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**Research Article**

Human fibroblasts synthesize several polypeptides that assort into the various forms of hexosaminidase (hex). We report here the occurrence of three newly identified, hexosaminidase-related polypeptides resolved by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis of immunoprecipitates from [35S]methionine-labeled cell extracts. These polypeptides, called band 2 (75,000), band 3 (70,000), and band 4 (63,000), were immunoprecipitated by an antiserum specific to placental hex I2. They are distinct from pre-alpha- (60,000) and pre-beta- (58,000) precursor polypeptides and the alpha- (56,000), beta a- (27,000), and beta b- (27,000) polypeptides of the mature hex A (alpha beta a beta b) and hex B (2[beta a beta b]). When fibroblast extracts were chromatographed on DEAE-Sepharose, bands 2, 3, and 4 were eluted together in fractions before hex A, in a position characteristic of serum and placental hex I2 and serum hex P. This suggests that bands 2, 3, and 4 might represent the polypeptides of a fibroblast hex I. The analysis of partial proteolytic digests of the radioactively labeled polypeptides revealed that bands 2 and 3, pre-beta, and beta a had several peptides in common, suggesting that they are structurally related to each other. However, bands 2, 3, and 4 were present in extracts of Tay-Sachs (pre-alpha and alpha deficiency) and Sandhoff cells (pre-beta, beta a, and beta b deficiency) and appeared later than pre-beta in pulse-chase […]



# Characterization of Polypeptides Serologically and Structurally Related to Hexosaminidase in Cultured Fibroblasts

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ABSTRACT Human fibroblasts synthesize several polypeptides that assort into the various forms of hexosaminidase (hex). We report here the occurrence of three newly identified, hexosaminidase-related polypeptides resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitates from [$^{35}$S]methionine-labeled cell extracts. These polypeptides, called band 2 (75,000), band 3 (70,000), and band 4 (63,000), were immunoprecipitated by an antiserum specific to placental hex $I_2$. They are distinct from pre-$\alpha$- (60,000) and pre-$\beta$- (58,000) precursor polypeptides and the $\alpha$- (56,000), $\beta_a$- (27,000), and $\beta_b$- (27,000) polypeptides of the mature hex A ($\alpha\beta_a\beta_b$) and hex B ($2[\beta_a\beta_b]$). When fibroblast extracts were chromatographed on DEAE-Sepharose, bands 2, 3, and 4 were eluted together in fractions before hex A, in a position characteristic of serum and placental hex $I_2$ and serum hex P. This suggests that bands 2, 3, and 4 might represent the polypeptides of a fibroblast hex I.

The analysis of partial proteolytic digests of the radioactively labeled polypeptides revealed that bands 2 and 3, pre-$\beta$, and $\beta_a$ had several peptides in common, suggesting that they are structurally related to each other. However, bands 2, 3, and 4 were present in extracts of Tay-Sachs (pre-$\alpha$ and $\alpha$ deficiency) and Sandhoff cells (pre-$\beta$, $\beta_a$, and $\beta_b$ deficiency) and appeared later than pre-$\beta$ in pulse-chase experiments. These results suggest that bands 2 and 3 occur independently of pre-$\beta$ and $\beta_a$ and are probably specified by different mRNA, whether from the same gene or distinct but homologous genes.

## INTRODUCTION

The major forms of hexosaminidase (hex)[1] in human tissues occur as multimers of $\alpha$- and/or $\beta$-polypeptide chains. Hex A is composed of one $\alpha$- and one $\beta_2$-subunit ($\alpha\beta_2$) and hex B has the structure $2\beta_2$ (1–4). Recently, we reported that the $\beta_2$ subunit is made up of two nonidentical polypeptide chains, $\beta_a$ and $\beta_b$, which have similar molecular weights of 25,000 but completely different peptide compositions (5).

There are two principle genetic disorders in which the expression of the $\alpha$- and $\beta$-chains are affected directly. In Tay-Sachs disease there is a deficiency of $\alpha$-chains resulting in the absence of hex A but retention of hex B; and Sandhoff disease is characterized by a deficiency of $\beta$-chains so that both forms of hexosaminidase are absent (6).

In addition to hex A and hex B, several other forms of hexosaminidase have been reported. These include hex S, an $\alpha$-chain dimer found in small amounts in Sandhoff disease (7), and hexosaminidases that are intermediate in charge between hex A and hex B. Among the latter are hex $I_1$ and hex $I_2$, first demonstrated by Price and Dance (8) after ion exchange chromatography of serum. One additional form of hexosaminidase, hex P, is found in the serum of pregnant women and is very similar or identical to hex $I_2$ (9). Although hex P appears to be antigenically related to hex B (8, 9), its polypeptide structure has not been investigated, and its relationship to the $\alpha$-, $\beta_a$-, or $\beta_b$-chains remains unknown. Intermediate peaks of hexosaminidase activity obtained by ion exchange chromatography have

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[1] *Abbreviations used in this paper*: CLA, cross-linked hex A; hex, hexosaminidase; NRS, normal rabbit serum; PAG, polyacrylamide gel.

also been identified in extracts of a number of other tissues including cultured fibroblasts (10–12). Their relationship to the serum hex $I_1$, $I_2$, or hex P is unknown.

