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Apparent Hexosaminidase B Deficiency in Two Healthy Members of a Pedigree

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SUMMARY

A family is described in which all members have decreased serum and leukocyte hexosaminidase activity. Two individuals, the mother and the younger daughter, have a normal ratio of hexosaminidase B (HEX B) to total hexosaminidase, but their serum enzymes display respectively partial or complete lability to heat. It is proposed that the proband is a double heterozygote for the Sandhoff allele and for an allele producing thermolabile β subunits.

INTRODUCTION

A number of healthy individuals with mutant alleles at the loci which control expression of the β hexosaminidases (E.C.3.2.1.30) have been described [1–6]. Studying these variants may help define the enzymatic boundary between the normal phenotype and the molecular pathology associated with the G_{M2} gangliosidoses.

In this study the serum and leukocyte β hexosaminidases of the proband, a healthy female adolescent, and her family, were investigated. Two pedigree members are heterozygous for an allele at the β subunit locus of the hexosaminidase isozymes resulting in the production of thermolabile subunits. Low total serum and leukocyte hexosaminidase values for all pedigree members suggest that an allele for Sandhoff disease may also be segregating in this family.

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MATERIALS AND METHODS

Subjects

The family investigated is composed of four individuals designated as P (proband), S (sibling), M (mother) and F (father). P was identified in a Tay-Sachs carrier screening clinic conducted in a Montreal high school. The parents are both Jewish. F is of Sephardic ancestry and was born in Iraq. M was born in England, although her ancestry is Ashkenazic. There are no collateral relatives living in North America. Neither F nor M have any knowledge of neurological disease among their respective ancestors or collaterals. S is a 20-year-old healthy female.

Control sera and leukocytes were obtained from laboratory personnel. Sandhoff carriers were all obligate heterozygotes.

Materials

Fluorogenic substrates and fluorescent standard, 4-methylumbelliferone were purchased from Koch-Light (Edmonton, Alberta). Crystalline bovine serum albumin (BSA) was a product of Sigma (St. Louis, Mo.). Diethylaminoethylcellulose was purchased from Bio-Rad (Richmond, Ca.). Other chemicals were from Fisher (Montreal, Quebec).

Analytical Methods

Preparation of samples for analysis. Serum samples were obtained by collection of 5 ml of blood in a non-heparinized syringe. The blood was allowed to clot for 30–60 min at 25°C and the clotted samples were centrifuged at 160 *g* for 10 min at 25°C. Serum samples were stored at −20°C pending analysis.

Leukocytes were obtained from 10 ml of heparinized blood according to Kaback's procedure [7] and stored at −20°C. Leukocyte supernatants were prepared by mixing pellets with 1 ml 0.1 M citrate-phosphate, pH 4.4. The suspension was then subjected to three cycles of freezing in an iso-propanol dry ice bath and thawing at 37°C. The supernatant was obtained after centrifugation at 600 *g* for 10 min at 4°C.

Enzyme and protein determinations. Assays for lysosomal hydrolases were performed using a Turner fluorimeter. Autoanalysis of serum hexosaminidase has been previously described both in its technical details [8] and in its criteria for genotype assignment [9]. Hexosaminidase activity was assayed manually according to Leaback and Walker [10]. α-L-fucosidase [11], β-galactosidase [12] and β-glucuronidase [13] were determined using optimum conditions established by others. Leukocyte enzyme activities are reported per mg of non-hemoglobin protein. Protein was determined by the method of Lowry [14] using crystalline BSA as standard.

Thermal inactivation studies. Thermal inactivation studies on serum hexosaminidases were performed at 55°C. Samples were heated for various time intervals in mixtures containing enzyme, .02–.04 nmoles/ml, human serum albumin (HSA) (Connaught Labs, Toronto, Ontario), 5 mg/ml, and sodium citrate-phosphate, 30 mM, pH 4.4, in volumes of 100 μl. Leukocyte HEX B was determined after incubation of supernatants for 2 hr at 50°C in mixtures containing HSA, 5 mg/ml and sodium citrate-phosphate, 70 mM, pH 4.4.

