Amer J Hum Genet 25:372–381, 1973

Absence of β-N-Acetyl-D-Hexosaminidase A Activity in a Healthy Woman

JACLYN VIDGOFF,[1] NEIL R. M. BUIST,[2] AND JOHN S. O'BRIEN[3]

INTRODUCTION

Tay-Sachs disease is an inborn error of lipid metabolism associated with a defect of hexosaminidase activity in the serum and tissues. Discovery of two β-N-acetyl-D-hexosaminidase isozymes [1, 2] and of the deficiency of the A form in patients with Tay-Sachs disease [3] has facilitated both mass screening of the high-risk Ashkenazic Jewish population and also antenatal diagnosis of affected infants [4–6].

Hexosaminidase A appears to be the only isozyme capable of catalyzing the first step in the catabolism of G_{M2}, the Tay-Sachs ganglioside [7–10]. Presumably hexosaminidase A deficiency causes ganglioside accumulation and symptoms of Tay-Sachs disease. Assay of the enzyme is based upon the thermolability of the more acidic isozyme, hexosaminidase A, whereas hexosaminidase B activity is thermostable [3, 11, 12]. Both of these isozymes are inactive in clinically indistinguishable Sandhoff's disease [13] which, unlike Tay-Sachs disease, is panethnic.

This report describes studies of a healthy, non-Jewish, 29-year-old mother of a child who died of Tay-Sachs disease. The finding of minimal heat-labile hexosaminidase A in the mother's serum or leukocytes appeared similar to findings reported in patients with Tay-Sachs disease.

Since our subject (hereafter referred to as the proband) had given birth to a child with classical Tay-Sachs disease, we assume that she was heterozygous for two abnormal genes, one for classical Tay-Sachs disease and the second presumably resulting in a functional gene product which lacks the physico-chemical properties of normal hexosaminidase A.

MATERIALS AND METHODS

The substrate 4-methylumbelliferyl-β-N-acetyl-D-glucosaminide was purchased from Pierce Chemical Company (Rockford, Illinois), and 4-methylumbelliferone from K and

Received November 3, 1972; revised February 8, 1973.

This study was supported by grants HD 00165, NB 08682, and GM 17702 from the U.S. Public Health Service.

[1] Department of Medical Genetics, University of Oregon Medical School, Portland, Oregon 97201.

[2] Departments of Pediatrics and Medical Genetics, University of Oregon Medical School, Portland, Oregon 97201.

[3] Department of Neurosciences, University of California at San Diego, La Jolla, California 92037.

K Laboratories, Inc. (Plainview, New York). All other chemicals were obtained in the best available grade from commercial sources.

Absorption measurements were made on a Hitachi-Perkin Elmer 139 and fluorescence measurements on an Aminco-Bowman fluorometer.

Plasma was derived from whole blood treated with heparin (100 units/5 ml blood). Leukocytes were isolated by the method of Kampine et al. [14] from 5 ml to a final volume of 1.0 ml. Tissue samples of the child with Tay-Sachs disease and an age-matched control who died of unrelated causes were homogenized in 3 vol of 0.1% bovine serum albumin to which an equal volume of 0.05 M sodium citrate, *p*H 4.3, was added.

Tissues were extracted with 19 vol of chloroform/methanol (2:1) according to the method of Folch et al. [15]; gangliosides were detected with a resorcinol spray [16] after silica gel thin-layer chromatography. The developing solvent was chloroform/methanol/2.5 M ammonia (60:35:8).

Hexosaminidase Assay

Assay for hexosaminidase was run in duplicate using a modification of the method of O'Brien et al. [12]. The buffer was 0.05 M sodium citrate, *p*H 4.3. Reactions were terminated with 3.0 ml of 0.2 M glycine/NaOH, *p*H 10.4. In the blanks, base was added before substrate. Fluorescence was read at 450 nm after excitation at 360 nm. Heat-inactivated samples had been preincubated at 50° C for 3 hr.

