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Chemical Characterization and Subunit Structure of Human *N*-Acetylhexosaminidases A and B†

B. Geiger and R. Arnon*

ABSTRACT: Human hexosaminidases A and B were purified from placentae, using two stages of affinity chromatography, to a high degree of purity. Each enzyme was purified 5000–6000-fold, and isolated in 25–40% yield. Enzyme preparations appeared homogeneous in the analytical ultracentrifuge and by acrylamide gel electrophoresis. Hexosaminidase A contained 1.65 residues of sialic acid per molecule, whereas no sialic acid was present in hexosaminidase B. The molecular weights of the A and B isozymes as determined by gel filtration and sedimentation equilibrium are 100 000 and 108 000, respectively. In 5 M guanidine-HCl each of the enzymes yielded a 50 000-dalton species, which can further be dissociated into 25 000-dalton polypeptide chains by reduction and alkylation. The hexosaminidase B yielded one type of polypeptide chain, denoted β, whereas the product from hexosaminidase A could be separated by ion-exchange chromatography into two species of chains, denoted α and β, in equal amounts. The amino acid compositions of the separated α and β chains were determined, and were found to correlate well with those of the intact enzymes. These findings enable the construction of a plausible model for the molecular structure of both enzymes. According to this model hexosaminidase A is composed of two subunits, α_2 and β_2, in which the two polypeptide chains are linked by a disulfide bridge. The structure of hexosaminidase B is, in parallel, $\beta_2\beta_2$. The suggested model is discussed in view of the accumulated information about the interrelationships between hexosaminidase A and B and the genetic metabolic disorders with which they are involved.

Hexosaminidase[1] exists in human tissues in two major isozymic forms A and B; deficiency in one of the isozymes or both results in severe inherited metabolic disorders, which are manifested as GM_2 gangliosidoses, e.g., Tay-Sachs or Sandhoff–Jatzkewitz diseases (Okada and O'Brien, 1969; Sandhoff et al., 1968; O'Brien, 1969). A large body of evidence is available on the biological properties of these isozymes, including their substrate specificity, as well as genetic aspects of their expression in various conditions (reviewed in Tallman, 1974; Neufeld et al., 1975). However, only limited information exists as to their fine molecular structure, or to the molecular relationship between the A and B isozymes, which is based on direct chemical characterization of the pure isoenzymes.

Various hypotheses were suggested to fit the relevant biological and genetic phenomena. The earliest hypothesis was suggested by Robinson and Stirling (1968) stating that hexosaminidase B may be the asialo form of hexosaminidase A, since a conversion from A to "B" was observed upon treatment with neuraminidase. This suggested model was ruled out lately

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[1] 2-Acetamido-2-deoxy-β-D glucoside-acetamidodeoxyglucohydrolase, EC 3.2.1.30.

by the findings that conversion of the A to the B form could be accomplished in the presence of merthiolate (an antibacterial agent present in most neuraminidase preparations), even without neuraminidase (Carmody and Rattazzi, 1974; Beutler et al., 1974). Tallman et al. (1974) suggested the hypothesis that the A and the B isozymes are conformers that share the same polypeptide chains, but differ only in the degree of cross-linking by disulfide bridges. Beutler and his colleagues, on the other hand, have offered several molecular models for the hexosaminidases assuming the existence of different subunits. Based on amino acid analyses they suggested a subunit composition of $(\alpha\beta)_n$ and $(\beta\gamma)_n$ for hexosaminidases A and B, respectively (Beutler and Srivastava, 1973; Srivastava and Beutler, 1974). In later publications they raised the possibility that hexosaminidase B is a homooligomer of β chains, whereas hexosaminidase A is composed of several α and β chains (Beutler and Kuhl, 1975). The exact number of subunits in the intact molecule was unclear, and varied from 4 to 8 (Tallman et al., 1974; Beutler et al., 1975; Beutler and Srivastava, 1973).

Information derived from immunochemical studies is consistent with a hypothesis suggesting a unique moiety present in hexosaminidase A, and not in B (Srivastava and Beutler, 1974; Ben-Yoseph et al., 1975; Bartholomew and Rattazzi, 1974), in addition to a considerable common antigenic structure which is manifested in high extent of cross-reactivity.

The present publication contains a direct biochemical investigation of the two hexosaminidase isozymes and their constituting subunits and polypeptide chains. This study was feasible due to the availability of the two isozymes in a pure form in relatively large amounts.

The findings enabled the suggestion of a molecular model into which all the data from genetic and biochemical studies, as well as the information on various variants of Tay-Sachs disease, may be fitted.

Experimental Procedure

Materials

Guanidine-HCl, urea, and bovine γ-globulin were purchased from Schwartz-Mann; Sepharose 4B, Sephadex G-150 and G-200, and dextran blue 2000 were from Pharmacia (Sweden); ovalbumin and lysozyme were from Worthington; 5,5′-dithiobis(2-nitrobenzoic acid), dithiothreitol (DTT),[2] and *N*-acetylneuraminic acid were obtained from Sigma; iodoacetic acid (BDH, U.K.) and iodoacetamide (Fluka, Switzerland) were recrystallized from benzene before use. 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide and methylumbelliferone were from Pierce. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were bought from Whatman. Iodo[2-^{14}C]acetic acid, 51 mCi/mmol, was from The Radiochemical Centre, Amersham (U.K.). Concanavalin A (two times crystallized, in saturated NaCl) was purchased from Miles-Yeda (Israel). All other reagents were of analytical grade or the best grade available.

Methods

Purification of Hexosaminidases A and B. Concanavalin A (Con A) was bound to CNBr-activated Sepharose 4B (Porath et al., 1967) in a ratio of 800 mg of Con A/100 ml of settled Sepharose.

2-Acetamido-*N*-(ϵ-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine (CNAG) was prepared and coupled to Sepharose 4B as described previously (Lotan et al., 1973; Geiger et al., 1974). The conjugated Sepharose contained 0.625 μmol of CNAG/gram of gel.

Human placentae were obtained from the Tel-Aviv municipality maternity hospital and frozen immediately following delivery.

Purification of hexosaminidases A and B was done on a large scale, starting with 100–200 kg of placental tissue in the different preparations. The purification followed the procedure described by us previously (Geiger et al., 1974, 1975) with several modifications and consisted of the following steps: (1) homogenization of the tissue in 4 volumes of ice-cold sodium phosphate buffer (0.01 M, pH 6.0) in a Waring commercial blender at top speed; (2) centrifugation of the homogenate in a sharpless, continuous flow centrifuge; (3) affinity chromatography on Sepharose-bound Con A (Geiger et al., 1975) followed by concentration and dialysis vs. 0.01 M sodium phosphate buffer, pH 6.0, in a hollow-fiber concentrator-dializer equipped with H1DP10 membrane cartridge; (4) DEAE-cellulose chromatography for the separation of isozymes A and B of hexosaminidase; (5) final purification of hexosaminidase A and B was achieved by ion-exchange chromatography on CM-cellulose and by gel filtration on Sephadex G-150, respectively (Geiger et al., 1975).