Chromatography and electrophoresis. Ion exchange chromatography of β-hexosaminidases was performed according to the procedure of Nakagawa et al. [15]. Polyacrylamide gel electrophoresis was carried out at pH 8.9 according to Davis [16] and at pH 4.5 by the procedure of Reisfeld et al. [17]. Hexosaminidase activity on electrophoretic gels was detected using the activity stain developed by Hayashi [18].

RESULTS

Serum Hexosaminidase Activity

Total and heat stable serum hexosaminidase of individuals participating in the Tay-Sachs screening program are routinely determined by autoanalysis as shown in figure 1.

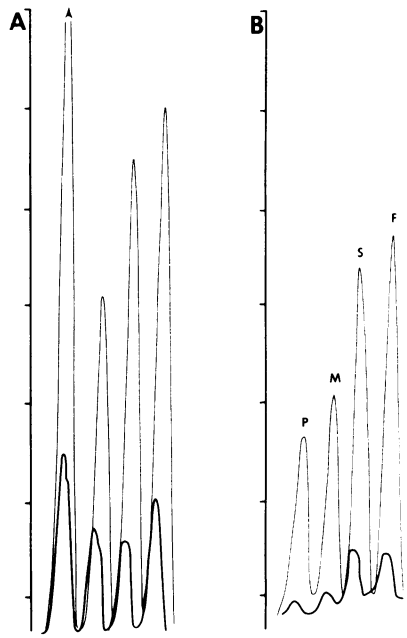

FIG. 1.—Total and heat stable hexosaminidase of controls and proband. Serum samples (.05 ml) were assayed simultaneously for total hexosaminidase (*light lines*) and heat stable hexosaminidase (*heavy lines*) using a Technicon autoanalyzer [8]. Peaks represent (*left* to *right*): *A*, two Tay-Sachs carriers and two homozygous normals. *B*, proband (*P*), mother (*M*), sib (*S*), and father (*F*).

The peak heights for total hexosaminidase (light lines) and heat stable hexosaminidase (heavy lines) for members of the pedigree (fig. 1*B*) indicated a considerable reduction in both values for all members of the family as compared to homozygous normal controls and heterozygotes for the Tay-Sachs allele (fig. 1*A*). The most dramatic reduction occurred in the heat stable component for individuals P and M. After storage for 4 months at −20°C, all hexosaminidase values for members of the pedigree were comparable with those obtained using fresh aliquots of serum. Values for P and S were confirmed by autoanalysis of serum from a second blood sample as well as by a manual assay procedure. Heat stable hexosaminidase was estimated after 15 min incubation at 55°C; P = 12.7%, S = 21%, homozygous normal control = 34%, and Tay-Sachs carrier = 59.3%.

The possibility that P's serum contained hexosaminidase activity inhibitor was ruled out by a mixing experiment. A mixture of an equal volume of P's serum and that of a homozygous normal control resulted in peak values for both total and heat stable hexosaminidase which were approximately equidistant between the values obtained for the two sera tested separately. The diminished enzyme activity in P's serum is therefore not due to the presence of an inhibitor.

Quantitative values for serum enzymes HEX A and HEX "B" (a mixture of HEX I and HEX B) can be obtained by integration and subtraction of peaks traced by the autoanalyzer. These values, when compared to HEX A and "B" values for

homozygous normal controls, obligate Tay-Sachs carriers, and obligate Sandhoff carriers, are used to assign genotypes to tested individuals. In figure 2 the serum HEX A and "B" values for pedigree members and individuals of known genotypes are plotted on a two-dimensional graph. Values for individuals P and M fall clearly within the region of the graph occupied by obligate Sandhoff heterozygotes. Individuals S and F have somewhat higher values for both HEX A and HEX "B," yet they do not appear to be within the homozygous normal group. With respect to HEX A, the values for S are 2.5 SDs from the mean of the normal controls, and F is 1.08 SDs from the normal mean. With respect to HEX "B" activity, S is 2.6 SDs and F is 2.08 SDs from the normal mean (sample size = 41). An insufficient number of obligate Sandhoff heterozygote samples prevents establishment of a statistical domain for this genotype.

