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Genetics

Interrelationship of hexosaminidases A and B: Confirmation of the common and the unique subunit theory

(β-*N*-acetylglucosaminidase/biochemical genetics/GM_2 gangliosidosis/subunit characterization/monospecific antiserum)

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ABSTRACT **Human kidney hexosaminidase A (β-*N*-acetylglucosaminidase; 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase; EC 3.2.1.30) is a heteropolymer of two immunologically distinct subunits designated as α and β. Hexosaminidase B, however, is a homopolymer comprised entirely of β subunits. When human kidney hexosaminidase A was dissociated into its subunits by *p*-hydroxymercuribenzoate, three distinct proteins having isoelectric points of pH 7.2, 5.4, and 4.3 were isolated. The fraction having an isoelectric point of pH 7.2, designated as β fraction, was electrophoretically and immunologically identical to hexosaminidase B and was enzymatically active. The proteins having isoelectric points of pH 5.4 and 4.3, designated as hexosaminidase Ai and α fractions, respectively, were enzymatically inactive and crossreacted with antiserum against hexosaminidase A and not with antiserum against hexosaminidase B. Upon incubation of *p*-hydroxymercuribenzoate-treated hexosaminidase A with dithiothreitol, hexosaminidase A activity, as well as antigenicity, was regenerated, indicating that α and β subunits hybridize to form hexosaminidase A. Antibodies raised in rabbits against β fractions reacted with both hexosaminidase A and B, whereas the antibodies against α and hexosaminidase Ai fractions reacted only against hexosaminidase A. This would indicate that both fractions are composed only of subunits unique to hexosaminidase A. The molecular weights of α, β, and hexosaminidase Ai fractions were estimated to be 47,000, 120,000, and 180,000 respectively, by Sephadex gel filtration. Upon urea-sodium dodecyl sulfate polyacrylamide electrophoresis, each of the three fractions dissociated into a single polypeptide having a molecular weight of approximately 18,000. It is concluded that *p*-hydroxymercuribenzoate dissociates hexosaminidase A, $(\alpha\beta)_3$, into its subunits, and the β subunits can reassociate to form relatively stable hexosaminidase B, $(\beta\beta)_3$, while the α subunits reassociate in both the dimeric state, α_2, and a polymeric state, α_8.**

The two major isozymes of lysosomal β-D-*N*-acetylhexosaminidase (hex) (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase; EC 3.2.1.30), namely, hex A and hex B, are widely distributed in human and other mammalian tissues. Both hex A and hex B catalyze the hydrolysis of G_{A2} globoside, whereas, only hex A catalyzes the hydrolysis of G_{M2} ganglioside. The inherited deficiency of hex A causes Tay-Sachs disease (1–4), while the deficiency of both hex A and hex B results in Sandhoff's disease (4, 5). Both of these inborn lipidoses involve accumulation of G_{M2} ganglioside in neuronal lysosomes. In Sandhoff's disease G_{A2} globoside is also deposited in the visceral tissues (5, 6). Since the demonstration of hexosaminidase involvement in G_{M2} gangliosidosis, several models have been proposed to explain the genetic relationship between Tay-Sachs and Sandhoff's diseases and the interrelationship of hex A and hex B (7–15). Biochemical, genetic, and immunological studies (12–20) suggest that a close structural relationship exists between hex A and hex B. The model that best explains the interrelationship of hex A and hex B and the genetic relationship between Tay-Sachs and Sandhoff's disease predicts that hex A is a heteropolymer of unique (α) and common (β) subunits and hex B is a homopolymer of common subunits (13–15). Tay-Sachs disease has been suggested to be due to a point mutation of the structural gene for the α subunits and Sandhoff's disease would be due to a point mutation of the structural gene coding for the β subunits (14, 15). This model explains the man–mouse somatic cell hybridization studies in which hex A, $(\alpha\beta)_3$, expression was always dependent on the presence of the gene controlling the expression of hex B, $(\beta\beta)_3$, whereas the gene controlling hex B seemed to segregate independently of the one controlling the structure of hex A (21, 22). However, somatic cell hybridization studies (23, 24), in which the structural gene for hex A also segregated independently of hex B, could not be explained by this model.