Analytical Polyacrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate. Polyacrylamide gels in different acrylamide concentrations were prepared in either tubes or as thin-layer slabs. The gel systems used were those described by Weber and Osborn (1969) in phosphate buffer or that of Laemmli (1970) in Tris-glycine buffer. Before the application to the electrophoresis gel, the samples were treated with either sodium dodecyl sulfate or with sodium dodecyl sulfate plus reducing agent, β-mercaptoethanol or dithiothreitol.

Analytical Ultracentrifugation. Sedimentation-equilibrium studies were carried out in the analytical ultracentrifuge (Model E, Beckman), equipped with photoelectric scanning absorption optical system (Schachman and Edelstein, 1966). Enzyme samples in concentrations of about 0.4 mg/ml were introduced into Kel-F double-sector cell of 12-mm optical path, and centrifuged at various speeds until equilibrium was observed. The molecular weight of the tested proteins was calculated from the plot of ln OD vs. the distance from the axis of rotation as described by Lamers et al. (1963). The solvent density, ρ, was measured directly, and the value of the partial specific volume was calculated from the partial specific volumes of amino acid residues (Vinograd and Hearst, 1962). The values that were calculated from amino acid analyses were used in the calculation of molecular weight of the native enzyme.

Amino Acid Analysis. Amino acid analyses of pure enzymes or of separated chains were performed in a Model 121 automatic amino acid analyser (Beckman). The samples were analyzed following acid hydrolysis in 6 N HCl for 22 h at 110 °C according to Moore and Stein (1954). Analysis of cysteine (after conversion to cysteic acid) was performed as described by Moore (1963). Tryptophan was analyzed after hydrolysis with methanesulfonic acid (Liu and Chang, 1971). Values for serine, threonine, tyrosine, and tryptophan were corrected for losses during hydrolysis.

Determination of Sialic Acid. The content of sialic acid was determined after partial acid hydrolysis (1 h in 0.1 N H_2SO_4 at 80 °C) by the thiobarbituric acid assay (Warren, 1959).

[2] Abbreviations used are: DTT, dithiothreitol; Con A, concanavalin A; CNAG, 2-acetamido-*N*-(ϵ-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine; DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, 2-amino-α-hydroxymethyl-1,3-propanediol; OD, optical density; CD, circular dichroism; BSA, bovine serum albumin; Nbs_2, dithionitrobenzene.

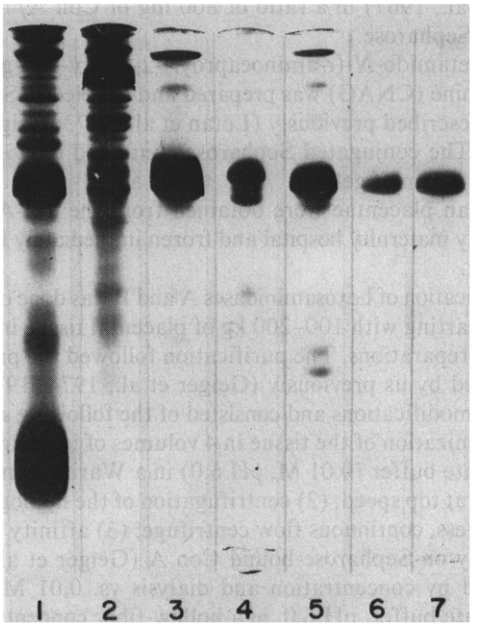

FIGURE 1: Polyacrylamide gel electrophoresis of hexosaminidases A and B at various stages of purification. The samples were not reduced prior to electrophoresis. (1) Crude placental homogenate; (2) eluate from Sepharose–Con A column; (3) eluate from affinity column (Sepharose–CNAG); (4) hexosaminidase A after DEAE-cellulose chromatography; (5) hexosaminidase B after DEAE-cellulose chromatography; (6) pure hexosaminidase A after CM-cellulose chromatography; (7) pure hexosaminidase B after gel filtration on Sephadex G-150.

TABLE I: Amino Acid Analysis of Pure Hexosaminidases A and B.

	Hexosaminidase A		Hexosaminidase B	
Amino acid	mol %[b]	mol of aa/mol of enzyme[c]	mol %	mol of aa/mol of enzyme
Lysine	5.38	47.8	5.65	54.7
Histidine	2.92	25.9	3.34	32.4
Arginine	4.64	41.2	4.57	44.3
Aspartic acid	9.76	86.7	9.54	92.4
Threonine	5.08	45.1	5.98	57.9
Serine	7.67	68.1	8.55	82.8
Glutamic acid	10.96	97.3	9.09	88.1
Proline	6.55	58.2	6.13	59.4
Glycine	7.48	66.4	6.52	63.2
Alanine	5.51	48.9	5.51	53.4
Cysteine + half-cystine[a]	1.43	12.7	2.54	24.6
Valine	5.99	53.2	4.99	48.3
Methionine	1.34	11.9	2.06	20.0
Isoleucine	3.86	34.3	4.07	39.4
Leucine	10.46	92.9	9.15	88.6
Tyrosine	4.21	37.4	5.05	48.9
Phenylalanine	5.24	46.5	5.53	53.6
Tryptophan	1.48	13.1	1.70	16.5
Sialic acid		1.65		UD[d]

[a] Determined as cysteic acid. [b] Expressed as mol of amino acid/100 mol of total residues in hydrolysate. [c] Expressed as the number of each residue in hexosaminidases A and B, the molecular weights taken for the calculations were 100 000 and 110 000, respectively. [d] Undetectable.

Quantitation of sialic acid was done using calibration curve of *N*-acetylneuraminic acid that was subjected to the same hydrolysis procedure.

Determination of Sulfhydryl and Disulfides. Free sulfhydryls in hexosaminidases A and B were determined in the presence and the absence of denaturating agents (guanidine-HCl or sodium dodecyl sulfate) using Nbs_2. The content of sulfydryls in enzyme solution at various concentrations was estimated from the absorbance at 412 nm, taking the value of E_{412} = 13 600 (Ellman, 1959).

Determination of both free sulfydryl and disulfide groups was done by alkylation of hexosaminidases A and B with ^{14}C-labeled iodoacetic acid before and after reduction. Nonspecific protein-bound radioactivity (which was low) was determined in enzyme samples by alkylation with cold iodoacetic acid prior to the radioactive labeling. These samples were taken as controls and the values obtained were subtracted from the experimental values. Radioactivity was measured in the Tri-Carb liquid scintillation spectrometer (Packard), after precipitation of the protein (0.5 mg of bovine γ-globulin was added as a carrier) with 10% trichloroacetic acid.

