitory effect of actinomycin D on* Bacillus subtilis *if it is present in a molar ratio of 3000 (thyroxine to actinomycin D).*

Actinomycin D has been extensively used for investigating RNA metabolism and protein synthesis in biological systems (*1*). While a few anomalous effects of actinomycin D have been reported (*2*), the general interpretation of its effect is that it inhibits DNA-dependent RNA synthesis. This inhibition is the specific result of binding of actinomycin D to DNA.

Studies involving subcellular binding of thyroxine (*3*) and actinomycin D (*4*) in tadpole liver nuclei showed that thyroxine is capable of interacting relatively strongly with actinomycin D. To define more clearly the nature of this interaction, we studied it in relation to its biological effect.

The concentration of crystalline actinomycin D was calculated from its extinction coefficient (E) at 440 mμ with a molar extinction coefficient value of 2.48×10^4 (*5*).

The absorption maximum of actinomycin D was shifted toward the higher wavelengths (Fig. 1), and absorption at 475 mμ increased while that at 440 mμ decreased after thyroxine was added.

Interaction between the two compounds was tested at *p*H 8, 9, and 10 (Fig. 2). Thyroxine is insoluble at a lower *p*H. To minimize any experimental error resulting from volume changes, the ratio of $E_{475\,m\mu}$ to $E_{440\,m\mu}$ was plotted against the molar ratio of actinomycin D to thyroxine. Smaller changes in the spectrum were observed in the system containing deoxyguanosine and actinomycin D. Deoxyguanosine has been used as a model compound in support of the hypothesis that the binding sites of actinomycin C (*6*) in DNA were the guanosine residues. The high affinity of guanine for actinomycin C in comparison with other constituents of the nucleic acids has been explained by the fact that it is a better electron donor than the pyrimidines and that its reactive center is different from that of adenine (*7*).

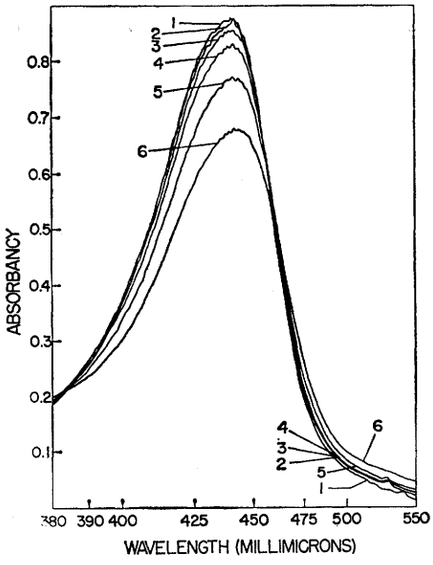

Fig. 1. Thyroxine binding to actinomycin D. Spectral changes of actinomycin D (20 μg/ml) after the addition of thyroxine solution were recorded by a Beckmann DK-2 spectrophotometer. The experiment was performed by successively adding 10, 10, 20, 50, and 100 μg of thyroxine to a solution of actinomycin D in 0.1*M* tris buffer, *p*H 8.0. Curve 1, actinomycin D; curve 2, actinomycin D and 10 μg of thyroxine; curve 3, as above and additional 10 μg of thyroxine; curve 4, as above and additional 20 μg of thyroxine; curve 5, as above and additional 50 μg of thyroxine; curve 6, as above and additional 100 μg of thyroxine.

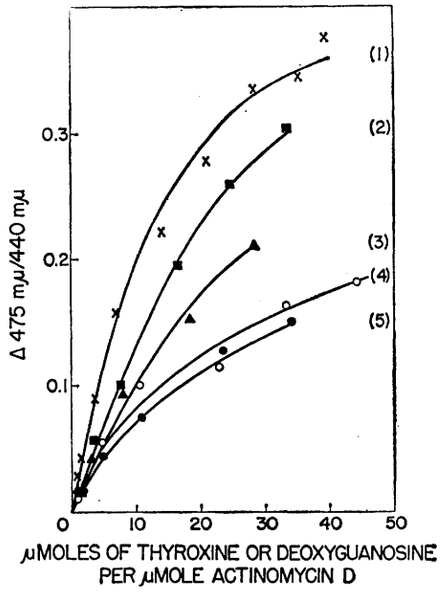

Fig. 2. Actinomycin D binding to thyroxine and deoxyguanosine. Ratios of the absorption at 475 and 440 mμ are plotted against the molar ratios of thyroxine to actinomycin D and deoxyguanosine to actinomycin D at different *p*H values. Curve 1, thyroxine and actinomycin D at *p*H 8; curve 2, thyroxine and actinomycin D at *p*H 9; curve 3, thyroxine and actinomycin D at *p*H 10; curve 4, deoxyguanosine and actinomycin D at *p*H 8; curve 5, deoxyguanosine and actinomycin D at *p*H 7.