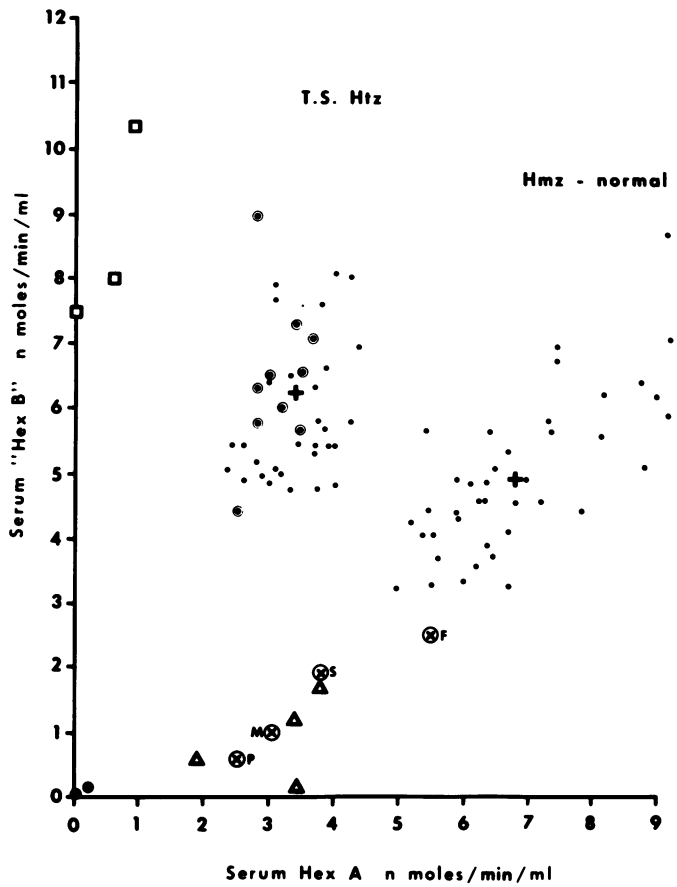

FIG. 2.—Serum HEX A and B activity in individuals of known genotypes and screenees. Serum HEX A and "B" (a mixture of HEX B plus HEX I) were determined by autoanalysis. • = tested individuals, ⊙ = obligate Tay-Sachs heterozygotes, + = mean values for carrier and homozygous normal group, □ = Tay-Sachs homozygotes, ● = Sandhoff homozygotes, △ = obligate Sandhoff heterozygotes, and ⊗ = members of proband's family. (Data used with permission of C. R. Scriver.)

Ion Exchange Chromatographic Separation of Serum Hexosaminidases

Sera (1.5 ml) of the four members of the family and of a homozygous normal control were dialyzed overnight against 300 mls of 10 mM NaH_2PO_4-NaOH, pH 6.0, and applied to DEAE cellulose columns using elution conditions developed by Nakagawa [15]. The chromatographic procedure resolves human serum hexosaminidases into B, I, and A components respectively (fig. 3). The chromatographic profiles for individuals S, M and F (not shown) were essentially identical to that for P. The correspondence of the peaks for the three isozymes among the five individuals indicated that there is probably no electrical charge difference between the enzyme proteins of members of the pedigree and those of a normal control. The recovery of applied enzyme sample after chromatography (about 87%) was the same for all serum samples. The distribution of enzyme activity between the three peaks eluting from the column was not markedly different. The data are summarized in table 1. Those differences in percent isozyme composition which are greater than experimental error are confined to the distribution of enzyme activity between the HEX B and I components with the percent HEX A of all five samples of fractionated sera being essentially identical. The isozyme ratios for P and her family are markedly different from homozygous normal controls when estimated by thermal fractionation, but not when measured by ion exchange chromatography.