Optical Methods. Absorbance measurements were done with Zeiss PMQ II spectrophotometer or with Cary Model 15 recording spectrophotometer. Changes in the fluorescence emission spectra of hexosaminidases in the presence of denaturants were determined in Hitachi-Perkin Elmer spectrofluorometer (Model MPF-3). Usually protein samples in concentration of ca. 0.07–0.08 mg/ml were tested. Circular dichroism (CD) spectra of the two isozymes were recorded in a Cary Model 60 spectropolarimeter equipped with Model 6002 circular dichrometer. The results are expressed as θ, the molar eliplicity (deg·cm^2·dmol^{-1}).

Chromatographic Procedures. Analytical gel filtration on Sephadex G-200 column (3 × 80 cm) was performed by ascending chromatography with a flow rate of 15 ml/h. The column was preequilibrated with phosphate-buffered saline and the enzyme sample (1 ml), containing in addition other protein markers, was applied to it. Absorbance at 280 nm as well as enzymatic activity were determined in the effluent fractions (5 ml). The molecular weight of the enzymes was calculated from the plot of log molecular weight vs. effluent volumes. Marker for the void volume was dextran blue 2000.

DEAE-cellulose chromatography for the separation of the polypeptide chain constituents of hexosaminidases was performed in 0.01 M sodium phosphate buffer, pH 6.0, containing 8 M urea. Elution from the column was performed with the same buffer containing 0.5 M NaCl.

Enzymatic Assay. Enzymatic activity of hexosaminidase was determined with the fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as described previously (Geiger et al., 1974). One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of methylumbelliferone/minute in the assay condition.

Results

Purification and Gross Chemical Characterization of Human Hexosaminidases A and B. Hexosaminidases A and B were purified to a homogeneous state according to the procedure described under Methods. The various stages in the purification of the isozymes and their separation from each other, as detected by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, are shown in Figure 1. The purity of the final enzyme preparations was established also by polyacrylamide gel electrophoresis without sodium dodecyl sulfate, showing one protein band coinciding with the band stained for enzymatic activity (Geiger et al., 1975), as well as by sedimentation velocity experiments in the analytical untracentrifuge, in which a symmetrical peak was observed.

TABLE II: Determination of Free Sulfhydryl Groups in Hexosaminidases A and B, Using Ellman's Reagent.[a]

Isozyme	mol of Enzyme/Sample	A_{412}	mol of SH[b]/Sample	mol of SH/mol of Enzyme
Hex A	2.72×10^{-9}	0.087	6.27×10^{-9}	2.30
	5.54×10^{-9}	0.190	13.69×10^{-9}	2.47
Hex B	3.04×10^{-9}	0	0	0
	6.08×10^{-9}	0	0	0

[a] Hexosamidase abbreviated as Hex. [b] The E_{412} for this calculation was 13 600.

TABLE III: Determination of Free Sulfhydryls and of Disulfides in Hexosaminidases A (Hex A) and B (Hex B) Assayed With ^{14}C-Labeled Iodoacetic Acid.

	Hex A (1.7×10^{-9} mol)		Hex B (2.2×10^{-9} mol)	
	net cpm/Sample ±SD[a]	mol of SH/mol of Enzyme	net cpm/Sample ±SD	mol of SH/mol of Enzyme
Free sulfhydryl[b]	2 048 ± 155	1.45	398 ± 37	0.22
Disulfides[c]	17 018 ± 1408	12.05	35 226 ± 206	19.27
Total[d]	17 957 ± 380	12.71	35 757 ± 708	19.56

[a] SD, standard deviation. [b] Free sulfhydryls were determined by alkylation of denatured enzymes with ^{14}C-labeled iodoacetic acid without reduction. [c] Disulfides were determined by alkylation of the denatured enzyme with cold iodoacetic acid (1 mM), reduction with DTT (20 mM), and subsequent alkylation with the radioactive iodoacetic acid (8.31×10^{11} cpm/mol). [d] Total content of free and bound SH groups was calculated from the radioactivity associated with the enzyme after complete reduction and alkylation with [^{14}C]iodoacetic acid.

The overall purification was 5800 for hexosaminidase A and 5100 for the B isozyme, with a total yield of 25–40%. The specific activities of hexosaminidases A and B in our assay system was 23.3 U/mg and 22.6 U/mg, respectively. The lyophilized enzyme preparations had absorbancy values at 280 nm of 1.40 and 1.21 for 1 mg/ml solutions of hexosaminidases A and B, respectively.

Amino acid composition of the two isozymes is given in Table I. The results are expressed both in mole percent and as the calculated number of residues of each amino acid in the whole protein molecule. Based on our results, which will be presented below, a value of 100 000 was taken as the molecular weight of hexosaminidase A, and 110 000 as that of hexosaminidase B.

Sialic acid content in both isozymes is also given in Table I. For its determination, samples of hexosaminidases A and B, in concentrations of 5.8 mg/ml and 4.7 mg/ml, respectively, were subjected to partial acid hydrolysis for 1 h. Preliminary study showed that both shorter and longer hydrolysis resulted in lower values for the sialic acid, due to either incomplete hydrolysis, or its partial destruction. As can be seen from Table I, 1.65 moles of sialic acid were found per mole of hexosaminidase A, whereas no sialic acid can be demonstrated in hexosaminidase B.

Determination of free sulfhydryl groups in hexosaminidase was performed by Ellman's reaction: solutions of hexosaminidases A and B (0.32 ml) at concentrations of 1.7 mg/ml and 2.1 mg/ml, respectively, were added to 0.64 ml of 7.5 M guanidine-HCl in 0.01 sodium phosphate buffer, pH 8.0 (or to the same buffer without the denaturant). Twenty microliters of Nbs_2 (4 mg/ml) was added and the absorbance at 412 nm determined within 3–5 min. As can be seen from Table II, in the presence of guanidine, 2.3–2.4 mol of SH could be demonstrated per mole of hexosaminidase A, whereas no free SH whatsoever could be detected in hexosaminidase B. The reaction did not proceed at all in the absence of denaturant. Determination of free SH in the presence of another potent denaturant, namely sodium dodecyl sulfate, gave lower values, but since it was documented that detergents may slightly interfere (8%) with the Ellman's reaction (Wang and Volin, 1968) the results with guanidine are considered more reliable. The sensitivity of the method enables us to state that hexosaminidase B does not contain any demonstrable free sulfhydryl (less than 0.1 SH/mole of protein).