After dilution of 100 μliters of sonicated leukocytes to 1.0 ml with citrate buffer, 50-μliter samples were assayed as above. The substrate (150 μliters) contained 0.05% bovine serum albumin.

Tissue extracts (10 μliters) were incubated with 200 μliters of 1.5 mM substrate containing 0.1% bovine serum albumin. All other conditions were as above.

To study the *p*H profile, plasma was diluted with 9 vol of 0.5 M sodium citrate, *p*H 4.0–5.0. Heat inactivation at 50° C for 3 hr was carried out on 100-μliter samples. An equal volume of 0.5 mM substrate in 0.05 M sodium citrate, *p*H 4.3, was incubated as described above.

To determine the effect of storage at various temperatures, aliquots of plasma were assayed either immediately or after 3-hr storage at 4° C, −15° C, or 50° C. Later, tests were run on plasma which had been preincubated at −15° C for 48 hr and then tested before and after heat inactivation.

Plasma from a control and the proband was mixed and assayed as described above.

Separation of Hexosaminidase A and B

A modification of the method of Dance et al. [17] was employed to separate hexosaminidase A and B by treatment of plasma with anion exchange resin. Whatman DE-52 cellulose was equilibrated in 0.01 M sodium phosphate buffer, *p*H 6.0. A 1-ml concentrated suspension of DEAE cellulose was centrifuged and the supernatant buffer discarded. The pellet was resuspended in 0.9 ml of cold 0.01 M sodium phosphate buffer, *p*H 6.0, to which 100 μliters of plasma was added. After mixing for 30–45 min at 4° C, the suspension was centrifuged and the supernatant (hexosaminidase B) diluted 1:1 with 0.05 M sodium citrate containing 0.4 M NaCl, *p*H 4.3. The pellet was washed with 2.0 ml of 0.01 M sodium phosphate buffer and the supernatant discarded. Elution of hexosaminidase A from the pellet was effected with 2.0 ml of a cold solution of 0.005 M sodium phosphate, 0.025 M citrate, and 0.2 M NaCl, *p*H 4.3. Aliquots (100 μliters) were assayed as described above, before and after heat inactivation.

Polyacrylamide Gel Electrophoresis

One-step polyacrylamide disc gel electrophoresis was performed using a modification of the method of Friedland et al. [18]. Gels were poured without a sucrose layer. The

electrode buffers were made according to a modification* of those of Davis [19]. A 25-μliter aliquot of leukocyte sonicate in 0.2 ml of 40% sucrose was layered over each tube. Electrophoresis and activity staining were carried out according to the method of Friedland et al. [18]. Gels were sprayed with 0.2 M glycine/NaOH, *p*H 10.4, and observed under a long-spectrum black light.

A standard curve of 4-methylumbelliferone was made using 10–100 μliters of 10^{-4} M standard in 100 μliters of water, 100 μliters of 0.05 M citrate buffer, *p*H 4.3, and 3.0 ml of 0.2 M glycine/NaOH, *p*H 10.4. Protein determinations were made according to the technique of Lowry et al. [20] using a Labtrol standard.

All experiments on plasma specimens were performed immediately; serum or leukocyte samples required more time for processing and were studied within a few hours. Plasma was used despite reports of heparin inhibition [2] because of the speed with which it could be processed. Clausen et al. [21] and O'Brien et al. [12] found no difference in hexosaminidase activity between plasma and serum samples. In this laboratory, heparinized plasma exhibited 75%–80% of the activity of serum derived from the same source. This inhibition did not seem significant enough to preclude its use, particularly since any influence of heparin on the proband's samples should also be exerted on the controls.