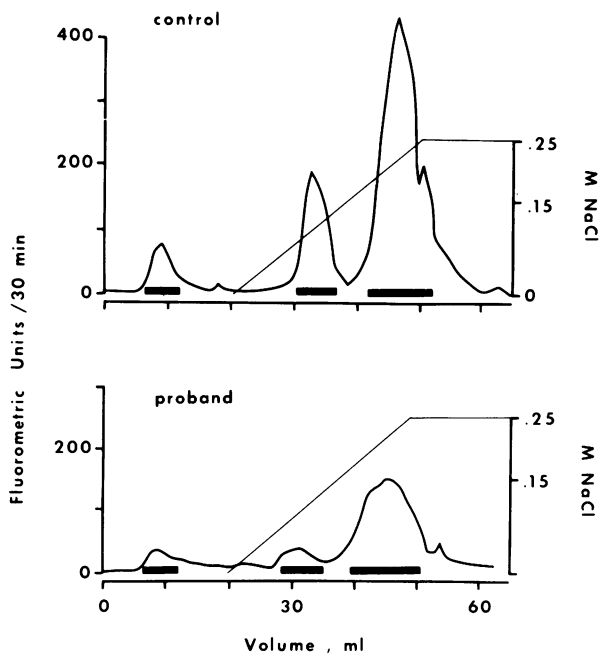

FIG. 3.—DEAE cellulose chromatography of serum hexosaminidase. Serum samples (1.5 ml) were dialysed and chromatographed on DEAE cellulose according to Nakagawa's procedure [15].

TABLE 1

COMPOSITION OF SERUM HEXOSAMINIDASES DETERMINED BY CHROMATOGRAPHY

Subjects	Total HEX nmoles/min/ml	% B	% I	% A
Control	8.2	7.0	19	74.0
Proband	3.7	9.8	11.6	78.4
Sib	6.4	8.9	14.5	76.6
Mother	4.0	11.0	11.7	77.2
Father	7.4	5.5	18.8	75.7

Thermostability of Isolated Serum Hexosaminidase Isozymes

The discrepancy between percent heat stable hexosaminidase and percent HEX "B plus I" for individuals P and M is resolved when the kinetics of thermal inactivation of the isolated serum isozyme components are investigated. Column fractions comprising isozymes HEX B, I, and A for the five individuals were pooled and concentrated by vacuum dialysis. The results of thermal inactivation experiments performed on these samples are shown in figure 4. At 55°C, the HEX B and I components of individuals S and F behave as heat stable enzyme activities. The thermal inactivation curves for these individuals are identical to those obtained with isolated HEX B and I peaks from the fractionated sera of a homozygous normal control and an obligate Sandhoff heterozygote. The HEX B and I isozymes of the proband, however, were rapidly and completely inactivated under the same conditions. With respect to thermal inactivation, P's HEX B and HEX I were undistinguishable from control HEX A ($t_{1/2}$ at 55°C HEX A_c = 180 sec, HEX B_p = 150 sec, HEX I_p = 210 sec). The thermal inactivation curves for both HEX B and I of individual M (mother of the proband) were biphasic. The initial phase corresponded to the inactivation of thermolabile isozymes similar to those of P. After complete inactivation of the labile components of HEX B and I, a second set of thermostable components are seen. By extrapolation of the "percent remaining" enzyme activity to time 0, the values for heat stable HEX B and I in M's serum are respectively 42% and 50%.