Quantitation of both free and S–S linked cysteine was performed using ^{14}C-labeled iodoacetic acid for carboxymethylation. The isozymes A and B of hexosaminidase in concentrations of 1.7 mg/ml and 2.4 mg/ml, respectively were incubated in 0.1 ml with 0.1% solution of sodium dodecyl sulfate for 30 min at 37 °C. For the determination of free sulfhydryls, samples in triplicates were allowed to react for 4 h with 50 mM ^{14}C-labeled iodoacetic acid (8.31×10^{11} cpm/mole). For the exclusive determination of disulfides, samples were alkylated with 1 mM of unlabeled iodoacetic acid, prior to reduction with 20 mM DTT for 60 min at 37 °C under nitrogen atmosphere and subsequent carboxymethylation with the radioactive iodoacetic acid. Total content of SH + S–S was calculated from the protein-bound radioactivity observed when the carboxymethylation was carried out after complete reduction with DTT without previous "cold" alkylation. Controls in which the enzyme was alkylated with 50 mM iodoacetic acid (unlabeled) prior to the addition of the isotope were in the range of 15 and 8% of the experimental values obtained for hexosaminidases A and B, respectively, and these were subtracted from the experimental values. The results are given in Table III. As shown in the Table III, hexosaminidase A contains 6 disulfide bridges and between 1–2 free SH groups, whereas hexosaminidase B contains about 10 disulfide bridges and no detectable free sulfhydryls. These values are somewhat lower than those expected from the total values for cysteine and half-cystine obtained in the amino acid analysis (Table I).

Subunit Structure of Human Hexosaminidases A and B. The molecular weights of the native, pure isozymes were determined by analytical ascending gel filtration on Sephadex G-200 column. Each chromatography was repeated three times with different protein markers including bovine γ-globulin, bovine serum albumin, ovalbumin, heavy and light chains of myeloma MOPC 315, and lyozyme. The results are depicted in Figure 2; in all the experiments hexosaminidase A migrated as a protein with molecular weight of 100 000–103 000, whereas hexosaminidase B had slightly faster migration indicating molecular weight of about 107 000.

The molecular weight of the native enzymes, as well as its subunits and polypeptide constituents, were further studied by sedimentation equilibrium in the analytical ultracentrifuge. The results are described in Figure 3. The size of native hexosaminidases in 0.1 M Tris-HCl buffer, pH 8.2, at 20 °C was found to be about 99 200 daltons for hexosaminidase A and 110 000 for B. Dissociation of the isozymes to their subunits was performed by exhaustive dialysis against 5 M guanidine-HCl in 0.1 M Tris-HCl, pH 8.2. The first dialysis (against 100

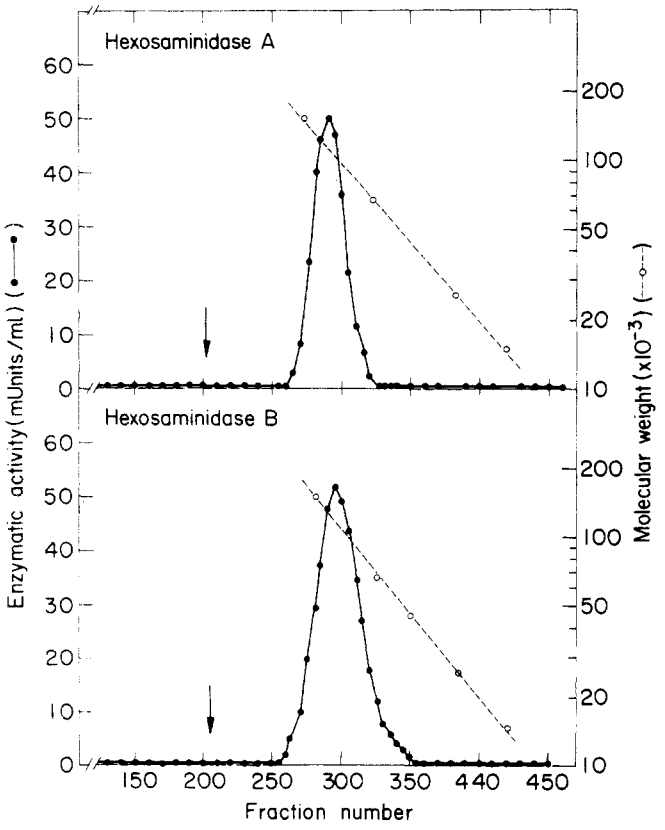

FIGURE 2: Analytical gel filtration of hexosaminidases A and B on Sephadex G-200. The protein markers used for calibration of these columns (in decreasing order of molecular weights) were: bovine γ-globulin, bovine serum albumin, ovalbumin, immunoglobulin light chain, and lysozyme.

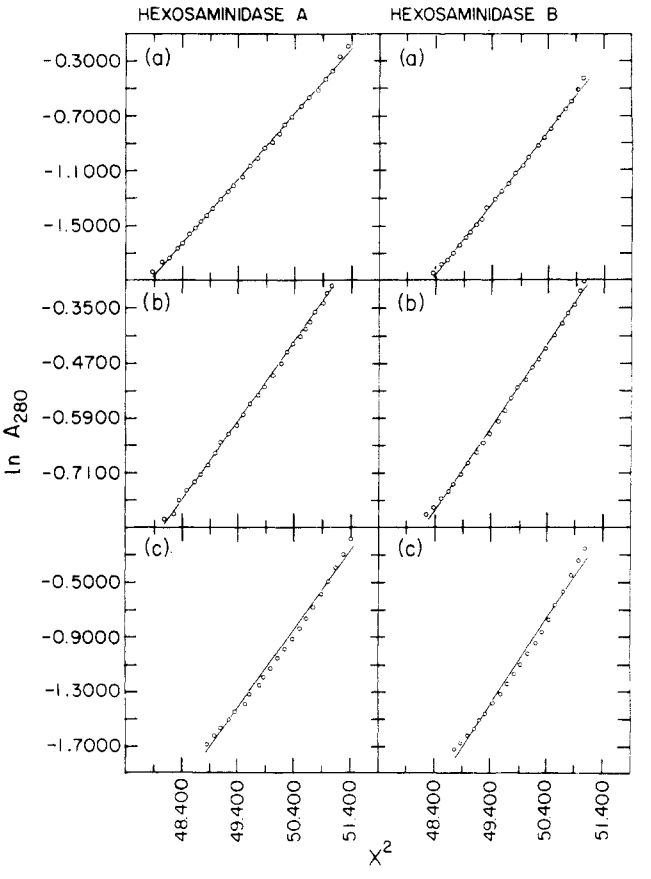

FIGURE 3: Sedimentation equilibrium of hexosaminidase A and B in the analytical ultracentrifuge. The inserted numbers denote the calculated molecular weights ±SD (standard deviation). The $\bar{v}$ value assumed for the calculations was 0.728. (a) Intact enzyme in 0.1 M Tris-HCl buffer, pH 8.2, run at 9000 rpm. (b) Samples obtained after dialysis against 5 M guanidine-HCl in Tris buffer, containing 10 mM iodoacetic acid, and subsequent exhaustive dialysis against 5 M guanidine solution, run at 9000 rpm. (c) Enzymes reduced with DTT (10 mM) in 5 M guanidine-HCl and alkylated at pH 8.2 with iodoacetic acid (25 mM). After alkylation the enzymes were dialyzed exhaustively against 5 M guanidine-HCl in 0.1 M Tris-HCl buffer, pH 8.2, run at 24 000 rpm.

volumes) was performed in the presence of 10 mM iodoacetic acid to prevent possible aggregation. Sedimentation equilibrium at 9000 rpm and at 14 000 rpm of both isozymes gave linear plots of ln OD vs. X^2 (Figure 3). The results indicated dissociation of both isozymes into subunits with about half the molecular weight of the original native enzyme.