We present evidence for the presence of a unique subunit in hex A. Homogeneous human kidney hex A has been dissociated into its subunits by treatment with *p*-hydroxymercuribenzoate and the subunits have been isolated. The unique subunit having a molecular weight of about 18,000 was isolated as a dimer, α_2, and as a polymer, α_8. The α_8 had the same electrophoretic mobility as hex A. Upon immunoelectrophoresis the α_2 and α_8 proteins did not react with antiserum against hex B. The antibodies raised against the α subunits were found to be monospecific for hex A and did not crossreact with hex B. In the presence of dithiothreitol the α subunits hybridized with β subunits to form enzymatically active hex A.

MATERIALS AND METHODS

4-Methylumbelliferyl-β-D-*N*-acetylglucosaminide was purchased from Koch-Light Co., England. Sephadex G-100 and G-200 were purchased from Pharmacia Co., Uppsala, Sweden. Agarose and other reagents were purchased from Sigma Chemical Co. Ouchterlony plates for double immunodiffusion studies were obtained from Hyland Laboratories. *p*-Hydroxy[^{203}Hg]mercuribenzoate was purchased from ICN, and Ampholine was purchased from LKB Productor. Protein was determined according to Lowry *et al.* (25), and hex A and hex B activities were determined by using 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide as substrate (17).

Dissociation of Hex A. Human kidney hex A and hex B were purified to homogeneity by a modification of Method I published earlier (17). The lyophilization step was omitted and an affinity chromatography step using concanavalin A insolubilized on beaded agarose was substituted (details of purification will be published elsewhere). The homogeneous preparations of hex A and hex B were found to have specific activities of 110 and 203 units/mg of protein, respectively, and moved, upon polyacrylamide disc electrophoresis, as a single protein band which corresponded to the enzyme activity band. For the dissociation of hexosaminidase, a 2 ml reaction mixture containing

Abbreviation: hex, hexosaminidase.

1 mg of homogeneous hex A or B, 25 mM citrate-phosphate buffer (pH 5.6), and 2 mM *N*-ethylmaleimide was incubated at 25° for 45 min. *p*-Hydroxy[^{203}Hg]mercuribenzoate (specific activity 96 mCi/g) was then added to bring the final concentration of *p*-hydroxymercuribenzoate to 15 μM and the reaction mixture was further incubated at 37° for 17 hr. This was designated as the conversion mixture and used for the isolation of subunits and for electrophoresis and immunological studies. In some experiments incubation with *N*-ethylmaleimide was omitted.

Thin-Layer Isoelectric Focusing. The method was essentially similar to that described (26). Sephadex G-75-120 (1.9 g), suspended in 25 ml of water and 1 ml of a 40% solution of Ampholine (pH 3–10), was poured on a 20 × 10 cm glass plate to a uniform thickness. Protein sample (250 μg in a total volume of 0.5 ml) was applied as a narrow streak in the middle of the plate. Paper wicks 1 cm wide were soaked in 2% (vol/vol) H_2SO_4 (anode) and 2% ethylenediamine (cathode) and electrodes were placed on top of the wicks. For the first 2 hr, 200 V were applied across the electrodes, after which voltage was increased to 500 and isoelectric focusing was continued for another 2.5 hr. After isoelectric focusing, 0.6 cm wide strips of the gel were cut and extracted with 2 ml of cold distilled water. The pH of each sample was measured, and aliquots from each were measured for radioactivity in Bray's solution (27) using a Beckman liquid scintillation counter (model LS-230). The peak fractions of radioactivity at pH 4.3 and 7.2 and the gel between pH 5 and 5.5, were pooled separately, extracted several times with water, and concentrated by ultrafiltration to about 0.5 ml.

Immunoelectrophoresis was performed on a 1% agarose gel in 20 mM phosphate buffer (pH 7.0) for 3 hr at 3 mA per slide. Slides were stained for enzyme activity as described earlier (14). After electrophoresis, antisera were applied in the center slots for immunodiffusion.