At 55°C, inactivation of serum HEX A is too rapid to allow comparisons between family members and controls. HEX A inactivation kinetics were therefore tested at 48°C. Table 2 summarizes the inactivation kinetics of the three isozyme species. At 48°C, all five isolated serum HEX A samples displayed a single phase inactivation curve. The HEX A samples of M and P were more labile than those of S, F, and the control. The differences between individuals, however, was not great, indicating that the thermo-responsiveness of HEX A is largely determined by the properties of the α subunits.

Leukocyte Hexosaminidase Activity

The values for leukocyte hexosaminidase activity for members of this family in relation both to each other and to controls are in accord with the results obtained from serum.

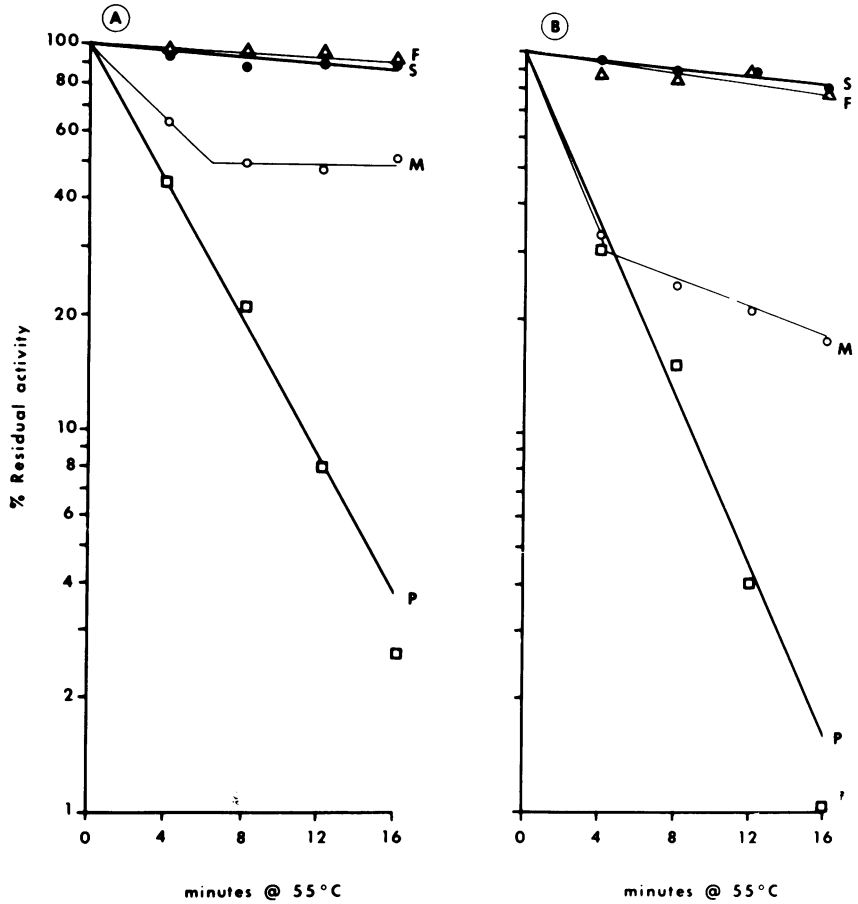

FIG. 4.—Thermal inactivation of serum HEX I and B. *A*, Pooled concentrated fractions of HEX I, and *B*, of HEX B, which were incubated in duplicate for indicated interval at 55°C. Heating was terminated by immersing tubes in an ice-H_2O bath. Heated samples were mixed with 1 mM 4 methyl-umbelliferyl *N*-acetyl-β-D-glucosaminide and incubated at 37°C for 30 min. □ = proband, ● = sib, ○ = mother, △ = father.