Separation of the polypeptide constituents of the enzymes was achieved by full reduction with 10 mM DTT for 4 h under atmosphere of nitrogen at 45 °C and subsequent alkylation with 25 mM iodoacetic acid at pH 8.2 (until negative reaction with Nbs_2 was observed). The solutions were dialyzed against 10^4 volumes of guanidine-HCl (5 M in 0.1 M Tris buffer, pH 8.2) and centrifuged at 24 000 rpm. The results show that each subunit of about 50 000 daltons can be split by reduction into two polypeptide chains with a size of about 25 000, suggesting that the two polypeptides which build each subunit are linked by S–S bridge or bridges. Corroborating data concerning the molecular weight of the subunits of hexosaminidase were obtained in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Dissociation of the enzyme into its individual polypeptide chains was achieved by reduction prior to the electrophoresis with β-mercaptoethanol (0.5 M) or with DTT (5 mM), whereas for separation of subunits which are not covalently bound, no reducing agent was applied. The results given in Figure 4 show that the nonreduced enzyme migrates as one band, the molecular weight of which is about 50 000 (calibrated with protein markers of known molecular weight including γ-globulin). Upon addition of reducing agent, the 50 000-dalton band dissociated into 25 000-dalton chains (Figure 4). The degree of dissociation into the 25 000-dalton unit was dependent on DTT concentration, and reached completion only in 20 mM DTT. The results in Figure 4 represent dissociation in 5 mM DTT and, therefore, show only partial formation of the dissociated polypeptide chains. This was found for both hexosaminidases, though hexosaminidase A started to dissociate in lower DTT concentrations than hexosaminidase B.

It should be mentioned, however, that upon aging, samples of hexosaminidase B aggregated and gave in sodium dodecyl sulfate gel (without reduction) a band corresponding to protein of 100 000 daltons or bigger (migrated between rabbit muscle glycogen phosphorylase b and bovine γ-globulin). Additional information concerning this phenomenon will be discussed later.

Characterization of the Separated Polypeptide Chains of Hexosaminidases A and B. Separation of the dissociated polypeptide chains was carried out by ion-exchange chromatography on a DEAE-cellulose column in 8 M urea. For that purpose, the samples of the enzymes, reduced with 40 mM DTT and alkylated with iodoacetamide in 6 M guanidine-HCl, were gradually dialyzed against 8 M urea in 0.01 M sodium phosphate buffer, pH 6.0, before the application to the column (the complete removal of the guanidine was ascertained by conductivity measurements). The unadsorbed material was eluted with the equilibrating buffer and the adsorbed material

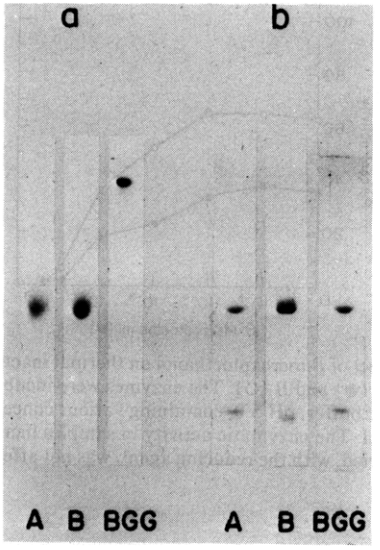

FIGURE 4: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of hexosaminidase A (A) and B (B) as well as bovine γ-globulin (BGG). (a) Nonreduced protein samples; (b) samples reduced in 5 mM DTT.

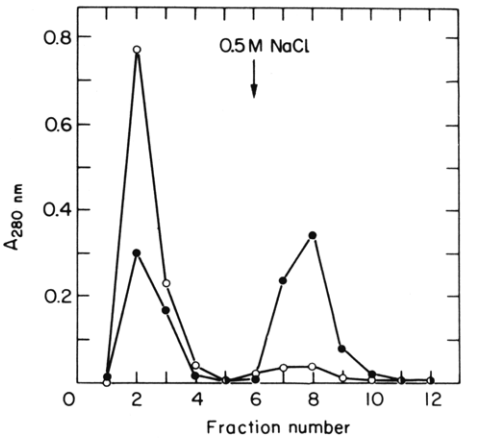

FIGURE 5: Separation of polypeptide chain constituents of hexosaminidase A (●) and B (○). The enzymes were reduced and alkylated in 5 M guanidine-HCl, dialyzed against 7 M urea in 0.01 M phosphate buffer, pH 6.0. The chains that absorbed to the column were eluted with 0.5 M NaCl.

was subsequently eluted with 0.5 M NaCl in the same buffer, or by a salt gradient with this buffer. As shown in Figure 5, the reduced and alkylated hexosaminidase A was separated into two peaks, one basic and one acidic, which exist in almost equal amounts. Hexosaminidase B, on the other hand, gave only one peak corresponding to the basic peak of the reduced and alkylated A isozyme. This peak contained about twice the amount of protein found in the basic peak obtained from an equal amount of hexosaminidase A. Using the terminology suggested previously by Beutler and Kuhl (1975) we denoted the basic polypeptide chain β and the acidic α. Amino acid analysis was performed on the separated α and β chains, after extensive dialysis to remove the urea. (Some precipitation occurred during the dialysis even though Triton X-100 was added.) Table IV gives the observed results of the amino acid analysis, alongside with the expected values for separate α and β chains, based on the assumption that hexosaminidase B contains four β chains and hexosaminidase A contains two β chains and two α chains, and calculated from the amino acid compositions of the intact isozymes (Table I). Due to the low levels of cysteine (as carboxymethylcysteine) and methionine, these amino acid could not be determined precisely in our samples, and therefore were not included in the table. As evident from the table, a good agreement was found between the expected values for the α and β chains, and the values obtained experimentally.

Physicochemical Studies with Hexosaminidases A and B. A circular dichroism spectrum was recorded for hexosaminidases A and B as seen in Figure 6. The spectrum of each isozyme did not change when the enzyme was in different buffers within the pH range of 4–8; moreover, no gross change could be observed upon addition of potent enzyme inhibitors like *N*-acetylglucosamine or *N*-acetylgalactosamine. As shown in Figure 6, identical spectrum for the A and B isozyme was found in the 210–230-nm region indicating identical or very similar α-helix content in both. On the other hand, differences were observed in the region corresponding to the optical activity of the aromatic chromophores, namely tryptophan, tyrosine, and phenylalanine.