RESULTS

Figure 1 shows the family pedigree; the proband is not Jewish and is of Irish and Dutch extraction. Serum hexosaminidase assays of both parents (I-2 and I-3)

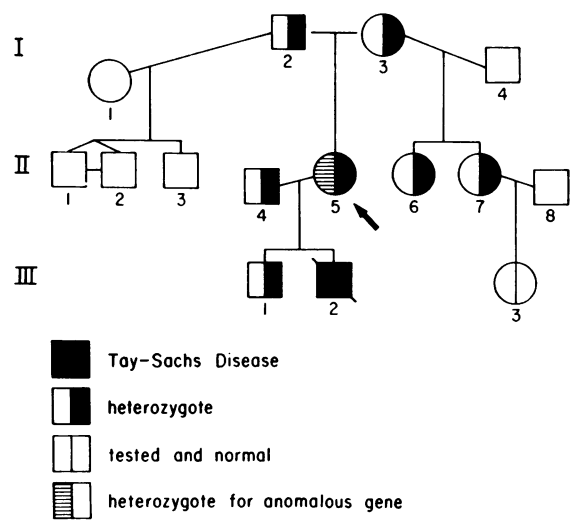

FIG. 1.—Pedigree; III-2 died in infancy of Tay-Sachs disease

of the proband (II-5) indicate they are apparent heterozygotes for Tay-Sachs disease. It is not known which carries the Tay-Sachs gene and which carries the atypical mutation. For simplicity, all heterozygotes are represented as carriers of Tay-Sachs disease.

* "Instructions for Polyanalyst," Buchler Instruments, Fort Lee, New Jersey.

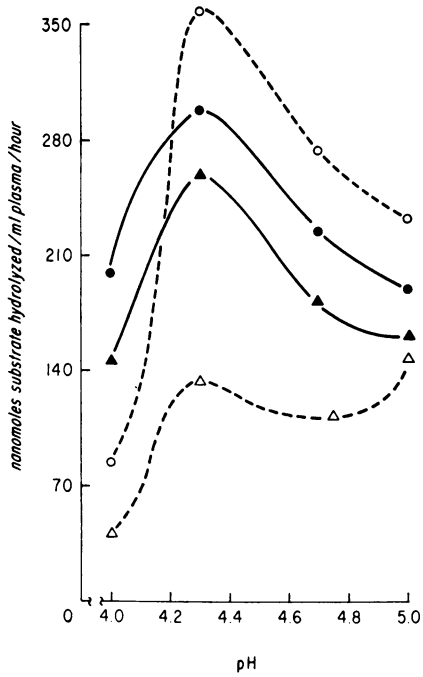

FIG. 2.—Plasma hexosaminidase activity of proband (●) and control (▲) before (▲——●) and after (△ - - - - - ○) 3 hr heating at 50° C as a function of *p*H in 0.27 M sodium citrate.

Control plasma samples had maximum activity at *p*H 4.3 and after heating decreased in activity at all *p*H values. The activity of the proband's plasma had the same *p*H optimum, but at three *p*H values activity of the fresh plasma hexosaminidase increased after heating (fig. 2). After heating at *p*H 4.0 for 3 hr, plasma hexosaminidase activity of the control and the proband showed similar losses.

Plasma and serum hexosaminidase activities of both the proband and controls increased after freezing the samples for 3 hr. Cooling at 4° C for 3 hr enhanced the activity of all samples except that of the proband (table 1). The magnitude of these changes was relatively small, and these experiments were performed only once.

The time course of heat inactivation of the enzyme in serum and leukocytes of controls and the proband is shown in figure 3. For the proband, there was an activity loss of 0%–15% in serum and 24% in leukocytes compared with a gain of 20% in fresh plasma under standard assay conditions. The gain in enzyme activity after heating was not evident in plasma samples which had been stored for 48 hr at —15° C. In the controls, all heated samples showed a significantly greater loss: 40%–50% in 18 carriers and 62%–75% in 40 controls (serum), and 44% in one carrier and 60%–78% in 13 controls (leukocytes).

Table 2 shows the results of the mixing experiment. The values observed upon combining the plasma of control and proband closely paralleled the prediction. Her plasma caused no relative inhibition of normal thermolability of control plasma.