Preparation of Antibodies. The antibodies against hex A and hex B were raised in rabbits as described (14). The antibodies against "α", hex "Ai", and *p*-hydroxymercuribenzoate-converted hex B (designated as "β") were raised in rabbit by intradermal injection of about 50 μg of protein crosslinked with glutaraldehyde (5%).

RESULTS

Incubation of hex A with *N*-ethylmaleimide and *p*-hydroxy[^{203}Hg]mercuribenzoate dissociated hex A into subunits. *N*-Ethylmaleimide was used to alkylate all the accessible sulfhydryl groups of hex A, so that the amount of *p*-hydroxy[^{203}Hg]mercuribenzoate bound to —SH groups inaccessible to *N*-ethylmaleimide could be determined. However, extensive dialysis of the conversion mixture against 10 mM phosphate buffer (pH 7.0) released all of the ^{203}Hg. During thin-layer isoelectric focusing, immunoelectrophoresis, or Sephadex gel filtration, some radioactivity initially bound to protein was released. Thus, the molar ratio of *p*-hydroxymercuribenzoate required to dissociate hex A into subunits could not be determined. In subsequent experiments the presence of *N*-ethylmaleimide in the conversion mixture had no effect on the dissociation of hex A.

Thin-layer isoelectric focusing

After thin-layer isoelectric focusing of the conversion mixture, the radioactivity was associated with two peaks having median isoelectric points of pH 7.2 and 4.3 (Fig. 1). The fraction focused at pH 7.2 was enzymatically active toward 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide and in immunoelectrophoresis corresponded to the mobility of kidney hex B. On the other hand, the sharp peak at pH 4.3 did not demonstrate any enzymatic activity. No appreciable radioactivity and no enzyme activity was found in the pH range of 5–5.5, which corresponds to the mobility of the control sample of kidney hex A. The fractions corresponding to hex B (pH 7.2) and hex A (pH 5.2–5.4) and the fraction having maximum radioactivity at pH 4.3 were extracted, pooled, concentrated, and designated as "β", hex "Ai" (enzymatically inactive hex A), and "α", respectively. Molecular weights of "α", "β", and hex "Ai" were estimated by Sephadex G-100 and G-200 gel filtration to be 47,000, 120,000, and 180,000, respectively. The molecular weight of hex "Ai" was determined by Sephadex gel filtration after the protein was radioiodinated.

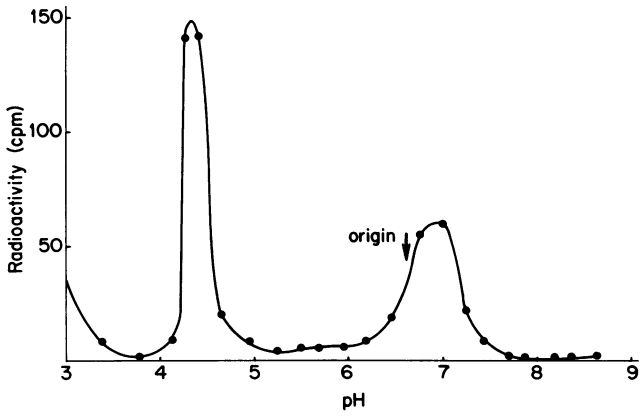

FIG. 1. Thin-layer isoelectric focusing of hex A incubated with *p*-hydroxymercuribenzoate. Details are described in the *text*. The peak to the left of the origin represents "α" fraction and corresponds to pI of 4.3. The peak to the right of the origin represents the "β" fraction and corresponds to pI range of 7.0–7.2.

Although the concentrated hex "Ai" fraction had no enzyme activity or radioactivity, a substantial amount of crossreacting material was detected upon double immunodiffusion which reacted with antiserum against hex A only. When the hex "Ai", "α", and "β" fractions were subjected to urea-sodium dodecyl sulfate-2-mercaptoethanol-polyacrylamide disc electrophoresis, along with reference samples of bovine serum albumin, ribonuclease A, and aldolase, complete dissociation of "β", "α", and hex "Ai" into subunits of molecular weights about 18,000 was observed. A comparison of the molecular weights of the subunits of "α" and hex "Ai" is presented in Fig. 2.