Further studies were carried out on the changes in the conformation of the enzymes during various procedures of dena-

TABLE IV: Amino Acid Composition of Separated α and β Polypeptide Chains of Hexosaminidases.

	mol of Amino Acid/Chain[a]				
	Expected[b]		Found		
Amino acid residue	β Chain	α Chain	Hex B β Chain	Hex A β Chain	Hex A α Chain
Lysine	13.4	10.0	13.2	15.4	10.3
Histidine	7.9	4.6	7.4	7.6	4.3
Arginine	10.9	9.4	11.3	11.6	9.2
Aspartic acid	22.7	22.6	22.9	23.5	22.7
Threonine	14.2	9.3	14.3	12.9	9.6
Serine	20.3	15.3	22.2	20.3	15.5
Glutamic acid	21.6	29.2	22.0	22.8	26.8
Proline	14.6	15.8	15.2	15.6	16.4
Glycine	15.5	19.2	17.0	17.1	20.4
Alanine	13.1	12.5	14.5	13.8	13.4
Valine	11.9	15.9	13.4	12.5	15.7
Isoleucine	9.7	8.2	10.6	10.5	9.4
Leucine	21.8	26.7	23.3	21.9	25.6
Tyrosine	12.0	7.5	11.9	11.5	8.2
Phenylalanine	13.2	11.1	13.5	12.7	11.9

[a] For this calculation β chains were estimated as 27 000 daltons, the size of α chain used here was 25 000 daltons. [b] The expected values for the amino acid composition of the separated chains were calculated from the analysis of the intact enzymes, assuming $\beta_2\beta_2$ structure for hexosaminidase B and $\beta_2\alpha_2$ for the A isozyme.

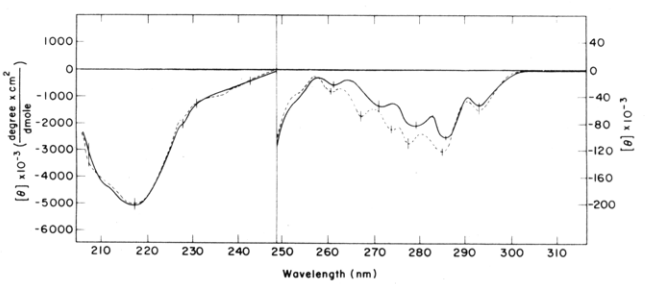

FIGURE 6: Circular dichroism spectrum of pure hexosaminidases A (- - -) and B (—) in 0.1 M phosphate buffer, pH 6.0.

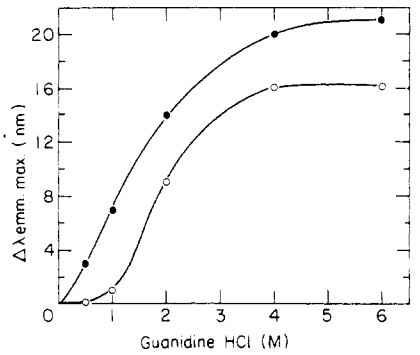

FIGURE 7: The shift in the fluorescence emission peak of hexosaminidases A (●) and B (○) upon addition of guanidine-HCl. The peak of emission of both untreated enzymes in 0.1 M Tris-HCl, pH 8.2, was 338 nm.

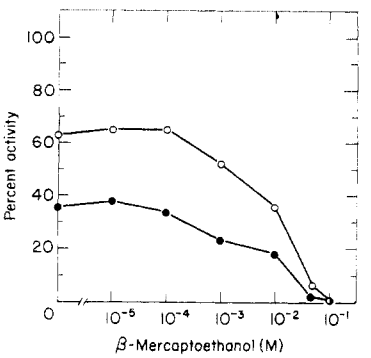

FIGURE 8: Effect of β-mercaptoethanol on thermal inactivation of hexosaminidases A (●) and B (○). The enzymes were incubated for 1 h at 55 °C, in citrate buffer, pH 5.0, containing various concentrations of β-mercaptoethanol. The enzymatic activity in samples incubated at 0 °C for the same period, with the reducing agent, was not affected (less than 5%).

TABLE V: Inactivation of Hexosaminidase A (Hex A) and B (Hex B) With Guanidine-HCl or Urea.

	% Inactivation[a]			
	Urea		Guanidine-HCl	
Denaturant Concn (M)	Hex A	Hex B	Hex A	Hex B
0	0	0	0	0
2	2	4	16	24
4	0	2	100	100
6	4	4	100	100
8	9	6	-	-

[a] The percent inactivation was calculated from the residual activity after treatment with the denaturant and subsequent 1:1000 dilution with denaturant free reaction buffer.

turation or inactivation. In fluorescence measurements, enzyme solutions (0.07–0.08 mg/ml final concentration) were incubated with various concentrations of guanidine-HCl in Tris buffer, pH 8.2. The excitation wavelength was 278 nm (which was found to be the peak of absorbance of both enzymes) and the emission spectrum in the range 280–400 nm was recorded. Upon addition of guanidine-HCl a concentration-dependent red shift was observed for both enzymes (Figure 7), but hexosaminidase B required higher denaturant concentrations for the full effect, thus suggesting that conformational changes accompanied by changes in the environment of the relevant chromophores, mainly tryptophan, are induced more easily in hexosaminidase A (when compared to B). It is noteworthy that the red shift in hexosaminidase B was accompanied by about 30% increase in the quantum yield, whereas no such increase, and even a small decrease, was found for hexosaminidase A.

Treatment with urea in concentration of up to 8 M caused only relatively small changes in λ emission maximum. In order to correlate the structural and denaturation profiles of the isozymes with the enzymatic activity, the effect of urea and both guanidine-HCl and sodium dodecyl sulfate on the catalytic activity of both isozymes was studied. Pure enzymes were diluted in phosphate buffer, pH 6.0, containing 0.1% bovine serum albumin (hexosaminidase free) and increasing concentrations of urea or guanidine-HCl. Following 60-min incubation at room temperature, the enzymes were diluted at least 1000-fold (a dilution in which the denaturant is not inhibitory at all) with the reaction buffer, and the activity was assayed (Table V). The results show that treatment with guanidine-HCl causes irreversible inactivation that is dependent on the denaturant concentration and reaches completion at 4 M solution. The effect of urea, on the other hand, was reversible, and even in samples treated with 8 M urea over 90% of the original activity was restored. When urea was present in the reaction mixture during the enzyme assay, almost complete inhibition of both isozymes occurred at concentrations of 1–1.5 M and over.

Sodium dodecyl sulfate was found to be an effective irreversible inactivator of the enzyme, but since this detergent is known to bind proteins strongly, it is difficult to evaluate the mechanism of inactivation by sodium dodecyl sulfate in terms of conformational changes in the enzyme molecule. It should be noted that sodium dodecyl sulfate as well as guanidine-HCl brings about dissociation of hexosaminidase isozymes into subunits, an effect which does not take place in the presence of 8 M urea.