Our laboratory has been examining the genetic and biochemical relationship of the various forms of hexosaminidase through an investigation of their synthesis. Cultured fibroblasts have proved to be an ideal tissue for these studies because their proteins are amenable to biosynthetic analysis and because they appear to express most, if not all, of the hexosaminidase-related proteins. In this study, we have investigated the biosynthesis in fibroblasts of several polypeptides serologically related to hexosaminidase. These include the $\alpha$-, $\beta_a$-, and $\beta_b$-polypeptides found in hex A and hex B as well as putative pre-$\alpha$- and pre-$\beta$-polypeptides recently described by Hasilik and Neufeld (13). In addition, we report the occurrence of three newly identified polypeptides, two of which show structural relatedness to the pre-$\beta$- and $\beta_a$-polypeptides by peptide mapping and all three of which may represent polypeptides of a fibroblast hex I.

## METHODS

*Hexosaminidase purification.* Hexosaminidase was purified from human placenta (4). Hex A, B, and $I_2$ were separated by DEAE-Sepharose chromatography at pH 6.0 with a salt gradient of 0–0.22 M NaCl (4). The detailed isolation and structural characterization of placental hex $I_2$ will be reported elsewhere.[2] Briefly, placental hex $I_2$ was one of four protein species separated by DEAE-Sepharose chromatography of the hexosaminidase purified by affinity chromatography. All four species were enzymatically active and included, in the order of elution, hex B, $I_1$, $I_2$, and A. The hex I were so designated only to conform to the nomenclature of Price and Dance (8) because they were eluted as species of intermediate charge between hex A and hex B. Their relationship to the hex I of serum or other tissues has not been investigated. The purity of the native enzymes used in this study, hex A, B, and $I_2$, was confirmed by polyacrylamide gel (PAG) electrophoresis with native conditions in which hexosaminidase activity was located by hydrolysis of naphthol-AS-BI-*N*-acetyl-$\beta$-D-glucosaminide (4) and protein was located by staining with Coomassie Blue. All three species showed enzyme activity and could be readily distinguished from one another with hex $I_2$ running between hex A and hex B.[2]

*Preparation of antisera.* Three antisera were used in this study. Rabbit anti-hex cross-linked hex A (CLA) was raised against a preparation of placental hex A that was cross-linked with glutaraldehyde according to the method of Srivastava et al. (14). It is a typical anti-hex A antiserum in that it reacts with both hex A and hex B and retains the ability to recognize hex A after preabsorption with hex B. The hex A used for the immunizations was prepared by two cycles of DEAE-Sepharose chromatography to assure that the preparation was devoid of hex $I_2$. The purity of the preparation was confirmed by sodium dodecyl sulfate (SDS)-PAG electrophoresis (4).

A second antiserum, called anti-hex (A + $I_2$) was raised in a rabbit with a DEAE-Sepharose-purified hexosaminidase preparation from placenta containing both hex A and hex $I_2$. It is similar to anti-hex CLA except that it retains the ability to precipitate hex $I_2$ after preabsorption with concanavalin A (Con A)-Sepharose-bound hex A or hex B. Thus, anti-hex (A + $I_2$) contains specific anti-hex A and anti-hex $I_2$ components. The characterization of the cross-reaction of anti-hex (A + $I_2$) with placental enzymes is reported elsewhere.[2]

A third antiserum, anti-hex S, was raised against a preparation of hex S prepared by the subunit dissociation of hex A with merthiolate and isolation of hex S by DEAE-Sepharose chromatography (4). It was used for the immunoprecipitation of pre-$\alpha$- (called band 6 in this study) and $\alpha$-polypeptides.

*Absorption of antiserum.* Anti-hex (A + $I_2$) was preabsorbed with placental hex A, B, or $I_2$ by washing the antiserum with an excess of antigen bound to Con A-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ). Typically, 500 $\mu$g of enzyme in 0.5–1.0 ml phosphate-buffered saline (PBS) was bound to 1 ml packed vol of Con A-Sepharose in a 5-ml plastic tube by rotating the tube at 4°C for 1 h. The resin-bound enzyme was recovered by centrifugation and washed with PBS. Undiluted antiserum, 0.5 ml, was added to the tube and it was rotated at 4° overnight. The suspension was centrifuged and the supernatants were combined to give ~1 ml of absorbed antiserum.

*Source and growth of cells.* All strains were subcultures of skin fibroblasts from normal individuals and patients with $GM_2$ gangliosidosis. The latter included strains 904 from a 13-mo-old male infant with the classical form of Tay-Sachs disease prevalent in Ashkenazi Jews; strain 905, his heterozygous mother; strain WG107 from a 3-yr-old female with the French Canadian variant of Tay-Sachs disease (Repository for Human Cell Strains, Children's Hospital, Montreal); and strain GM317 from a 14-mo-old female infant with Sandhoff disease (Human Genetic Mutant Cell Repository, Camden, NJ). Fibroblast monolayers were grown in $\alpha$-medium supplemented with 15% fetal calf serum (15) without antibiotics at 37°C in a 5% $CO_2$:95% air atmosphere.