Immunoelectrophoresis

In immunoelectrophoresis the electrophoretic mobilities of the fractions obtained from isoelectric focusing and designated as "β" and hex "Ai" were similar to hex B and hex A, respectively. The "α" fraction was, however, more anodal than hex A. All three fractions gave precipitin arcs against antiserum against hex A, but only the "β" fraction gave a precipitin line against antiserum against hex B. Thus, the "β" fraction appeared to be enzymatically, electrophoretically, and immunologically identical to hex B.

Upon immunoelectrophoresis of the conversion mixture, the enzyme activity was observed only in the hex B position (Fig. 3). Based on the crossreactivity with antiserum against hex A, the conversion mixture separated into three distinct fractions corresponding to fractions from thin-layer isoelectric focusing. The "α" and hex "Ai" fractions did not demonstrate enzyme activity and crossreacted with antiserum against hex A but not with antiserum against hex B, whereas the "β" fraction was enzymatically and immunologically similar to hex B.

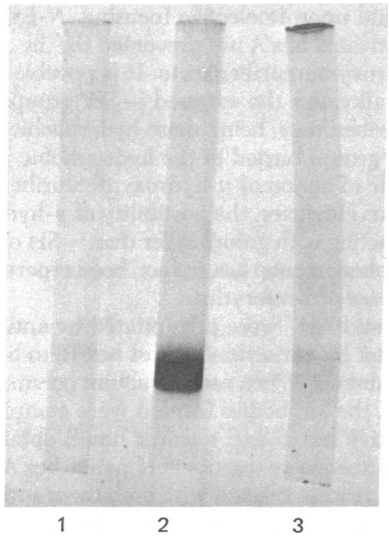

FIG. 2. Sodium dodecyl sulfate urea-2-mercaptoethanol polyacrylamide gel electrophoresis of "α" and hex "Ai" fractions. Gel 1 = "α" fraction; gel 2 = ribonuclease A; gel 3 = hex "Ai" fraction.

Reconstitution of hex A

The results from thin-layer isoelectric focusing and immunoelectrophoresis of the focused fractions indicate that *p*-hydroxymercuribenzoate treatment dissociates hex A into its subunits, α and β. Since hex A activity was completely lost and a significant amount of hex B activity appeared, it is presumed that the β subunits recombine to form enzymatically active hex B. In an attempt to regenerate the enzyme activity of hex A in the conversion mixture, it was incubated with 10 mM dithiothreitol in 20 mM potassium phosphate buffer (pH 7.0) at room temperature for 15 min. The sample was then subjected to immunoelectrophoresis. A significant amount of hex A activity was observed in the sample incubated with dithiothreitol (Fig. 3). The regenerated hex A was antigenically similar to authentic hex A in its crossreactivity towards antiserum against hex B. However, treatment of the gel with dithiothreitol, after electrophoresis of the conversion mixture, did not regenerate the enzyme activity or the antigenicity of hex "Ai" towards antiserum against hex B. Also, incubation of the isolated "α", "β", and hex "Ai" fractions or the incubation of all the fractions isolated from thin-layer isoelectric focusing between pH 3.5 and 10.0 with dithiothreitol failed to regenerate hex A activity or antigenicity.