TABLE 1

ACTIVITY OF PLASMA HEXOSAMINIDASE AS A FUNCTION OF PREINCUBATION TEMPERATURE

SAMPLE	IMMEDIATE ASSAY	PREINCUBATION* TEMPERATURE		
		+4° C	−15° C	+50° C
Anomalous carrier	298	290	336	361
	309	276	336	358
Classical carrier	184	197	215	152
	187	194	219	138
Control	263	277	305	135
	263	280	302	131

NOTE.—Values expressed as nanomoles substrate hydrolyzed per milliliter plasma per hour.
* 3 hr.

Figure 4 summarizes the distribution of hexosaminidase activity upon treatment of plasma with DEAE cellulose. Activities before and after heating are shown for the total plasma, for the DEAE supernatant (hexosaminidase B), and for the DEAE pellet (hexosaminidase A). Despite the wash before elution, there was still

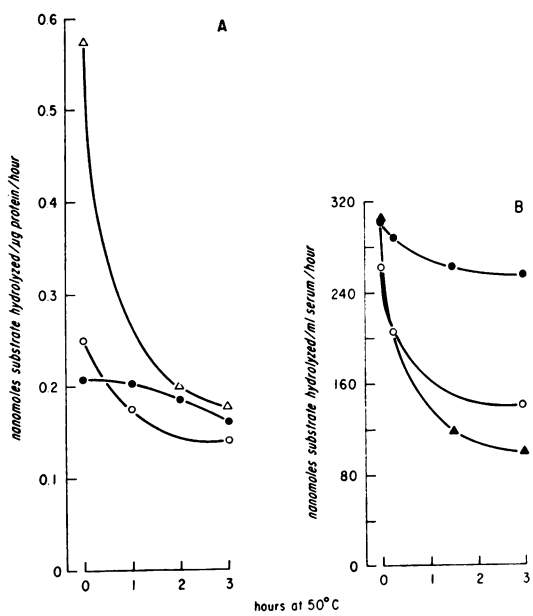

FIG. 3.—*A*, Time course of heat inactivation of hexosaminidase in leukocytes. Nanomoles of substrate hydrolyzed/μg protein/hr versus hours at 50° C of proband (●), Tay-Sachs heterozygote (○), and control (△). *B*, Time course of heat inactivation of hexosaminidase in serum. Nanomoles of substrate hydrolyzed/ml serum/hr versus hours at 50° C of proband (●), Tay-Sachs heterozygote (○), and control (▲).

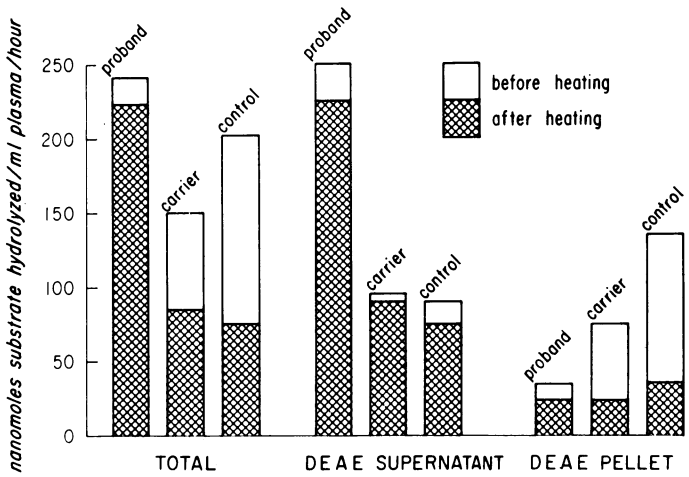

FIG. 4.—DEAE-cellulose anion exchange fractionation of plasma hexosaminidase. Crosshatched areas represent activity remaining after heating.

heat-stable activity in all pellets. This occurred with serum samples also. Total recoveries exceeded 100% whether the samples applied were plasma or serum.

Polyacrylamide disc gel electrophoresis of leukocyte hexosaminidase revealed the absence of the fast-moving, acidic hexosaminidase A in the proband.