*[$^{35}$S]Methionine labeling of fibroblasts.* For isotopic labeling experiments, the cells were grown to early confluence in 75-cm$^2$ tissue culture flasks, usually two flasks per experiment. One day before radioactive labeling, the medium was removed from the flasks and replaced by 10 ml of $\alpha$-medium containing 10 $\mu$M methionine (final concentration) and 15% dialyzed fetal calf serum. The next day, the medium was replaced by 5 ml of the same medium supplemented with 50 $\mu$Ci/ml of [$^{35}$S]methionine (800–1,200 Ci/mmol, Amersham Corp., Arlington Heights, IL). After 15 h incubation, the radioactive medium was removed and the flasks were washed twice with PBS. The fibroblasts were harvested by scraping with a rubber policeman. They were washed twice with PBS and lysed by freeze-thawing three times in 0.5 ml of buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1 M KCl, and 0.005 M $MgCl_2$. The lysed cells were homogenized in the presence of an equal volume of carbon tetrachloride by using a glass homogenizer and centrifuged for 1 min in a microfuge (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY). The supernatant was transferred to a tube containing 1 ml (packed vol) of Con A-Sepharose previously washed five times with a buffer containing 0.01 M sodium phosphate pH 7.0 and 0.5 M NaCl. The tubes were rotated at 4°C for

[2] Mahuran, D. J., F. Tsui, R. A. Gravel, and J. A. Lowden. Manuscript in preparation.

1–2 h to bind glycoproteins. The unbound material was removed and the resin was washed extensively with the same buffer to remove any remaining unbound material. The Con A-Sepharose-bound proteins were eluted by washing five times, each with 0.5 ml of 15% α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, MO) in the sodium phosphate-NaCl buffer. The eluates were combined and dialyzed overnight against at least 200 vol of PBS at 4°C.

The procedure was modified for analysis of the hexosaminidases by DEAE-Sepharose chromatography. 80 flasks of cells were used for the radioactive labeling requiring a total of 20 mCi of [$^{35}$S]methionine. The protein fraction was eluted from 20 ml of Con A-Sepharose, dialyzed against 0.01 M sodium phosphate, pH 6.0, and chromatographed through a 10-ml column of DEAE-Sepharose previously equilibrated with the same buffer. Proteins were eluted with a 1-liter gradient of 0–0.2 M NaCl in phosphate buffer and 5.5-ml fractions were collected into tubes containing 0.5 ml of human serum albumin (5 mg/ml). After measurement of hexosaminidase activity, fractions were pooled and concentrated by rebinding to Con A-Sepharose. The eluted proteins were dialyzed against PBS in preparation for immunoprecipitation.

For the pulse-chase experiments, fibroblasts were labeled with 100 μCi/ml of [$^{35}$S]methionine during a 1-h pulse. The medium was exchanged for a chase medium consisting of α-medium and 15% fetal calf serum (methionine at 0.1 mM) for the indicated times up to 72 h. Separate sets of two flasks were harvested after the pulse and after each chase period.

*Immunoprecipitation of radioactive cell extracts.* The dialyzed extracts (Con A-Sepharose binding fractions) were divided into three aliquots (0.5–1.0 ml each) and were preabsorbed twice with normal rabbit serum (NRS). This was accomplished by adding 25 μl NRS to each aliquot and incubating at 4°C from 4 h to overnight. After incubation, immune complexes were precipitated with 250 μl (packed vol) of PBS-washed Pansorbin (formalin-fixed *Staphylococcus aureus* containing protein A; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) by incubating at 4°C for an additional 2–4 h followed by centrifugation for 1 min in a microfuge (16). To the NRS-cleared extracts, either 10 μl of antiserum (20 μl of preabsorbed antiserum) or NRS was added and the extracts were incubated at 4°C for 4 h. Subsequently, 10 vol (100 or 200 μl relative to antiserum) of washed Pansorbin was added and the suspension was incubated an additional 2 h and centrifuged for 1 min. The pellet was washed three times with PBS with centrifugation after each step and solubilized by adding 20 μl of 10% SDS and 80 μl of 0.065 M Tris-HCl pH 6.8 followed by immersion in a boiling water bath for 3 min. This suspension was centrifuged as above and the supernatant was recovered. A 5-μl sample of the supernatant was removed for scintillation counting. The remaining solution was reduced and alkylated (4) and precipitated in 5 vol of acetone. The precipitate was solubilized in 10 M urea and subjected to SDS-PAG electrophoresis (17). Radioactive bands were visualized by fluorography (18).

In one experiment for the immunoprecipitation of pre-α- and α-polypeptides, the immunoprecipitate obtained by reaction of an extract from radiolabeled normal cells with anti-hex (A + $I_2$) was solubilized, diluted 20-fold with PBS, and subjected to a second immunoprecipitation with anti-hex S antiserum (13).

*Peptide mapping.* The peptide patterns of hexosaminidase-related polypeptides were examined by the Bordiev and Crettol-Jarvinen (19) two-dimensional gel procedure essentially as described previously (5). Briefly, radiolabeled polypeptides were prepared and resolved into individual species by SDS-PAG electrophoresis as described above. Individual lanes containing the separated polypeptides were excised from the gel and subjected to partial proteolytic digestion with *S. aureus* protease V8 in the stacking gel of a second SDS-PAG with subsequent separation of peptides by electrophoresis into the second gel.