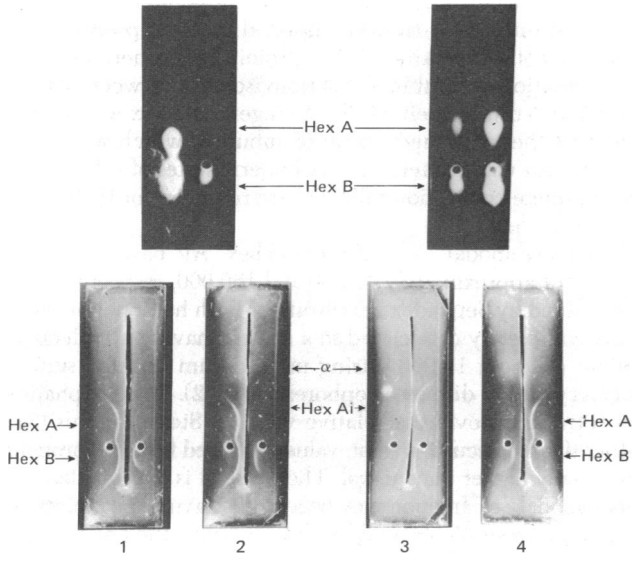

FIG. 3. Immunoelectrophoresis of hex A incubated with *p*-hydroxymercuribenzoate (conversion mixture) and treated with dithiothreitol. Details of the method are described in the *text*. Upper gels were stained for enzyme activity before immunodiffusion. The gel on the left is the same as gel 1; the gel on the right is the same as gel 3. Gel 1: left well, kidney hex A and hex B; right well, conversion mixture; center slot, antiserum against hex A. Gel 2: same as gel 1 except antiserum against hex B in center slot. Gel 3: left well, conversion mixture incubated with dithiothreitol; right well, kidney hex A and hex B; center slot, antiserum against hex A. Gel 4: same as gel 3 except antiserum against hex B in center slot. Gels 1 to 4 are unstained.

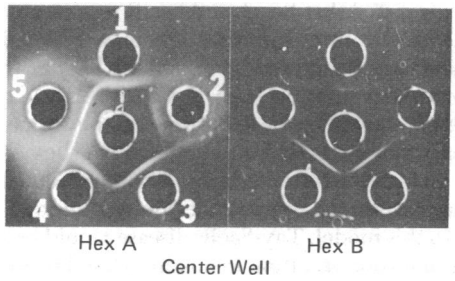

FIG. 4. Double immunodiffusion with antisera against "α", "Ai", and "β" tested against kidney hex A and B. Antisera: well 1, against hex A; well 2, against "α"; well 3, against hex B; well 4, against "β"; and well 5, against hex "Ai".

Immunodiffusion studies with "α", hex "Ai", and "β" antisera

In double immunodiffusion studies antisera against "α" and against hex "Ai" gave precipitin lines with hex A (Fig. 4), whereas hex B did not crossreact with either antiserum. As observed with antiserum against hex B, the antiserum against "β" gave precipitin lines with both hex A and hex B. Spurs could be seen between wells containing antiserum against hex A (well 1) and wells containing antisera against hex "Ai" (well 5) and against "α" (well 2) when hex A was in the center well. In addition, arcs exhibiting identity of some antigenic determinants could be seen in these wells. Spurs were observed between wells containing antisera against "α" and against hex B (well 3), as well as between wells containing antisera against hex "Ai" and against "β" (well 4). However, a double spur between wells 2 and 3 was not observed because of low titer of antiserum against "α". Complete fusion of arcs between the wells containing antisera against hex B (well 3) and against "β" (well 4) was also apparent. Thus, on the basis of the reactivity of their respective antisera with hex A and B, "α" and hex "Ai" share the unique antigenic determinant that is present in hex A and absent in hex B and "β".

DISCUSSION

Several models have been proposed to explain the interrelationship of hex A and hex B. The earliest model postulated that hex B was the precursor of hex A, which was formed by the addition of neuraminic acid residues to hex B by a specific sialyl transferase (7–10). This model explained the conversion of hex A to hex B by treatment with commercial neuraminidase preparations of *Vibrio cholerae*. However, it was found that hex A was converted to hex B, not by neuraminidase but by merthiolate (thimerosal) present as a preservative in commercial preparations (19, 20). Various other mercurials such as *p*-hydroxymercuribenzoate and $HgCl_2$ were also found to convert hex A to hex B (19). Another model, proposed by Tallman *et*