A possible mechanism of another inactivation procedure, namely heat inactivation, was studied in a different experiment; hexosaminidase A (the heat-labile isozyme) and hexosaminidase B were diluted to about 0.2 munits/ml in citrate buffer, pH 5.0, containing 0.1% BSA, and were heated for 1 h at 55 °C in the presence of various concentrations of β-mercaptoethanol, ranging from 10^{-5}–10^{-1} M. Identical enzyme samples were kept in an ice bath during the incubation period. At the end of the heat-inactivation step, substrate was added and the activity was determined. The reducing agent, even in the highest concentrations (10^{-1} M), had only a very small effect on the enzymatic activity; thus, hexosaminidases A and B, which were treated with 0.1 M β-mercaptoethanol and kept in ice, retained 93.7 and 96.2% of the original activity. The heat inactivation, on the other hand, was dramatically amplified by the reducing agent as shown in Figure 8.

Discussion

The extensive purification of hexosaminidases A and B to a high degree of homogeneity enabled the chemical characterization of both isozymes. This molecular characterization included determination of the amino acid composition, the contents of sialic acid, the presence of disulfide bridges, and free sulfhydryl groups in both isozymic forms of the enzyme. Moreover, the purified enzyme preparations lended themselves to reliable determination of molecular weight of the native enzyme, as well as of the dissociated subunits and polypeptide chains. Whereas hexosaminidase B yielded only one species of dissociated polypeptide chains, two species were obtained from hexosaminidase A, one of which was identical with that

present in the B isozyme.

In view of these findings we have used the terminology suggested by Beutler and Kuhl (1975), namely, we denote the two species of polypeptide chains as α and β, respectively, and we suggest herewith a molecular model for the enzymes, as depicted in Figure 9. In the following, we shall consider the experimental results presented in this communication and discuss them in relation to their compatibility with the suggested model.

The molecular weight obtained for hexosaminidase A is about 100 000, whereas hexosaminidase B appeared slightly larger, about 108 000. In both cases a good agreement was found between the values obtained by ultracentrifugal analysis and gel filtration. These values for molecular weights are in accord with values reported previously by Verpoorte (1974), but they are lower than the values of around 130 000 obtained by gel filtration in other studies (Sandhoff and Wässle, 1971; Tallman et al., 1974). It should be noted, however, that Srivastava and his colleagues (1974) also obtained by gel filtration a molecular weight of 140 000 but their sedimentation-equilibrium experiments led to a value of 100 000 which according to their own conclusion is more plausible. This molecular weight is in agreement also with the values obtained by us for the subunits and dissociated polypeptide chains (Figures 3 and 4).

Dissociation without reduction resulted in two subunits for each isozyme, with a molecular weight of 50 000. This dissociation occurred in sodium dodecyl sulfate and in 5 M guanidine but not in 8 M urea. These subunits could be further dissociated only after reduction and alkylation. In this case only one species of polypeptide chain was obtained from hexosaminidase B, whereas hexosaminidase A yielded two species (Figure 5), one of which is identical with the chain obtained from the B isozyme. This is in accord with the suggested model of $\beta_2\beta_2$ for hexosaminidase B and $\alpha_2\beta_2$ for hexosaminidase A. These results corroborate the data of Tallman et al. (1974) who obtained dissociation of both A and B isozymes into monomeric subunits following reduction, but the dimeric subunits without reduction. The phenomenon of nondissociative hexosaminidase B reported previously by these authors was also noticed by us when aged preparations of the enzymes were used, but not with freshly prepared enzyme, which dissociated readily.

The number of disulfide bridges, as determined by reduction and alkylation with radioactive iodoacetic acid, is different for the A and B isozymes. According to the model it is implied that five disulfide bridges (inter- or intrachain) are present in each β_2 subunit, whereas only one disulfide bridge connects the two α chains in the α_2 dimer. In addition, each α chain contains one free sulfhydryl group that is absent in the β chain. Whether this SH group contributes to the particular physicochemical and enzymatic properties of hexosaminidase A is too early to determine.

The sialic acid content of the two isozymes is different—whereas no such residues are found in hexosaminidase B, there are on the average 1.65 residues in the A isozyme, implying one residue per α chain. These data correlate well with the more acidic character of hexosaminidase A, but they do not come up to the high values suggested originally by Robinson and Stirling (1968) to account for the conversion of the A to the B isozyme.

The difference in the amino acid compositions between the two isozymes may also point to a slightly higher acidity of hexosaminidase A, even though this is not conclusive due to our incomplete discrimination between glutamic and aspartic acids and their corresponding amides. It should be noted that

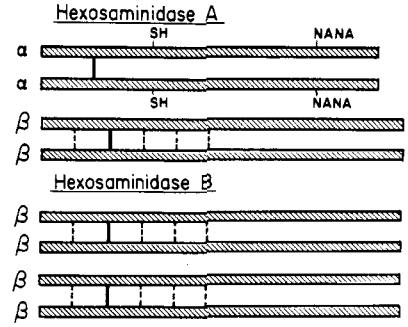

FIGURE 9: Suggested molecular structure of hexosaminidase A and B. Solid lines connecting two chains represent S–S bridge, broken lines represent either intra- or interchain S–S bridges. In the α chains of hexosaminidase A, in addition to the interchain S–S bridge, one sulfhydryl group (SH) and one sialic acid residue (NANA) are present.

the values for cysteic acid obtained after performic acid oxidation are indeed higher for hexosaminidase B than the values for hexosaminidase A, in accord with the analyses of disulfide bonds. The definite difference in amino acid composition rules out the suggestion of Tallman et al. (1974) that the two isozymes are conformers. The high degree of purity of our enzyme preparations and the good reproducibility of results with six independent determinations of three different preparations of each isozyme give good confidence to the validity of these data. It should be mentioned that the amino acid composition reported here is somewhat different from those reported by Beutler and Srivastava (1973) and by Srivastava et al. (1974), or by Verpoorte (1974). These differences might be due to variations in degree of purity of the enzyme preparations.

The most instructive information that can be obtained from the amino acid analyses are the results for the composition of the separated polypeptide chains. In this case it is evident from the data that the isolated β chains have an identical molar residue ratio to those found for the intact hexosaminidase B. The observed composition of the isolated α chain is, likewise, in a very good agreement with the expected calculated values (Table IV). The distinct differences in amino acid compositions between the two isolated chains is a conclusive proof that these are two different species. These findings are therefore compatible with our suggested model and this also rules out the possibility raised by Beutler and Srivastava (1973) that hexosaminidase B is composed of two different polypeptide chains denoted β and γ, whereas hexosaminidase A consists of α and β chains.

The model described above fits well with the data obtained from immunological studies that demonstrate that hexosaminidase A bears all the antigenic determinants present in hexosaminidase B and, in addition, unique determinants which can lead to specific antiserum reactive exclusively with hexosaminidase A (Beutler and Srivastava, 1973; Ben-Yoseph et al., 1975).

The relationship between manifestation of hexosaminidases and several genetic disorders has prompted in several laboratories studies with somatic cell hybrids of human and murine or hamster cells. From such studies it was concluded that the two isozymes require different chromosomes for their expression (Gilbert et al., 1974a,b), and that hexosaminidase A is never expressed in the absence of hexosaminidase B (Lalley et al., 1974). These results would indeed be anticipated assuming that hexosaminidase A contains both α and β chains, whereas hexosaminidase B contains only β chains, and therefore can be expressed independently of hexosaminidase A.