Because of the possibility that the proband's child was affected with a condition other than classical Tay-Sachs disease, his autopsy tissue was analyzed. The brain ganglioside pattern showed G_{M2} accumulation, and the brain hexosaminidase studies revealed a decrease in activity of only 6% upon heating at 50° C for 4 hr compared with 52% for an age-matched control (table 3). The biochemical and clinical findings in the infant were diagnostic of Tay-Sachs disease.

TABLE 2

MIXING EXPERIMENT

Sample	Before Heating	After Heating
Individual samples:		
Anomalous carrier	298	361
	309	358
Control	263	135
	263	131
Mixed samples:		
Predicted values	283	246
Actual values	266	227
	273	231

NOTE.—Values expressed as nanomoles substrate hydrolyzed per milliliter plasma per hour.

TABLE 3

BRAIN HEXOSAMINIDASE OF CHILD WITH TAY-SACHS DISEASE

Sample	Before Heating	After Heating
Tay-Sachs disease	1.62	1.55
	1.66	1.55
Control	1.09	0.535
	1.14	0.535

NOTE.—Values expressed as nanomoles substrate hydrolyzed per microgram protein per hour.

DISCUSSION

The healthy adult who is the subject of this report is an obligate carrier of Tay-Sachs disease, yet, instead of having about half normal levels of hexosaminidase A, she had little or no detectable amounts of this heat-labile enzyme; previously, it was assumed that total hexosaminidase A deficiency was incompatible with normal life.

The abnormal results are summarized in table 4. The *p*H optimum and activity of total hexosaminidase after freezing were normal; by all other criteria, her hexosaminidase behaved either uniquely or as would be expected in Tay-Sachs disease.

TABLE 4

SUMMARY OF HEXOSAMINIDASE STUDIES

Type of Study	Control	Carrier	Proband
Plasma hexosaminidase A:B	60:40	40:60	—10:110*
*p*H profile:			
Untreated samples (optimum)	4.3	4.3	4.3
Heat treated (optimum)	4.3	...	4.3
Heat effect at:			
*p*H 4.0	Decrease	...	Decrease
*p*H 4.3	Decrease	...	Increase
*p*H 4.7	Decrease	...	Increase
*p*H 5.0	Decrease	...	Increase
Effect of storage:			
Freezing	Increase	Increase	Increase
Refrigeration	Increase	Increase	Decrease
Heating	Decrease	Decrease	Increase
DEAE-cellulose:			
Supernatant	Heat stable	Heat stable	Heat stable
Pellet	Mostly heat labile	Some heat labile	Little activity
Acrylamide disc gel electrophoresis	Two bands	Two bands	One slow band

* Apparent negative value because of heat-induced increase in activity.

Although the proband's serum and leukocyte hexosaminidase activity decreased somewhat upon heating, her freshly drawn blood exhibited heat activation instead of destruction; this heat-induced augmentation of hexosaminidase activity may be peculiar to her enzyme or may reflect a normal phenomenon usually masked by overwhelming heat denaturation in control samples. Hultberg [22] and Murphy and Craig [23] reported "activation" by conversion of hexosaminidase A into a B-like form by neuraminidase; heat may partially convert one isozyme into the other and partially denature the labile species. Normal samples did not show heat activation even after as short an interval as 15 min at 50° C (J. Vidgoff, unpublished observation). An alternative explanation of heat activation is the destruction of a natural inhibitor; similarly, this might explain the stimulation of activity by freezing. The DEAE cellulose column purification of hexosaminidase from a child with Tay-Sachs disease resulted in more than 100% recovery [24] as if an inhibitor had been removed; this was confirmed in the experiments reported here. Spontaneous destruction of an inhibitor could be responsible for the failure of heat activation of the proband's enzyme after storage for 2 days at —15° C. On the other hand, instability of her enzyme might explain this observation; instability was evidenced by a slight decrease in hexosaminidase activity following a short period of refrigeration. Control samples exhibited a small increase in activity after similar treatment.