al. (12), suggested that hex A and hex B represented different conformational states of the same protein, with hex B at a lower energy level and separated by an energy barrier from hex A, which was in a metastable state. Since hex A could be partially converted to hex B by heat treatment, they postulated that additional energy was required to unfold the hex A conformer from its metastable state so that it could overcome the energy barrier and attain the more stable configuration of hex B. According to this model, Tay-Sachs disease would be the expression of a mutation resulting in an amino acid substitution at a point critical for the metastable folding, rendering hex A less stable than usual. Sandhoff's disease would be the expression of a mutation of the active site leading to inactivation of both hex A and hex B. However, the following observations negate the conformational theory: (*i*) a monospecific antiserum against hex A can be obtained by repeated absorption with hex B (14). (*ii*) The structural gene controlling the expression of hex B is located on the fifth chromosome, whereas genes on both fifth and fifteenth chromosomes are necessary for the expression of hex A (21, 24). (*iii*) Tay-Sachs and Sandhoff's fibroblast heterokaryons express normal hex A activity (28). (*iv*) Amino acid composition of hex A and hex B is different (18). (*v*) The carbohydrate content of hex A and hex B is very different (our unpublished observations). (*vi*) A subunit unique to hex A has now been isolated and characterized. An alternate explanation may, however, be given for the first two observations, such as the possibility of conformational dependent antigenic determinants and that a conformer could result by interaction with a prosthetic group under the control of a gene on chromosome 15. The conformational theory, however, does not explain the last four observations.

The third model proposed by us and by Robinson (13–15) predicts that both hex A and hex B share a common subunit and that hex A has, in addition, a unique subunit. Therefore, hex B would be a homopolymer of common subunits $(\beta\beta)_3$, whereas hex A would be a heteropolymer $(\alpha\beta)_3$. In addition to explaining the genetic origin of both Tay-Sachs and Sandhoff's diseases by point mutations of the α and β genes, respectively, this theory best explains most of the data obtained from structural, immunological, and somatic cell hybridization studies (12–22). On the other hand, studies of man-hamster (23) and man–mouse somatic cell hybridization (24) have shown that both hex A and hex B segregate independently of each other. However, in all these studies only electrophoresis was used to identify the hybrids of hex A and hex B. Lalley *et al.* (21, 22) however, using immunological techniques during their somatic cell hybridization studies, have demonstrated that hex A expression was always dependent on hex B expression, whereas hex B segregated independently of hex A. In fact, they have demonstrated the formation of heteropolymers between man and mouse enzyme (22). As a result of this finding, it is apparent that the observed independent segregation of both isozymes (23, 24) may be due to nonspecific species hybridization of subunits, rather than the independence of human hex A or hex B expression. Our model would predict the independent segregation of hex B but not of hex A.

The association of α and β subunits in hex A appears to be weak since hex A can be converted to hex B by heat treatment (8), freeze thawing in 3 M NaCl (29), or by treatment with merthiolate or *p*-hydroxymercuribenzoate (19, 20). The present results demonstrate that during dissociation of hex A by *p*-hydroxymercuribenzoate, the common β subunits readily recombine to form enzymatically active hex B. The α subunits, however, aggregate to form two enzymatically inactive homopolymers, which are easily separated upon electrophoresis at pH 8.0 and upon isoelectric focusing. *N*-Ethylmaleimide neither inactivated hex A nor prevented the dissociation of hex A by *p*-hydroxymercuribenzoate. It is possible that *N*-ethylmaleimide alkylates the exposed —SH groups while *p*-hydroxymercuribenzoate, being more hydrophobic, may alkylate other —SH groups buried in the hydrophobic regions of hex A. The mode of action of *p*-hydroxymercuribenzoate on hex A is not clear. However, the possibility of *p*-hydroxymercuribenzoate reacting with groups other than —SH cannot be ruled out. Such a phenomenon has, in fact, been reported by Klapper (30) in the case of hemerythrin.

The "α" and "Ai" were precipitated by antiserum against hex A and not by antiserum against hex B, indicating the absence of β subunits. When each α subunit polymer was injected into rabbits, the antibodies formed were completely inactive towards native hex B and towards hex B obtained by *p*-hydroxymercuribenzoate treatment of native hex A. On the other hand, hex A readily reacted with both these antisera. The pattern of precipitin lines in double immunodiffusion studies (Fig. 4) indicates the presence of additional antigenic determinants in hex A, presumably on the β subunits not present in either of α polymers. As expected, antisera against hex B and "β" show a complete line of identity against hex A or hex B without the appearance of any spurs. Thus, these observations confirm that "α" and hex "Ai" are the polymers of the subunit present only in hex A and that "β" is the polymer of the common subunit.