Table 3 compares the leukocyte-specific activities for HEX A and B as well as specific activities for three other lysosomal hydrolases of family members with normal controls. Specific activities of α-L-fucosidase, β-galactosidase and β-glucuronidase of all pedigree members fall within the range established by the six normal controls, indicating that the mutation(s) segregating within the pedigree involves a specific enzyme defect and not a generalized defect of lysosomal function, such as I-cell disease [19]. Figure 5 compares both specific activity of leukocyte HEX B and percent of HEX B determined by thermal fractionation with values for individuals of known genotypes. Since percent of heat stable hexosaminidase is a valid assessment of percent of HEX B for S and F, the classification of these individuals as Sandhoff heterozygotes is not unreasonable. M and P, however, cannot be classified as such, based on the

TABLE 2

KINETICS OF THERMAL INACTIVATION OF SERUM HEXOSAMINIDASES

SUBJECTS	T½ AT 55°C		T½ AT 48°C
	B seconds	I seconds	A seconds
Control	3000	> 5000	1890
Proband	150	210	1320
Sibling	3600	> 5000	1500
Mother	90/3300	150/ > 5000	900
Father	2460	> 5000	2190

distribution of leukocyte or serum hexosaminidase activities between heat stable and heat labile components. Polyacrylamide gel electrophoresis of P and control leukocyte supernatant protein reveals both HEX A and B isozymes after incubation with activity stain. While the staining procedure is, at best, semiquantitative, the slower migrating band, corresponding to HEX B, accounts for 30%–60% of the total stainable activity in both gels compared to the 5%–6% HEX B found with the thermal fractionation procedure. Polyacrylamide gel electrophoresis at pH 4.5 gave essentially identical results: the distribution of leukocyte hexosaminidase activity between HEX A and B isozymes was similar to that of normal controls, and no evidence for electrophoretic variation between normal and P's hexosaminidases could be detected. Homozygous normal control and P's leukocyte hexosaminidase activity did not differ with respect to pH optimum, Km, or response to incubation in the presence of sulfhydryl compounds.

DISCUSSION

While a number of pedigrees showing heteroalleism at the loci controlling expression of human hexosaminidases have been reported, the pedigree investigated here is, to our knowledge, a novel instance of such mutations ascertained through a

TABLE 3

LEUKOCYTE LYSOSOMAL ENZYME ACTIVITIES IN FAMILY OF PROBAND AND CONTROLS

SUBJECTS	NMOLES FLUOROGENIC SUBSTRATE CLEAVED/HR/MG PROTEIN				
	HEX A	HEX B	β-galactosidase	α-L-fucosidase	β-glucuronidase
Controls:					
1	1074	420	18.6	144	99
2	···	···	42.5	68	408
3	1230	534	23.8	153	241
4	1398	552	25.9	217	260
5	1380	666	72.9	···	759
6	1314	456	···	···	···
Family:					
Proband	689	18.4	89.2	94	639
Sibling	362	65.8	36.6	59	434
Father	745	136	40.5	91	308
Mother	826	7.9	49.5	78	322

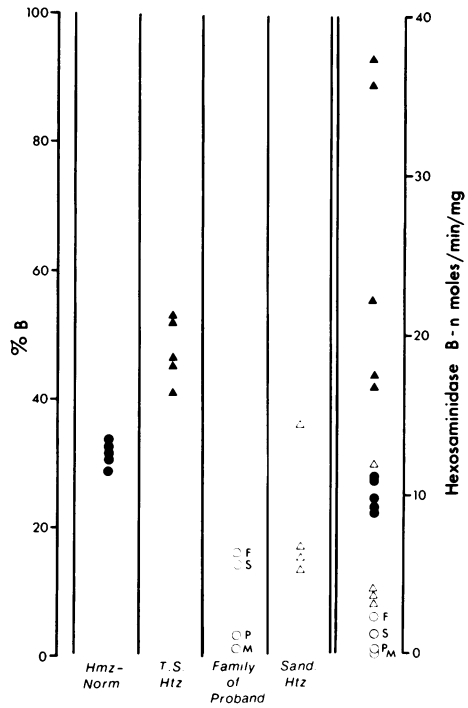

FIG. 5.—Specific activity of leukocyte HEX B in family of proband and controls.