## RESULTS

Hexosaminidase-related polypeptides produced by fibroblasts were examined by growing cells in [$^{35}$S]methionine overnight and immunoprecipitating radiolabeled proteins with anti-hex (A + $I_2$) or anti-hex CLA. The immune complexes were solubilized and examined by SDS-PAG electrophoresis (Fig. 1). In all studies, preimmune serum or NRS was used to identify nonspecifically precipitated bands including two high molecular weight polypeptides (>100,000) (lane 1, Fig. 1). The polypeptides precipitated with immune sera are shown in lanes 2–6. Anti-hex CLA precipitated two polypeptides in addition to the α- and β-chains (lane 2, Fig. 1). These polypeptides, designated bands 5 and 6, are similar in apparent molecular weights (60,000 and 58,000, respectively) to the pre-α- and pre-β-polypeptides described by Hasilik and Neufeld (13). The β-chain band includes the distinct $\beta_a$- and $\beta_b$-polypeptides that are not resolved by SDS-PAG electrophoresis (5) (see peptide studies below).

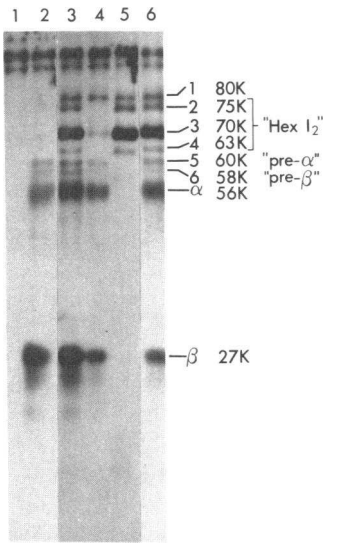


FIGURE 1 SDS-PAG electrophoresis of hexosaminidase-related polypeptides immunoprecipitated from fibroblasts radioactively labeled with [$^{35}$S]methionine. Details are given in Methods. The antiserum used for each lane is as follows: lane 1, precipitation with NRS; lane 2, anti-hex CLA; lane 3, anti-hex (A + $I_2$); lane 4, anti-hex (A + $I_2$) preabsorbed with placental hex $I_2$; lane 5, anti-hex (A + $I_2$) preabsorbed with placental hex A; and lane 6, anti-hex (A + $I_2$) preabsorbed with hex B. K; ×1,000.

When immunoprecipitation was carried out with anti-hex (A + $I_2$), several bands, in addition to bands 5, 6, $\alpha$, and $\beta$, were observed (lane 3, Fig. 1). These included four low mobility species (band 1–4) ranging in molecular weight from 80,000 to 63,000.

In order to determine if these extra bands were hexosaminidase-related, anti-hex (A + $I_2$) serum was absorbed with various forms of placental hexosaminidase. After preabsorption with Con A-Sepharose-bound hex A (lane 5, Fig. 1), the remaining antiserum no longer reacted with bands 5, 6, $\alpha$, or $\beta$. However, it could still precipitate bands 1–4, indicating that these polypeptides contained antigenic sites distinct from those of the $\alpha$- and $\beta$-chains of hex A. This experiment also confirmed that the absorption of anti-hex (A + $I_2$) by hex A was complete.

Similarly, when anti-hex (A + $I_2$) was absorbed with hex B (lane 6, Fig. 1), the remaining antiserum also precipitated bands 1–4. However, bands 5 and $\alpha$ could still be precipitated as could reduced amounts of bands 6 and $\beta$, presumably owing to reaction of the absorbed antiserum with the $\alpha$-chain determinants of hex A heteropolymers ($\alpha\beta_2$) and a possible pre-hex A (containing bands 5 and 6) heteropolymer. Thus, bands 1–4 contained antigenic sites independent of those of hex B.

When the anti-hex (A + $I_2$) serum was preabsorbed with placental hex $I_2$, the results were more complex. The remaining antiserum precipitated band 1, reduced amounts of bands 5, $\alpha$, and $\beta$, and a greatly diminished amount of band 3. None of the bands 2, 4, or 6 was precipitated. This experiment indicated that at least bands 2, 3, 4, and 6 shared antigenic determinants found on placental hex $I_2$.

The results of the immunoprecipitation experiments indicated that anti-hex (A + $I_2$) is composed of at least two distinct components in terms of their reactivity toward fibroblast proteins. One of these, behaving like an anti-hex A, reacted with bands 5, 6, $\alpha$, and $\beta$. The other antiserum component, removable by absorption with placental hex $I_2$, appeared wholly responsible for bands 2, 3, and 4 precipitation. This latter component also appeared to show some reaction toward bands 5, 6, $\alpha$, and $\beta$ because these bands were reduced in quantity when the hex $I_2$-absorbed antiserum was used. Such a result might have been expected if the hex $I_2$ used for the absorption of the antiserum had been contaminated with hex A or hex B. However, as indicated in Methods, the hex $I_2$ preparation was judged free of such contaminants when examined by PAG electrophoresis of the native protein, a procedure that can readily distinguish among hex A, B, and $I_2$. Therefore, these results indicate that bands 2, 3, and 4 have one class of antigenic sites distinct from those on bands 5, 6, $\alpha$, or $\beta$ because they did not react with the anti-hex A component of the antiserum. Secondly, bands 2, 3, and 4 appear to have a second class of antigenic sites that they share in common with similar sites on bands 5, 6, $\alpha$, or $\beta$.

Band 1 could not be eliminated in the immunoprecipitation experiments by preabsorbing the antiserum with hex A, B, or $I_2$. As such, its identity remained unknown and it was not characterized further.

