DEFICIENCY OF SPHINGOMYELIN-CLEAVING ENZYME ACTIVITY IN TISSUE CULTURES DERIVED FROM PATIENTS WITH NIEMANN-PICK DISEASE

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Summary: The activity of the enzyme which catalyzes the hydrolysis of sphingomyelin has been assayed in tissue cultures derived from the skin and bone marrow of patients with different forms of Niemann-Pick disease. Compared with the values obtained with control cells, the activity of this enzyme was markedly reduced in two forms of Niemann-Pick disease.

Niemann-Pick disease (NPD) is an inherited syndrome that is characterized by the accumulation of sphingomyelin and cholesterol in many tissues throughout the body and occurs in several forms (Fredrickson, 1966). Previous studies in this laboratory have shown that fibroblasts from patients with Niemann-Pick disease have an elevated sphingomyelin content (Uhlendorf et al., 1966). A deficiency of the enzyme which catalyzes the hydrolysis of sphingomyelin has been demonstrated in liver and kidney from patients with two forms of this disorder (Brady et al., 1966 Schneider and Kennedy, 1967). We have now found that the content of sphingomyelin-cleaving enzyme (Sph'ase) is markedly depressed in fibroblasts derived from patients with these same two forms of the disease.

MATERIALS AND METHODS

Cultures were obtained from four patients with the fatal, infantile form (Type A)in the clinical classification of Crocker (1961), four others whose disease began late in childhood (Type B), and one with a protracted course of visceral and neural involvement (the Nova Scotia variant or Type D). The patients ranged from six months to 24 years of age. The 21 control cultures were obtained from 16 individuals ranging in age from 10 months to 26 years. Included among the control patients was one patient each with G_{M1} gangliosidosis, Tay-Sachs disease, and Hurler's syndrome.

Sphingomyelin labeled with ^{14}C in the methyl carbon atoms of the choline portion of the molecule was employed in all assays. The sphingomyelin was either chemically synthesized (Kanfer et al., 1966) or obtained from fibroblasts grown in the presence of ^{14}C-methyl-methionine and ^{14}C-methyl-choline. The biosynthesized sphingomyelin was purified by standard techniques (Sweeley, 1963). Orthonitrophenyl-β-D-galactopyranoside, p-nitrophenyl phosphate, and p-nitrophenyl-N-acetyl-β-galactosaminide were purchased from Mann Research Laboratories. Cutscum (isooctylphenoxypolyoxyethanol) was obtained from Fisher Scientific.

Fibroblasts derived from skin and bone marrow were grown under the conditions previously described (Uhlendorf et al., 1966). The fibroblast preparations were sonicated for two periods of one minute each in 2.5% Cutscum. All samples were then frozen until assayed; repeated freezing and thawing did not affect enzymatic activity.

The activity of (Sph'ase) in the fibroblast preparations was

assayed by measuring the formation of the CCl_3COOH soluble product, ^{14}C-phosphorylcholine, released from the acid-insoluble ^{14}C-methyl-sphingomyelin substrate; activity was expressed as mμmoles of sphingomyelin hydrolyzed per million cells per hour.

The incubation mixtures contained aliquots of the various tissue culture preparations, 25 μmoles of sodium acetate buffer of pH 5.1, 75 mμmoles of ^{14}C-methyl-sphingomyelin (400,000 dpm/μmole), 100 μg of sodium cholate, 200 μg of Cutscum, and water in a final volume of 0.2 ml. After incubation for 90 minutes at 37°, the reaction was terminated by the addition of 0.1 ml of human serum albumin (100 mg/ml), 0.1 ml of 100% CCl_3COOH, and 0.8 ml of cold water. The supernatant solutions obtained by centrifugation were decanted and the precipitates washed once in 1 ml of cold 20% CCl_3COOH. Aliquots of the combined supernatant solutions were assayed by liquid scintillation spectrometry, employing a Triton-toluene counting solution (Benson, 1966). Corrections for quenching were made by the automatic external standard technique.

The capacity of the various tissue culture preparations to hydrolyze the several nitrophenyl derivatives was assayed by measuring spectrophotometrically the formation of nitrophenol. The incubation mixture contained known quantities of fibroblasts, 25 μmoles of sodium acetate buffer of pH 5.1, 100-400 μg of the specific nitrophenyl derivative, 200 μg of Cutscum, and water in a final volume of 0.2 ml. After incubation for 90 minutes at 37°, the reaction was terminated by the addition of 0.1 ml of human serum albumin (100 mg/ml) and 0.5 ml of 4% CCl_3COOH. The supernatant

solutions obtained by centrifugation were pipetted into 0.4 ml of 1M Na_2CO_3 and the optical density determined at 415 mμ.

