THE METABOLISM OF SPHINGOMYELIN, II. EVIDENCE OF AN ENZYMATIC DEFICIENCY IN NIEMANN-PICK DISEASE

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The accumulation of excessive quantities of sphingomyelin in tissues of patients with Niemann-Pick disease was demonstrated by Klenk in 1934[1, 2] and has been amply confirmed by other investigators.[3–5] A study by Crocker and Mays[6] indicated that the rate of biosynthesis of sphingomyelin in tissues from these patients appeared to be essentially normal. These findings suggested that the metabolic lesion in this condition might be of a catabolic nature.

We have recently obtained evidence for the presence of a specific enzyme in liver tissue which catalyzes the hydrolysis of sphingomyelin.[7] The enzyme was partially purified from rat liver tissue, and the products of the reaction were demonstrated to be phosphorylcholine and ceramide. The most highly purified enzyme preparations did not catalyze the hydrolysis of lecithin or phosphatidylethanolamine. Lecithin, however, appeared to be a competitive inhibitor of the reaction. Similar enzymatic activity could be detected in human liver tissue. The present report describes experiments in which the level of the sphingomyelin-cleaving enzyme in liver and kidney tissue obtained from six patients with the classic infantile form of Niemann-Pick disease[8] is compared with the values found in a liver biopsy sample from a normal human adult and liver and kidney tissue of patients with various other disorders.

Materials and Methods.—Sphingomyelin labeled with C^{14} in the methyl carbon atoms of the choline portion of the molecule was synthesized as described previously.[7] Samples of liver and kidney tissue were homogenized in 10 vol of 0.25 *M* sucrose with an all-glass TenBroeck homogenizer and centrifuged at 600 × *g* for 12 min. Suitable aliquots of the supernatant suspensions were assayed in order to ensure that the level of sphingomyelin-cleaving activity detected was proportional to the amount of protein in the samples. Separate aliquots of the enzyme preparations were extracted with 25 vol of chloroform-methanol solution 2:1 (v/v), and the amount of the various phospholipids was determined by quantitative thin-layer chromatographic analysis.[9] The determination of sphingomyelin in the various preparations by this procedure permitted an accurate calculation of the dilution of labeled substrate by endogenous sphingomyelin.

The incubation mixtures contained aliquots of the various tissue preparations, 24 μmoles of potassium acetate buffer, pH 5.0, 67.6 mμmoles of sphingomyelin-C^{14} (325,000 cpm per μmole), 0.25 mg of sodium cholate, 50 μg of Cutscum (isooctylphenoxypolyoxyethanol), and water in a final volume of 0.2 ml. After incubation for 1 hr at 37°, 0.8 ml of cold water was added to each tube followed by 0.1 ml of human serum albumin (100 mg per ml), and 0.1 ml of 100% trichloroacetic acid (TCA) solution. Sphingomyelin is insoluble in dilute TCA. Phosphorylcholine is completely soluble under these conditions, and this characteristic has been used previously for the determination of phosphorylcholine.[10] The supernatant solutions obtained by centrifugation were decanted and the precipitates resuspended in 1 ml of cold 10% TCA solution. After centrifugation, the combined supernatant solutions were extracted with 1 ml of ether to remove any residual turbidity, and aliquots of the aqueous phase were taken for radioactivity determination by liquid scintillation spectrometry.[11]

Results and Discussion.—The enzyme which catalyzes the hydrolysis of sphingomyelin could be demonstrated in human liver tissue obtained by surgical biopsy

or autopsy. The range of enzymatic activity was 4.4–11.1 mμmoles of sphingomyelin cleaved per mg of protein per hour (Table 1). The average value was 6.6 mμmoles/mg of protein/hour. The quantity of sphingomyelin in several of the tissue samples was determined and was found to be less than 2 per cent of the amount of labeled sphingomyelin added to each of the assay mixtures. Therefore, no correction for endogenous dilution was made in these preparations. Enzyme preparations of tissues obtained from patients with Niemann-Pick disease showed a drastic reduction in sphingomyelin-cleaving activity. The values ranged from 0 to 0.89 mμmole/mg of sphingomyelin hydrolyzed/mg of protein/hour. The average was 0.46 mμmole/mg of protein/hour. The quantity of endogenous sphingomyelin in these enzyme preparations varied from 28 to 188 per cent of the amount of added sphingomyelin-C^{14}, and the values reported for enzymatic activity in all of the examples of Niemann-Pick disease have been corrected for dilution of labeled substrate by assuming complete mixing with the endogenous sphingomyelin.

The diminished activity of the sphingomyelin-cleaving enzyme in the Niemann-Pick preparations probably cannot be attributed to inhibition by excess sphingomyelin. Experiments with the partially purified enzyme from rat liver[7] indicated no substrate inhibition at a sphingomyelin concentration as high as 2.7×10^{-3} M. In the present experiments, the highest concentration of endogenous sphingomyelin in the incubation mixtures was 3.03×10^{-4} M. Thus, the final maximal sphingomyelin concentration was 6.4×10^{-4} M, a level at which substrate inhibition does not occur in the rat. Studies in which sodium cholate or Cutscum was omitted from the incubation mixtures indicated that the enzyme in preparations from the Niemann-Pick patients was not inhibited or inactivated by these materials.