Results of the mixing experiment eliminated the possibility of heat stabilization by a loosely bound, diffusible factor. In control samples partitioned with DEAE cellulose, the supernatant fraction contained hexosaminidase B almost exclusively while the resin predominantly bound hexosaminidase A. In this system, the sample from the proband showed the presence of large amounts of hexosaminidase B in the supernatant and only small amounts of enzyme retained by the resin. The level of activity of retained enzyme did not differ radically from that of heat-stable (hexosaminidase B) activity in the same fraction of control samples. The failure to find significant levels of enzyme in this fraction and the absence of heat-labile hexosaminidase were consistent with the lack of activity migrating toward the anode upon polyacrylamide disc gel electrophoresis.

Since the proband did not demonstrate the expected intermediate levels of hexosaminidase A, we assume she had another mutant gene allelic to the Tay-Sachs mutation which resulted in a functional variation of normal ganglioside metabolism. Perhaps the functional enzyme was extremely labile (for which there was minimal evidence), was heat stable and uncharged, or acted only upon the natural substrate. It is conceivable that she had an isozyme of hexosaminidase A which was confined to the brain, but hexosaminidase A, which normally is present in many tissues, is absent in all tissues which have been studied in Tay-Sachs disease. It is likely, therefore, that the electrophoretic variants which have been found in a number of tissues [24–26] arise from postribosomal modification of enzymes coded by a common cistron.

The most likely explanation is that the proband was a heterozygote for two different mutant alleles: one mutation was the classical Tay-Sachs gene and the other

resulted in a functional gene product which recognized the natural substrate but not the synthetic one. The precedent for such substrate specificity was a case of Tay-Sachs "Variant AB" in which both hexosaminidases hydrolyzed synthetic substrates but not the natural substrate [27]. We plan to test this hypothesis by assaying the proband's tissues using G_{M2} as substrate.

Until the causes of these unusual biochemical findings are resolved, antenatal counseling is impossible for this family: the classical Tay-Sachs genotype and the proband's genotype are indistinguishable. Moreover, carriers of either abnormal gene cannot be differentiated. This finding suggests that the true incidence of Tay-Sachs carriers cannot be estimated using current screening procedures which rely on artificial substrates.

SUMMARY

Tay-Sachs disease is an autosomal recessive condition caused by hexosaminidase A deficiency. Studies were made on the blood of a healthy, non-Jewish woman whose child had died of Tay-Sachs disease. Instead of demonstrating the characteristically intermediate levels of this enzyme, this carrier had minimal hexosaminidase A activity. Conventional methods of distinguishing hexosaminidase B from A, including differential heat inactivation and techniques based upon charge differences, failed to demonstrate hexosaminidase A activity. Possible explanations for these anomalous biochemical findings are proposed.

ACKNOWLEDGMENTS

We are grateful to Drs. William Brady, Howard Huntington, and Larry Lewman who provided us with postmortem tissues and to Drs. Nancy Kennaway and Robert Koler who reviewed the manuscript.

ADDENDUM

After this paper was written, we learned of a similar case reported by R. Navon, B. Padeh, and A. Adam (*Amer J Hum Genet* 25:287–293, 1973).

REFERENCES

1. Caygill JC, Roston CPJ, Jevons FR: Purification of β-acetylglucosaminidase and β-galactosidase from ram testis. *Biochem J* 98:405–409, 1966
2. Robinson D, Stirling JL: N-acetyl-β-glucosaminidases in human spleen. *Biochem J* 107:321–327, 1968
3. Okada S, O'Brien JS: Tay-Sachs disease: generalized absence of a β-D-N-acetylhexosaminidase component. *Science* 165:698–700, 1969
4. Kaback MM, Zeiger RS, Gershowitz H: A pilot program in the control of genetic disease (abstr.). *Pediat Res* 6:261, 1972
5. Delvin EE, Scriver CR, Pottier A, Clow CL, Goldman H: Maladie de Tay-Sachs: dépistage et diagnostic prénatal. *Un Med Canada* 101:683–688, 1972
6. O'Brien JS, Okada S, Fillerup DL, Veath ML, Adornato B, Brenner PH, Leroy JG: Tay-Sachs disease: prenatal diagnosis. *Science* 172:61–64, 1971
7. Kolodny EH, Brady RO, Volk BW: Demonstration of an alteration of ganglioside metabolism in Tay-Sachs disease. *Biochem Biophys Res Commun* 37:526–531, 1969
8. Sandhoff K: The hydrolysis of Tay-Sachs ganglioside (TSG) by human N-acetyl-β-D-hexosaminidase A. *Fed Europ Biochem Soc Letters* 11:342–344, 1970