The subunit structure suggested here can explain also the

long recognized data about the unidirectional conversion of hexosaminidase A to B under appropriate conditions (e.g., Robinson and Stirling, 1968; Tallman et al., 1974; Carmody and Rattazzi, 1974). It might be envisaged that in these cases dissociation has taken place between the α_2 and β_2 subunits, with subsequent reassociation to form partly a $\beta_2\beta_2$ complex, namely hexosaminidase B. This type of association is probably thermodynamically favored under these particular conditions to other reassociations such as $\alpha_2\alpha_2$ or back to $\alpha_2\beta_2$. These results were corroborated recently by the very elegant experiments of Beutler and Kuhl (1975) that demonstrate that repeating freezing and thawing cycles of hexosaminidase A on the one hand and of mixtures of hexosaminidases B and S on the other hand gave rise to all three molecular species. The same mechanism is probably operative in this case. This procedure, namely freezing and thawing, is known to cause the dissociation of noncovalently bound subunits in proteins (Chilson et al., 1965; Markert, 1963).

A major point of interest in the studies on hexosaminidase is the elucidation, on a molecular level, of the difference between the A and B isozymes. It was known for a long time that hexosaminidase A is much more sensitive than the B form towards physical inactivation by heat (O'Brien et al., 1970) or acid (Saifer and Rosenthal, 1973). In all the experiments reported in this paper, which utilized various physicochemical techniques, hexosaminidase A appeared more liable to undergo molecular changes than hexosaminidase B. For example, heat inactivation is amplified by very mild reducing conditions; similarly, the red shift of the fluorescence observed upon addition of guanidine-HCl to both enzymes was more prominent for the A isozyme than for the B. These findings, in addition to the observation that heat inactivation is extremely dependent on the enzyme concentration (R. Navon, personal communication), support the assumption that this phenomena may be due to dissociation, and prevention of subsequent reassociation.

The interest in human hexosaminidases arose mainly due to its relevance to several genetic disorders resulting in lipid storage diseases, including Tay-Sachs disease, Sandhoff-Jatzkewitz disease, and others. The isozyme pattern manifestated in these diseases can be explained in the light of the suggested molecular structure of the hexosaminidases. Patients with the classical form of Tay-Sachs disease do not synthesize α chains and hence have no hexosaminidase A. Patients with Sandhoff's disease do not synthesize the β chain and, therefore do not have any hexosaminidase A or B, but manifest small amount of the recently described hexosaminidase S which probably contain only α chains, $\alpha_2\alpha_2$ (Beutler et al., 1975; Ikonne et al., 1975). Moreover, hybridization experiments between Tay-Sachs and Sandhoff's disease cells showed complementation in the de novo production of hexosaminidase A (Galjaard et al., 1974; Thomas et al., 1974; Rattazzi et al., 1974) that can be accommodated with the hypothesis of an association of subunits α_2 and β_2 originating from the different cells. The interesting variant originally described by Navon et al. (1973) and that was shown to contain only about 5% of hexosaminidase A (Navon et al., 1975) may be defective either in the synthesis of α chains or in the mechanism of association of the subunits to form the intact hexosaminidase molecule. These pathological conditions are therefore compatible with the subunit model we suggest.

Acknowledgments

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Synergistic Activation of Adenylate Cyclase by Guanylyl Imidophosphate and Epinephrine†

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ABSTRACT: A kinetic analysis of the synergistic activation of turkey erythrocyte adenylate cyclase by 1-catecholamines and guanylyl imidodiphosphate (Gpp(NH)p) is described. We have found that the role of the catecholamine hormone is to facilitate the activation of the enzyme by the guanyl nucleotide according to the following mechanism:

$$R \cdot E + G \rightleftharpoons R \cdot EG$$

$$R \cdot EG + H \rightleftharpoons HR \cdot EG \xrightarrow{k} HR \cdot E''G$$

where R is the receptor, E the enzyme, G the guanyl nucleotide effector, and H the hormone. The binding steps are fast and reversible but the conversion of the inactive enzyme E to its active stable form (E″) occurs with a rate constant of $k = 0.7$ min^{-1}. This step is essentially irreversible in the presence of high Gpp(NH)p concentrations. In the absence of β-agonist (1-catecholamine) and at low free Mg^{2+} concentrations, the activation of the enzyme is insignificant. At high Mg^{2+} concentration the conversion of E to E″ occurs slowly in the absence of hormone, probably by another pathway. Thus, the presence of a guanyl nucleotide at the allosteric site is obligatory but not sufficient to induce the conversion of the inactive enzyme to its active form. The process of enzyme activation requires both Gpp(NH)p and hormone and under these conditions is essentially irreversible. The permanently active enzyme is stable in the absence of hormone and Gpp(NH)p and its high catalytic activity is stable for many hours. However, hormone and ATP induce a conversion of the high activity to the low activity form. Thus, it seems that both the process of enzyme activation by Gpp(NH)p and its reversal are hormone dependent. Both processes are blocked by the β-blocker propranolol.

Recently it was demonstrated that GTP[1] plays a key role as a regulatory ligand in the action of hormone-activated adenylate cyclases (Rodbell et al., 1971a,b; Londos et al., 1974). It has also been shown that the GTP analogues Gpp(NH)p and to a lesser extent $Gpp(CH_2)p$ are superior to GTP in stimulating hormone-activated adenylate cyclases (Londos et al., 1974, and references therein). It was suggested that the activation of adenylate cyclases is brought about by the synergistic action of Gpp(NH)p and hormone, namely that the activation of adenylate cyclase by hormone and Gpp(NH)p when present together have a greater combined stimulatory effect than the sum of their individual effects. This synergistic effect is seen with GTP analogues stable to phosphotransferase reactions such as Gpp(NH)p and $Gpp(CH_2)p$. Rodbell and his group demonstrated that the role of the hormone is to facilitate the rate of adenylate cyclase activation by guanine nucleotides (Rodbell et al., 1974) in a number of adenylate cyclases from different sources. More recently, Rodbell and his group proposed a generalized model for the interrelationships between hormone and guanine nucleotides in their action as activators of adenylate cyclases (Salomon et al., 1975; Lin et al., 1975; Rendell et al., 1975).

Similar to many other hormone-stimulated adenylate cyclases (Londos et al., 1974), it was demonstrated that 1-epinephrine stimulated adenylate cyclase from nucleated erythrocytes is also activated by Gpp(NH)p (Bilezikian and Aurbach, 1974; Schramm and Rodbell, 1975; Pfeuffer and Helmreich, 1975). We have studied in some detail the interaction between the hormone binding site and the guanyl nucleotide regulatory site to gain a better understanding on the

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[1] Abbreviations used are: Gpp(NH)p, guanylyl imidodiphosphate; GTP, GDP, guanosine tri- and diphosphate; ATP, adenosine triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.