*Characterization of bands 2, 3, and 4.* Because bands 2, 3, and 4 were found to be antigenically related to placental hex $I_2$, we investigated whether these bands behaved like the placental enzyme by ion exchange chromatography. Fibroblasts were radiolabeled with [$^{35}$S]methionine and the Con A-Sepharose binding portion of the cell extract was examined by DEAE-Sepharose chromatography. The eluted fractions were pooled according to enzyme activity, concentrated by rebinding to Con A-Sepharose, and immunoprecipitated by anti-hex (A + $I_2$). The resulting polypeptide patterns obtained from SDS-PAG electrophoresis are shown in Fig. 2. Two major peaks of enzyme activity were revealed, one in fraction 1 and the other in fractions 7–9, corresponding to hex B and A, respectively, as confirmed by the demonstration of $\beta$-chains in the former and $\alpha$- and $\beta$-chains in the latter fractions by SDS-PAG electrophoresis. Bands 2, 3, and 4 were clearly visible in fractions 4–7 by SDS-PAG electrophoresis with a peak in fraction 6. The eluting concentrations of NaCl (0.06–0.14 M) were similar to those which have eluted placental and serum hex $I_2$ during chromatography at pH 6.0 at a position just before hex A (12, 14). However, no enzyme activity was detected at this position in the present experiment.

*Peptide analysis.* We next set out to determine if bands 2, 3, and 4 shared structural homology with the known hexosaminidase polypeptides. Radiolabeled polypeptides initially separated by SDS-PAG electrophoresis were subjected to partial digestion with protease V8 and peptides were separated in the second electrophoretic dimension. For the analysis of peptide maps, electrophoretic lanes were cut out from photographs and aligned for comparison (Fig. 3). Bands 2, 3, $\beta_a$, and $\beta_b$ were immunoprecipitated from a radiolabeled normal cell extract. Their peptide patterns came from a single gel and were aligned as on that gel (Fig. 3B). Bands 5 and 6 could not be sufficiently resolved during electrophoresis so alternate procedures were used for the preparation of their peptide maps. Because band 5 is not appreciably synthesized in Tay-Sachs disease, band 6 was prepared by anti-hex (A + $I_2$) precipitation of an extract of the cells of a Tay-Sachs variant that had been radiolabeled for 1 h (described below, Fig. 5). The peptide pattern could be accurately aligned in Fig. 3B by using the peptide patterns from the first gel as a reference. Finally, bands 5 and $\alpha$ were derived from an immunoprecipitation of an extract of radiolabeled normal cells by