RESULTS AND DISCUSSION

The results obtained in a total of sixteen NP cultures and the cultures from controls are shown in Tables I and II. There was a pronounced diminution of the activity of the sphingomyelin-cleaving enzyme in the tissue cultures from patients with both Types A and B of Niemann-Pick disease. The activity was decreased in both skin and bone marrow fibroblasts. Sph'ase activity in cells from the Type D patient was within the normal range. These results in tissue culture cells are in accord with previous findings in solid tissue (Brady et al., 1966; Schneider and Kennedy, 1967).

TABLE 1

Level of Sphingomyelin-Cleaving Enzyme in Bone Marrow Tissue Culture Preparations

Condition of Patient	Number	Enzymatic Activity (units per million cells)*
NPD Type A†	4	0.1 ± 0.1‡
NPD Type B	2	3.2 ± 0.9
NPD Type D	1	30.0
Controls	9	52 ± 10

* One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 mμmole of sphingomyelin per hour using the conditions described in the text.

† Types A, B, and D refer to the clinical classification system of Crocker (Crocker, 1961)

‡ Standard deviation.

The conditions of incubation are described in the text.

TABLE 2

Level of Sphingomyelin-Cleaving Enzyme
in Skin Tissue Culture Preparations

Condition of Patient	Number	Enzymatic Activity (units per million cells)*
NPD Type A†	4	0.1 ± 0.1‡
NPD Type B	4	2.0 ± 0.7
NPD Type D	1	37.0
Controls	12	35.1 ± 6.2

* One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 mμmole of sphingomyelin per hour using the conditions described in the text.

† Types A, B, and D refer to the classification system of Crocker.

‡ Standard deviation.

The conditions of incubation are described in the text.

The amount of added ^{14}C-methyl-sphingomyelin exceeded the amount of endogenous substrate by 10-20-fold. Moreover, mixtures of control and NP cells cleaved precisely the amount of sphingomyelin expected from the mixtures. These experiments indicate that an inhibitor was not present in the Niemann-Pick cells and that the low Sph'ase activity in the NPD tissue cultures could not be attributed to dilution of the labeled substrate by endogenous levels of sphingomyelin. These experiments also indicate that the low Sph'ase activity in the NPD cells is not due to depressed levels of an enzyme activator.

Since Sph'ase and several acid hydrolases are lysosomal enzymes (Weinreb et al., 1968; Wattiaux et al., 1963; Sellinger et al., 1960; Frohwein and Gatt, 1967), it was of special interest to determine

the activities of β-N-acetyl-galactosaminidase, β-galactosidase, and acid phosphatase in the tissue culture cells. The values for each of the hydrolases in NPD cells fell within the range obtained in the control cells.

The very low values of Sph'ase in Types A and B of Niemann-Pick disease confirm a preliminary experiment with amnion (Uhlendorf et al., 1966) suggesting that these two disorders can be diagnosed by tissue culture before other abnormalities of the disease become clinically evident. Presumably the depressed Sph'ase activity accounts for the elevated sphingomyelin content found in these two forms of NPD. The nature of the enzyme defect is unknown. It is quite possible that the enzymatic defect is not the same in the several forms of NPD since they appear to be due to different mutations. An examination of presumed heterozygotes is now underway to determine the possibilities of phenotyping by tissue culture.

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REFERENCES

Benson, R. H. Analyt. Chem. 38: 1353, 1966.
Brady, R. O., Kanfer, J. N., Mock, M. B., and Fredrickson, D. S. Proc. Nat. Acad. Sci. 55: 366, 1966.
Crocker, A. C. J. Neurochem. 7: 69, 1961.
Fredrickson, D. S. In The Metabolic Basis of Inherited Disease, edited by Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., New York, McGraw-Hill, 1966, pp. 586-617.
Frohwein, Y. Z., and Gatt, S. Biochemistry 6: 2775, 1967.
Kanfer, J. N., Young, O. M., Shapiro, D., and Brady, R. O. J. Biol. Chem. 241: 1081, 1966.

Schneider, P. B., and Kennedy, E. P. J. Lipid Res. 8: 202, 1967.
Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A., and DeDuve, C. Biochem. J. 74: 450, 1960.
Sweeley, C. C. J. Lipid Res. 4: 402, 1963.
Uhlendorf, B. W., Holtz, A. I., Mock, M. B., and Fredrickson, D. S. In Proceedings of the Third International Conference on the Sphingolipidoses, edited by Aronson, S. M., and Volk, B. W., New York, Pergamon Press, 1966, pp. 443-453.
Wattiaux, R. In Ciba Foundation Symposium on Lysosomes, edited by DeRuck, A. V., and Cameron, M. P., Boston, Little, Brown & Co., 1963, p. 176.
Weinreb, N. J., Brady, R. O., and Tappel, A. L. Biochim. Biophys. Acta 159: 141, 1968.