Lecithin has been shown to be a competitive inhibitor of the sphingomyelin-cleaving enzyme.[7] Since lecithin also accumulates to some degree in tissues of Niemann-Pick disease, the amount of lecithin in these preparations was also determined. The amount of lecithin was never more than one sixth of that of sphingomyelin in these preparations. Since the K_i for lecithin is approximately equal to the K_m for sphingomyelin, the quantity of sphingomyelin in the enzyme preparations would preclude any marked competitive inhibition of the sphingomyelin-cleaving activity by lecithin. Rather, it is conceivable that the increased quantities of lecithin in tissues of patients with Niemann-Pick disease could be due to a similar inhibition of a specific lecithinase by the excessive quantities of sphingomyelin.

Further evidence for the specificity of the enzyme involved in Niemann-Pick disease was obtained with the use of liver tissue from patients with a different sphingolipidosis. In Gaucher's disease an increase of glucocerebroside occurs in various tissues, and an attenuation of the activity of the enzyme which catalyzes the cleavage of this substance has been demonstrated.[12] The activity of the sphingomyelin-cleaving enzyme in the liver preparations from two patients with this disease was within normal values.

A survey of the level of sphingomyelin-cleaving enzyme in various tissues of the rat indicated that next to liver, kidney preparations exhibited the highest specific activity.[7] An enzyme preparation from a sample of human kidney tissue frozen for 3 years showed considerable hydrolytic activity toward sphingomyelin. A similar preparation of kidney tissue from a patient with Niemann-Pick disease failed to show any sphingomyelin-cleaving activity. This observation suggests that there is

TABLE 1

LEVEL OF SPHINGOMYELIN-CLEAVING ENZYME IN HUMAN TISSUE PREPARATIONS

Tissue	Source	Patient	Diagnosis	Age (yr)	Sex	Enzymatic activity (units/mg of protein)*
Liver	Fresh (necropsy)	MH	Rheumatic heart disease	55	M	4.7
"	" "	RD	" " "	45	M	4.4
"	" "	NK	Neuroblastoma	64	M	10.3
"	" "	CD	Tetralogy of fallot	10	F	4.9
"	" "	JD	Mitral stenosis	48	M	11.1
"	" "	NN	Rheumatic heart disease	44	F	4.4
"	Biopsy	DK	Normal	63	F	9.5
"	Frozen, 3 yr	KS	Heart disease	5	F	8.9
"	" 3 "	KW	Septal defect	7	F	4.4
"	" 1 "	JV	Aβ-lipoproteinemia	11	M	4.5
"	" 4 "	ZK	Gaucher's disease	31	M	4.6
"	" 3 "	WC	Infantile Gaucher's disease	1	M	7.0
						Mean 6.6 ± 2.6†
Kidney	" 3 "	KW	Septal defect	7	F	4.4
Liver	" 2 "	KP	Niemann-Pick disease‡	2	F	0.0
"	" 3 "	NW	" " "	3	F	0.54
"	" 1/2 yr	PW	" " "	3	M	0.61
"	" 2/3 "	ND	" " "	3	M	0.26
"	Biopsy	DB	" " "	9 mo.	M	0.46
"	"	ES	" " "	9 "	M	0.89
						Mean 0.46 ± 0.31†
Kidney	Frozen, 2 yr	KP	" " "	2	F	0.0

* 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 of incubation described in the text.

† Standard deviation.

‡ Clinical details covering the first four patients with Niemann-Pick disease appear elsewhere in a review[8] coded as nos. 163(KP), 70(NW), 71(PW), and 164(ND). The biopsy sample on DB, an infant with history entirely compatible with the infantile form of the disease, was generously provided by Dr. Janet Cuttner, Mt. Sinai Hospital, New York. The biopsy sample on ES was supplied by Dr. Robert Kaye, Children's Hospital, Philadelphia. The proportion of total liver phospholipid phosphorus represented by sphingomyelin in these six patients was 60, 59, 55, 49, 51, and 62%, respectively. The normal value is less than 10%.

a generalized diminution of sphingomyelin-cleaving enzyme activity in various tissues of patients with Niemann-Pick disease.

Summary.—The level of the enzyme which catalyzes the hydrolysis of sphingomyelin has been determined in tissue samples from patients with Niemann-Pick disease and compared with tissue specimens from other human sources.

It appears that the metabolic lesion in the classic infantile form of Niemann-Pick disease is attributable to a drastic attenuation or loss of activity of the enzyme which catalyzes the cleavage of sphingomyelin.

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*EFFECT OF ACETOXYCYCLOHEXIMIDE AND OF AN ACETOXYCYCLOHEXIMIDE-PUROMYCIN MIXTURE ON CEREBRAL PROTEIN SYNTHESIS AND MEMORY IN MICE**

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Puromycin affects memory in mice[1] and, as shown by Agranoff *et al.*,[2] in goldfish. It has been tentatively proposed that the destruction of memory (simple maze learning) in mice by puromycin depends upon the degree and duration of inhibition of protein synthesis produced by this antibiotic.[3–5] There is the possibility, however, that some other action of puromycin might be responsible for loss of memory. For this reason, analogues of puromycin have been tested but they have been found to be without effect on memory.[4] Acetoxycycloheximide has been used in the experiments reported here. This antibiotic has been observed to produce profound inhibition of protein synthesis *in vivo*[6] by inhibiting transfer of amino acid from sRNA to polypeptide.[7, 8] Puromycin has a different mode of action, being incorporated into the carboxyl ends of growing polypeptide chains and causing their premature release.[9, 10]