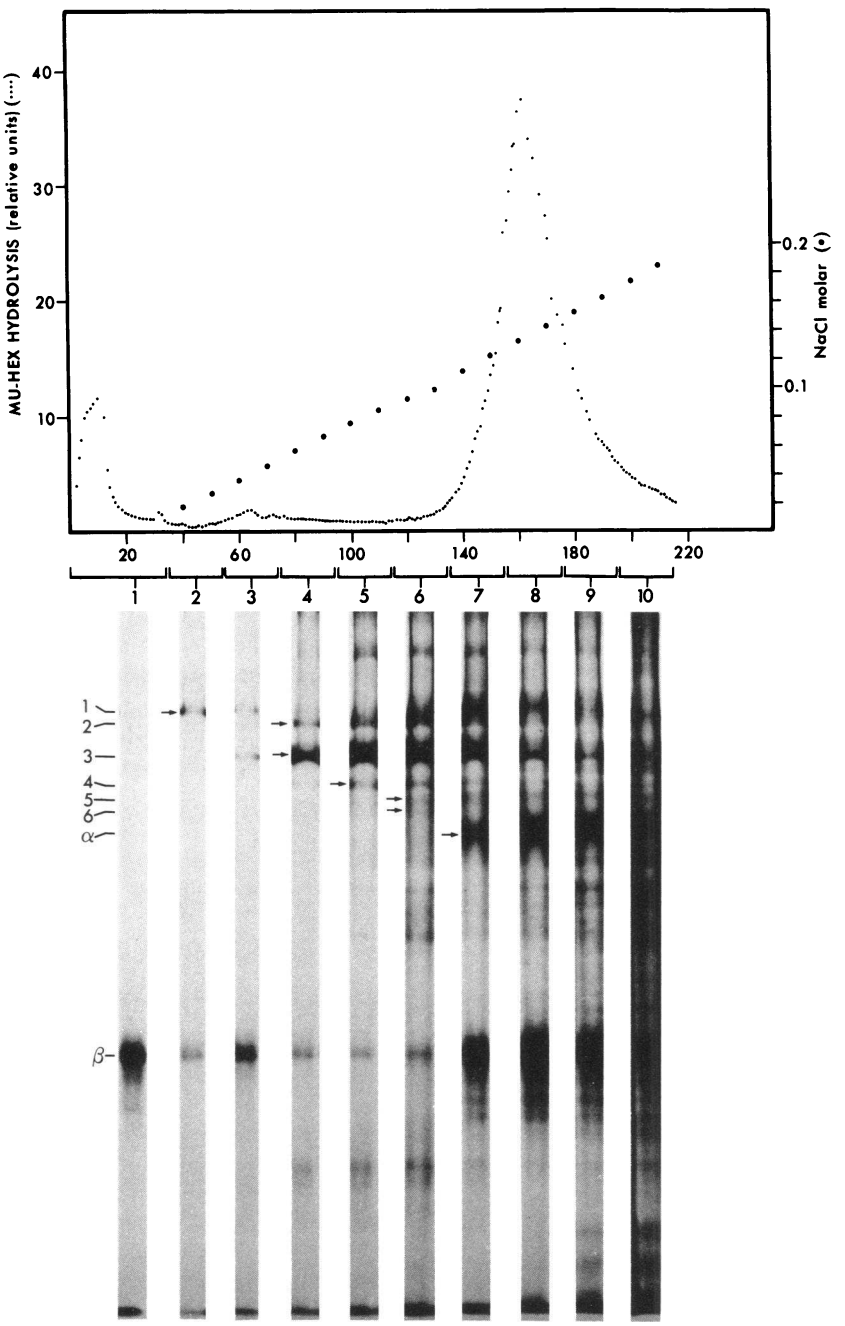


FIGURE 2 SDS-PAG electrophoresis of immunoprecipitates of fractions pooled from DEAE-Sepharose chromatography of a [$^{35}$S]methionine-labeled fibroblast extract. Fractions were collected from the DEAE-column (upper panel, fractions 1–220) and assayed for hexosaminidase activity. The fractions were combined to give pooled fractions 1–10. These were concentrated and precipitated with anti-hex (A + $I_2$) and resolved by SDS-PAG electrophoresis (lower panel). Each lane corresponds to the pooled fraction number indicated above it. Polypeptide bands (numbered on left side of lower panel) were identified by comparison with the polypeptide pattern in Fig. 1.

using anti-hex S, which reacts with $\alpha$-chain determinants (13).

These experiments revealed an apparent structural relationship among bands 2, 3, 6, and $\beta_a$ (Fig. 3B). First, the peptide patterns of bands 2 and 3 appear virtually identical to each other and might differ only

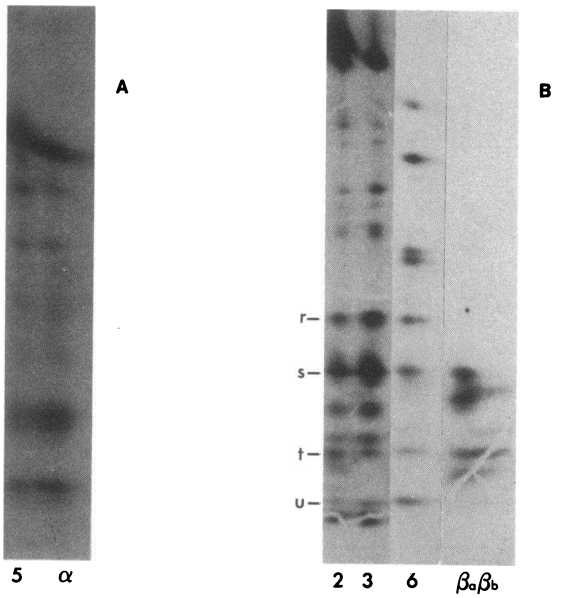


FIGURE 3 SDS-PAG electrophoresis of peptides obtained by partial digestion with protease V8 of hexosaminidase-related [$^{35}$S]methionine-labeled polypeptides. Polypeptides were separated by SDS-PAG electrophoresis as in Fig. 1. Lanes were cut out of the gels and applied to the top of a second SDS-PAG and overlayed with protease V8. Digestions were done in the stacking gel of the second dimension gel and the peptides were subsequently resolved by electrophoresis. Details are given in the text. The numbers and letters given at the bottom on the figure correspond to the polypeptides indicated in Fig. 1. r, s, t, and u are labels for four peptides discussed in text.

by a terminal peptide or perhaps by a difference in oligosaccharide side chain. Secondly, both bands 2 and 3 show four peptides, labeled r, s, t, and u, which may be identical to peptides of similar mobilities from band 6. This is clearly the case for peptides r and s and possibly so for peptides t and u, given that the analyses required separate gels. Finally, two of these peptides, s and t, comigrated with bands seen in the digests of the $\beta_a$-chain. Similar results have been obtained for these polypeptides derived from several fibroblast strains. We therefore conclude that bands 2, 3, 6, and $\beta_a$ show varying degrees of structural homology. Band 4 never produced more than one or two peptides after protease digestion and these could not be matched to the peptides of any other protein.

Band 5 and the $\alpha$-chain did not show homology with the $\beta_a$ group of polypeptides but they did appear to be structurally homologous to each other (Fig. 3A). Their peptide maps were virtually identical except that band 5 appears slightly larger than the $\alpha$-chain, a result providing structural evidence in support of the contention of Hasilik and Neufeld (13) that band 5 is a precursor of the $\alpha$-chain.

Fig. 3B also shows a partial resolution of the peptide maps of the $\beta_a$- and $\beta_b$-chains. As described elsewhere for placenta (5), these polypeptides comprise the $\beta_2$ subunit of hexosaminidase and generate markedly different peptide patterns after protease V8 digestion in experiments in which the polypeptides were fully separated by isoelectric focusing. Although the peptide patterns are nearly merged in the present experiment (Fig. 3B), the unrelated structure of these two chains is apparent. In addition, no evidence of shared peptides was found between $\beta_b$ and any of the other polypeptides.