mass screening program. In the absence of an index case of G_{M2} gangliosidosis, the assignment of genotypes in this pedigree becomes somewhat problematic. The pedigree shown in figure 6 represents a hypothesis based on the data presented above. The proposed model addresses itself to the following considerations: (1) Both consanguinity and illegitimacy can be ruled out. (2) There is clear evidence for a heat labile allele in M and its transmission to P. (3) M also possesses an allele which produces HEX B and I components with normal response to heat. This allele is apparently transmitted to S but not to P. (4) S and F both have low ratios of heat stable/total hexosaminidase, a reported characteristic feature of Sandhoff heterozygotes [20, 21]. Total hexosaminidase activity in the serum and leukocytes of S and F is also consistent with reported values for the Sandhoff heterozygote genotype.

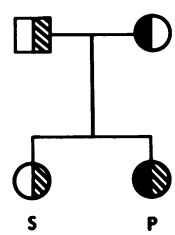

FIG. 6.—Proposed pedigree. *Black* = allele conferring heat labile HEX B. *Hatched* = Sandhoff allele.

The proposed assignment of genotypes raises the following problem: M is presumed to be heterozygous for a normal allele. She also carries an allele which is believed to confer normal enzyme activity when the estimation is performed prior to heating, yet total hexosaminidase values for M are consistently lower than that of F, the proposed Sandhoff heterozygote. This anomaly could be explained by a nonadditive mode of allelic interaction between the normal and thermolabile gene products, possibly at the level of HEX A assembly. An additive mode of allelic interaction would be expected to produce species of HEX A in the heterozygote in proportions 25% $\alpha_2\beta_2$* (β* = heat labile gene product of the mutant allele), 50% $\alpha_2\beta$*β and 25% $\alpha_2\beta_2$. The turnover number of the $\alpha_2\beta$*β species would be expected to be intermediate between that of the other two species. In a nonadditive mode either a disproportionately large amount of $\alpha_2\beta_2$* would be formed, or the HEX A species $\alpha_2\beta$*β would have a turnover number comparable to the $\alpha_2\beta_2$* species. The proposed models, however, cannot be tested with the serum and leukocyte samples available to us.

Given a model in which there are equal amounts of the two β subunit types, with no restrictions on their association to form the homotetrameric structure of HEX B [22], five distinct types of tetramers may be formed. The proportions of each type will be given by the binomial theorem. The thermal inactivation curve for such a mixture of enzymes would be very complex. If, for example, a single heat labile β subunit conferred heat lability on the enzyme of which it is a monomer, then an individual heterozygous for this allele would be expected to have only 6.25% of the HEX B fraction displaying normal thermal inactivation kinetics. If a minimum of two thermolabile subunits per enzyme molecule were required for observation of the heat labile HEX B phenotype, then 31.25% of the B fraction should show normal inactivation kinetics. In fact, the data presented in figure 5 are more consistent with nonrandom association between subunits. The inactivation curves for serum HEX B and I of individual M are most easily explained by the presence of two distinct species of both HEX B and I, one being composed entirely of stable subunits and the other entirely of heat labile subunits.

Other reports have appeared in the literature concerning the production of abnormal β subunits. Momoi et al. [23] studied a Japanese child of consanguinous parents who died with symptoms of classical Tay-Sachs disease. Biochemical investigations revealed that along with a complete deficiency of serum HEX A, the serum HEX B component was thermolabile. The authors suggest, since a double mutation is unlikely, the molecular lesion may be due to an inability of the altered β subunits to combine with α subunits to produce functional HEX A.

The mutation termed "Hexosaminidase Paris" described by Dreyfus et al. [24] also produces defective β subunits. In this case the β subunits preferentially associate with α subunits and no HEX B is detectable.

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