The reconstitution of hex A by addition of dithiothreitol in the *p*-hydroxymercuribenzoate-treated conversion mixture indicates that α and β subunits under appropriate conditions can reunite to form hex A that is enzymatically, electrophoretically, and immunologically similar to the native hex A. However, once these subunits are separated by gel electrophoresis or by thin-layer isoelectric focusing, it is not possible to reconstitute hex A by incubation with dithiothreitol. Inability to reconstitute hex A from the isolated reaction product was probably not due to any missing protein component because the incubation of combined fractions isolated between pH 3.5 and 10 with dithiothreitol failed to regenerate hex A. It is possible that the monomeric form of subunits, which will be in equilibrium with dimeric and polymeric states of subunits in the complete conversion mixture, are required for the reconstitution of hex A.

The more anodal "α" fraction and hex "Ai" have molecular weights of approximately 47,000 and 180,000, respectively, as determined by Sephadex gel filtration. Both homopolymers of α are completely dissociated to a protein having a molecular weight of about 18,000 during urea-sodium dodecyl sulfate polyacrylamide disc electrophoresis (Fig. 2). Since Sephadex gel filtration provides a relative value of Stoke's radius (31) rather than molecular weight, values obtained for glycoproteins are usually higher than actual. Therefore, it is possible that the more anodal "α" fraction may be a dimer having the molecular weight of about 36,000 and hex "Ai" may be the polymer α_8. The dissociation of hex A can be empirically presented as:

$$\underset{\text{hex A}}{(\alpha\beta)_3} \longrightarrow \underset{\text{"}\alpha\text{"}}{\alpha_2} + \underset{\text{hex "Ai"}}{\alpha_8} + \underset{\text{hex B}}{(\beta\beta)_3}$$

We had indicated earlier that α-polymer is probably not stable (14). The present data, however, show that α_2 and α_8 are stable, at least *in vitro*, but not enzymatically active. Also, for the reconstitution of hex A activity by dithiothreitol, the presence of the entire conversion mixture, which contains "β" subunits, was essential. This would indicate that the enzyme activity site is on the "β" subunits. The "α" subunits in hex A

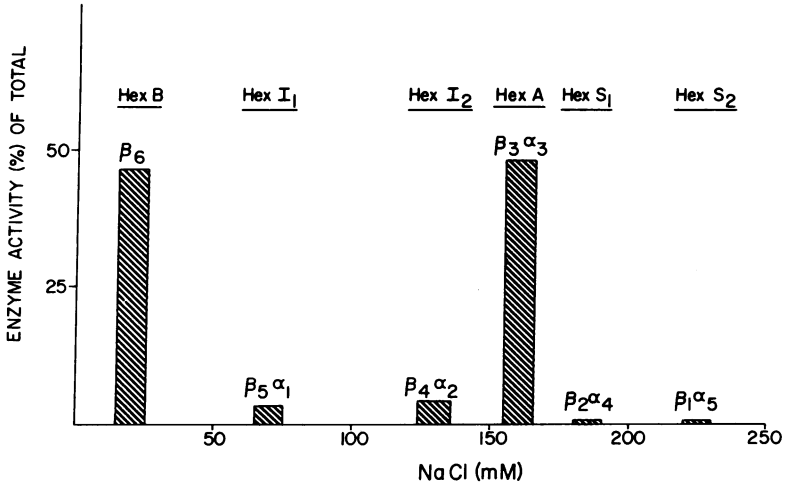

FIG. 5. Subunit composition of human hexosaminidase isozymes. Diagrammatic presentation of the elution pattern of human liver and kidney hexosaminidase isozymes from DEAE-cellulose (DE-52) column.

may function as specifiers directing the enzyme activity toward G_{M2} ganglioside. It was proposed earlier (32) that hex S may represent an altered ratio of α and β subunits as compared to hex A. We now extend this hypothesis to explain various isozymes of hexosaminidase. According to our model, at least six isozymes having a subunit composition of $\alpha_1\beta_5$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_4\beta_2$, and $\alpha_5\beta_1$ are predicted. In fact, all six isozymes are present in normal human liver and kidney (Fig. 5) (32–36).

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