*Polypeptides of mutant strains.* Having demonstrated antigenic and structural similarities among bands 2, 3, and 4 and hexosaminidase-related polypeptides, we examined their expression in fibroblast strains derived from patients with Tay-Sachs or Sandhoff disease (Fig. 4). In Tay-Sachs disease cells, the polypeptides immunoprecipitated by anti-hex (A + $I_2$) show a pattern like that of normal cells except that band 5 and the $\alpha$-chain are completely missing (lane 1, Fig. 4). The heterozygous parent of this patient gave a normal polypeptide pattern except that a reduced amount of $\alpha$-chain was detected (lane 3, Fig. 4) (con-

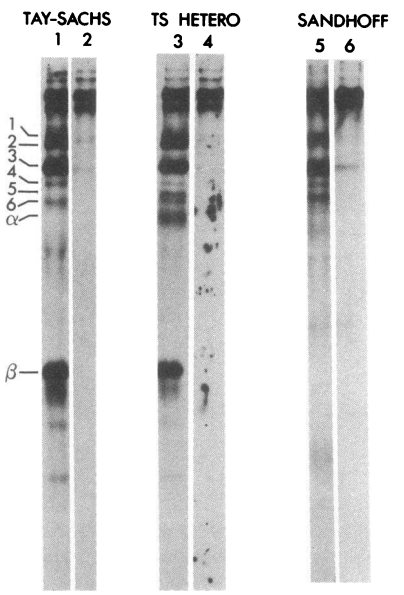


FIGURE 4 SDS-PAG electrophoresis of hexosaminidase-related polypeptides immunoprecipitated from mutant cell extracts radioactively labeled with [$^{35}$S]methionine. The disorders examined are identified at the top of the gel and include strain 904, Tay-Sachs disease; strain 905, mother of strain 904 identified as "TS Hetero"; and strain GM317, Sandhoff disease. Lanes 2, 4, and 6 are from precipitations with NRS. Lanes 1, 3, and 6 are from precipitations with anti-hex (A + $I_2$). A corresponding normal control is shown in lane 3, Fig. 1.

trast the ratio of $\alpha$ to $\beta$ intensity in the heterozygote with the ratio for the normal pattern in lane 3, Fig. 1). In Sandhoff disease cells, band 6 and the $\beta$-chains are missing and the amount of $\alpha$-chain is greatly reduced (lane 5, Fig. 4). Although the levels of band 2 in normal and mutant strains was highly variable, bands 2, 3, and 4 all appeared to be synthesized in mutant strains (a total of six Tay-Sachs and four Sandhoff strains examined).

Our results confirm the band 5 and $\alpha$-chain deficiency in Tay-Sachs disease and the band 6 and $\beta$-chain deficiency in Sandhoff disease (13). Despite the peptide relatedness of bands 2, 3, 6, and the $\beta_a$-chain, bands 2 and 3 were detected in the Sandhoff cells whereas the other bands were not. In addition it is evident that both the $\beta_a$- and $\beta_b$-chains are absent in Sandhoff disease because no radioactivity was detected in the $\beta$-chain position in any of the strains examined.

*Pulse-chase experiments.* The higher molecular weight of bands 2 and their structural relatedness to bands 6 and $\beta_a$ led us to examine the biosynthetic relationship of these polypeptides in pulse-chase experiments to determine if a precursor-product relationship could be demonstrated. Fibroblasts of a Tay-Sachs variant were used for this experiment in order to observe the behavior of band 6 without it being obscured by band 5 or the $\alpha$-chain. Cells were radioactively labeled with [$^{35}$S]methionine for 1 h and chased with unlabeled medium for up to 72 h. Polypeptides were immunoprecipitated from duplicate cultures after the pulse and different intervals of chase (Fig. 5). Band 6 was the earliest discernible band and appeared during the 1-h pulse. It also appeared to occur as a doublet and both bands disappeared after 2–4 h chase. Concurrently by 2 h of chase, the $\beta$-chains first appeared and persisted throughout the remainder of the chase period ($\beta_a$ and $\beta_b$ could not be distinguished from one another). The two bands of the band 6 doublet gave identical peptide patterns after protease V8 digestion of the 1-h pulse lane (lane 6, Fig. 3B). These results suggest that band 6 is converted to a $\beta$-chain although it cannot be determined from this experiment whether only $\beta_a$ or both $\beta_a$ and $\beta_b$ are derived from band 6.

None of bands 2, 3, or 4 appeared after a 1-h pulse (Fig. 5). Band 2 appeared after ~2–4 h chase and decreased in intensity over the 72-h period. By 16–24 h, band 4 appeared and was maintained to at least 72 h. Band 3 appeared by 4 h of chase and persisted throughout the experiment. Experiments with normal cells, different Tay-Sachs variants, and Sandhoff cells gave essentially identical results. Thus, bands 2 and 3 were first detected well after the initial appearance of band 6. Therefore, any biosynthetic relationship between bands 2, 3, and 4 with band 6 or $\beta_a$ was not revealed.