9. SANDHOFF K, WÄSSLE W: Anreicherung und Charakterisierung zweier Formen der menschlichen N-acetyl-β-D-hexosaminidase. *Hoppe Seyler Z Physiol Chem* 352: 1119–1133, 1971
10. TALLMAN JF, JOHNSON WG, BRADY RO: The metabolism of Tay-Sachs ganglioside: catabolic studies with lysosomal enzymes from normal and Tay-Sachs brain tissue. *J Clin Invest* 51:2339–2345, 1972
11. DANCE N, PRICE RG, ROBINSON D, STIRLING JL: β-galactosidase, β-glucosidase and N-acetyl-β-glucosaminidase in human kidney. *Clin Chim Acta* 24:189–197, 1969
12. O'BRIEN JS, OKADA S, CHEN A, FILLERUP DL: Tay-Sachs disease: detection of heterozygotes and homozygotes by serum hexosaminidase assay. *New Eng J Med* 283:15–20, 1970
13. SANDHOFF K, ANDREAE U, JATZKEWITZ H: Deficient hexosaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. *Path Europ* 3:278–285, 1968
14. KAMPINE JP, BRADY RO, KANFER JN, FELD M, SHAPIRO D: Diagnosis of Gaucher's disease and Niemann-Pick disease with small samples of venous blood. *Science* 155: 86–88, 1966
15. FOLCH J, LEES M, SLOANE STANLEY GH: A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509, 1957
16. SVENNERHOLM L: Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim Biophys Acta* 24:604–611, 1957
17. DANCE N, PRICE RG, ROBINSON D: Differential assay of human hexosaminidases A and B. *Biochim Biophys Acta* 222:662–664, 1970
18. FRIEDLAND J, SCHNECK L, SAIFER A, POURFAR M, VOLK BW: Identification of Tay-Sachs disease carriers by acrylamide gel electrophoresis. *Clin Chim Acta* 28: 397–402, 1970
19. DAVIS BJ: Disc electrophoresis. II. Methods and application to human serum proteins. *Ann NY Acad Sci* 121:404–427, 1964
20. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
21. CLAUSEN J, MELCHIOR JC, PAERREGAARD P: Enzymic differentiation between different types of Tay-Sachs disease of similar clinical appearance. *Europ Neurol* 7:56–64, 1972
22. HULTBERG B: N-acetylhexosaminidase activities in Tay-Sachs disease. *Lancet* 2:1195, 1969
23. MURPHY JV, CRAIG L: Neuraminidase-induced changes in white blood cell hexosaminidase A. *Clin Chim Acta* 42:267–272, 1972
24. ROBINSON D, JORDON TW, HORSBURGH T: The N-acetyl-β-D-hexosaminidases of calf and human brain. *J Neurochem* 19:1975–1985, 1972
25. HOOGHWINKEL GJM, VELTKAMP WA, OVERDIJK B, LISMAN JW: Electrophoretic separation of β-N-acetylhexosaminidases of human and bovine brain and liver and of Tay-Sachs brain tissue. *Hoppe Seyler Z Physiol Chem* 353:839–841, 1972
26. YOSHIKAWA J, KOIDE H, YAMANAKA M: Demonstration of the multiplicity of N-acetyl-β-glucosaminidases by electrophoresis. *Biochem Biophys Res Commun* 46: 11–15, 1972
27. SANDHOFF K, HARZER K, WÄSSLE W, JATZKEWITZ H: Enzyme alterations and lipid storage in three variants of Tay-Sachs disease. *J Neurochem* 18:2469–2489, 1971