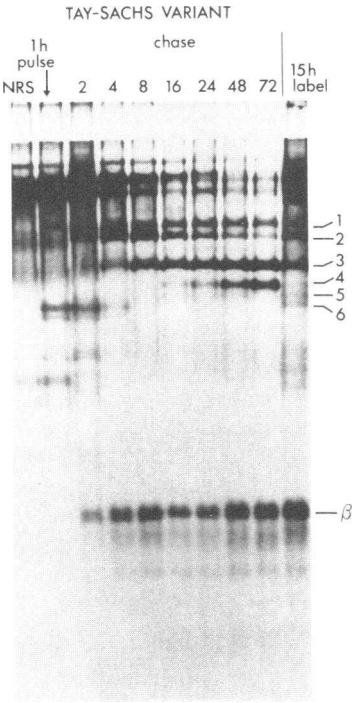


FIGURE 5 Pulse-chase experiment showing the biosynthesis of the $\beta$-related polypeptides in a Tay-Sachs variant, strain WG107, with a nearly complete absence of band 5 (pre-$\alpha$) biosynthesis and a complete deficiency of $\alpha$-chain synthesis. The NRS control corresponds to a normal rabbit serum precipitation of the 1-h pulse. The numbered lanes refer to the different chase periods for the indicated times in hours. The lane at the far right is from an overnight labeling similar to those used to produce Figs. 1 and 4 and was used for the identification of the polypeptide bands in the pulse and chase lanes. Band 6, shown as a doublet of very close bands, was examined by peptide mapping and is the source of the band 6 peptide map in Fig. 3.

## DISCUSSION

Three high molecular weight polypeptides, identified as bands 2, 3, and 4 by SDS-PAG electrophoresis, appear to be antigenically and structurally related to the hexosaminidases and may correspond with a cellular fibroblast version of a hex I. Several lines of evidence suggested a relationship to the hexosaminidases: (*a*) immunoprecipitation of bands 2, 3, and 4 could be blocked by preabsorption of the antiserum with placental hex $I_2$; (*b*) bands 2, 3, and 4 were eluted together from DEAE-Sepharose before hex A, as characteristic of placental and serum hex $I_2$ and serum hex P (9, 12, 14); and (*c*) two of the polypeptides, bands 2 and 3, were shown to be structurally related to band 6 (pre-$\beta$) and $\beta_a$ by peptide analysis of partial proteolytic digests. Despite these links to the hexosaminidases, there was no enzymatic activity associated with fractions of fibroblast extracts chromatographed on DEAE-

Sepharose containing bands 2, 3, and 4. If any enzyme activity were associated with these polypeptides, it might not have survived the extreme protein dilution and several days required to conduct the experiment. Similar difficulties have been experienced with placental hex $I_2$ in that it rapidly loses hexosaminidase activity during storage for short periods of time (2). Nevertheless, confirmation that the newly identified polypeptides correspond to a form of hexosaminidase would best be achieved through the demonstration of enzyme activity.

A hex I form of hexosaminidase has previously been documented in fibroblasts and aminocytes (10, 12). Nakagawa et al. (12) eluted an intermediate peak of enzyme activity from extracts of both cell types after DEAE-cellulose chromatography at a similar pH and salt concentration as required for bands 2, 3, and 4 in this study. Their peak contained ~3% of the total hexosaminidase activity in both cell types from normal individuals.

What is the relationship among all the hex I of the serum, placenta, cultured cells, and other tissues? Because the name is an arbitrary designation related to the chromatographic migration of hexosaminidase species of intermediate charge, they need not necessarily be related. Clearly, we must examine the structural and antigenic relatedness of the enzymes from all these sources to determine if they are related and if they contain the high molecular weight polypeptides described in this study. Preliminary studies of the polypeptides of placental hex I preparations show that, like their fibroblast counterparts, they also contain polypeptides with apparent molecular weights from 66,000–75,000 (2). Perhaps the antigenic similarity of serum hex P and hex B (9) is analogous to the structural relatedness of the high molecular weight bands 2 and 3 with the $\beta_a$-chain of hex B in fibroblasts. Therefore it is possible that the hex I from all of these sources could represent a separate group of polypeptides that are structurally related to hex B through the $\beta_a$ polypeptide.

Bands 2 and 3 probably originate independently of band 6. Although they are structurally related, band 6 appeared before bands 2 or 3 in pulse-chase experiments. Thus, the higher molecular weight bands 2 and 3 are not likely to be precursors of band 6. Support for this notion also came from Sandhoff disease cells in which band 2 and 3 were synthesized, whereas band 6 and $\beta_a$ were not. The results imply that these polypeptides originated from different mRNA. Either one gene specifies mRNA of different coding lengths or there might be two independent but homologous genes encoding this group of polypeptides.

Bands 2, 3, and 4 are probably derived from the same origin. Band 3 appeared later than band 2 and had one or two fewer peptides, suggesting that at least some of band 2 is clipped to give rise to band 3. Further, the data suggest that band 4 may also be derived from band 2 with the switch occurring at 16–48 h of chase. This latter point can not be substantiated, however, because band 4 never showed more than one or two peptides, too small a number for comparison.

The origin of the $\beta_b$-chain remains as enigma. The simplest conjecture is that $\beta_a$ and $\beta_b$ are derived by a proteolytic clip approximately in the center of band 6 (5, 13). The absence of all three polypeptides, $\beta_a$, $\beta_b$, and band 6, in Sandhoff disease is consistent with this notion. If this model were correct, at least one common peptide would be expected between $\beta_b$ and its precursor. Although we failed to detect such a peptide homology, it is possible that putative common peptides do not contain any methionine residues and so would not have been radioactive. Resolution of this question will require a more direct structural comparison of band 6 with $\beta_a$ and $\beta_b$.

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