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Specific Determination of *N*-Acetyl-β-D-hexosaminidase Isozymes A and B by Radioimmunoassay and Radial Immunodiffusion

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The two major isozymes of *N*-acetylhexosaminidase, namely hexosaminidases A and B were quantitatively determined in tissues and biological fluids of both normal individuals and Tay-Sachs patients. The determination was carried out by two sensitive immunoassays: radial immunodiffusion, using chromogenic substrate, and radioimmunoassay, which were developed in this study. For this pupose we used either a cross-reactive antiserum which reacts to a similar extent with both isozymes, or an antiserum reacting exclusively with hexosaminidase A (obtained by selective immunoadsorption). This enabled the quantitisation of the two isozymes separately, or in the presence of each other, in purified enzyme preparations or in tissue homogenates, affording a direct positive determination of hexosaminidase A. The results demonstrated that normal tissues contain the two isozymes in comparable amounts, whereas tissues of Tay-Sachs patients lack hexosaminidase A or any material which carries the A-specific antigenic determinants. The possible applications of these assays and their potential use in diagnosis are discussed.

N-Acetyl-β-D-hexosaminidase (here abbreviated hexosaminidase) is an enzyme that hydrolyzes the terminal *N*-acetylglucosaminyl or *N*-acetylgalactosaminyl moieties from other sugars. Its natural substrates are various glycolipids and polysaccharides, but its activity is conveniently assayed using synthetic substrates, in which the amino sugar is linked to a chromophoric or fluorophoric group. The enzyme exists in human tissues in two major isozymic forms, A and B, which differ from each other mainly in their electrophoretic mobility, A being more acidic than B, as well as in their thermal stability, B being more stable [1]. There are several inherited disorders, manifested mainly as lipid storage diseases, which are characterized by the absence of either one or both of hexosaminidase isozymes, *e.g.* Sandhoff's disease, in which both isozymes are missing [2] and Tay-Sachs disease, in which only the basic isozyme, B, is present [3]. The conclusion about the absence of hexosaminidase A in Tay-Sachs tissues was drawn both from the absence of enzymic activity in the electrophoretic region corresponding to hexosaminidase A and from the absence of the heat-labile form [4]. These findings serve today as the basis for the diagnosis of Tay-Sachs disease, by heat-inactivation assay on leucocyte extract, serum, or other biological fluids of suspected patients [4–6].

The absence of hexosaminidase A in Tay-Sachs tissues was observed also by the use of an immunological approach. Thus, Srivastava and Beutler [7] reported that no material cross-reacting with a specific anti-hexosaminidase A serum was present in Tay-Sachs liver. Bartholomew and Rattazzi [8] reached the same conclusion in an indirect way by using Tay-Sachs tissue for absorption, they obtained specific antiserum towards hexosaminidase A, whereas all anti-hexosaminidase B antibodies were absorbed. In view of the above, it seemed worthwhile to develop more sensitive assays for the determination of both A and B isozymes, for use in either biochemical studies of hexosaminidase, or in immunodiagnosis of storage disorders.

We wish to report the development of two most sensitive assays, namely radial immunodiffusion and radioimmunoassay, for the quantitative determination of both hexosaminidase A and B, either separated or in the presence of each other.

Enzyme. *N*-Acetyl-β-D-hexosaminidase or β-2-acetamido-2 deoxy-D-glucoside acetamidodeoxyglucohydrolase (EC 3.2.1.30).

MATERIALS AND METHODS

Materials

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (U.S.A.). Sephadex G-25, Sephadex G-50 and Sepharose 4B were from Pharmacia (Sweden). Concanavalin A (×2 crystallized, in saturated NaCl solution) was obtained from Miles-Yeda (Israel). 4-Methylumbelliferyl-*N*-acetyl-β-D-glucosaminide and methylumbelliferone were from Pierce Chemical Co. (U.S.A.). Naphthol-AS-BI-*N*-acetyl-β-D-glucosaminide and fast garnet GBC were from Sigma (U.S.A.). Agarose was obtained from L'Industrie Biologique Francaise (France), and ^{125}I was from The Radiochemical Centre, Amersham (U.K.).

Lactoperoxidase was purchased from Sigma.

Enzymes

Hexosaminidase A and B were prepared from human placentae and purified by affinity chromatography according to a procedure described previously [9] and consisting of the following steps.

a) The use of Sepharose-bound concanavalin A (J. F. Tallman, personal communication) equilibrated with phosphate-buffered saline for selective absorption of hexosaminidases from crude homogenate (elution was performed with 10% α-methyl glucoside in 0.5 M NaCl).

b) Separation of A and B isozymes on DEAE-cellulose [2].

c) Affinity chromatography on Sepharose-bound ligand 2-acetoamido-*N*-(ε-amino-caproyl)-2-deoxy β-D-glucopyranosylamine).

d) Further purification of hexosaminidase A on CM-cellulose column (0.04 M citrate buffer, pH 4.4, and elution in 0.5 M NaCl), and of hexosaminidase B on Sephadex G-150. The overall purification was 5100-fold for A and 5800-fold for B. Homogeneity of the enzyme preparations was established by sedimentation in a Beckman model E analytical ultracentrifuge equipped with schlieren optics, and by polyacrylamide gel electrophoresis on 5.3% gels according to Hayase [11]. The gels were developed by staining for proteins in 0.25% Coomassie brilliant blue, or by specific staining for enzymic activity with the substrate naphthol-AS-BI-*N*-acetyl-β-D-glucosaminide and fast garnet GBC salt, as described elsewhere [12].

Antisera

Antisera against purified hexosaminidase A and B were raised in goats. 1 mg of each isozyme in phosphate-buffered saline was emulsified with equal volume of complete Freund's adjuvant and injected intradermally at multiple sites. The animals were boosted after 10 days, and bled at 1-week intervals, from the jugular vein. Specific anti-hexosaminidase A IgG fraction was prepared from anti-hexosaminidase A serum by exhaustive absorptions with Sepharose-bound hexosaminidase B [12], and subsequent precipitation with 40% saturated, neutralized $(NH_4)_2SO_4$ solution. The antisera were tested by double diffusion in agar gels against enzyme preparations, as reported previously [12]. Anti-goat IgG was prepared in rabbits by immunization with goat anti-lysozyme purified antibodies.

Enzymic Assays

The enzymic activity of purified or crude preparations of hexosaminidases was assayed with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as described previously [5]. An enzyme unit was defined as the amount of enzyme capable of hydrolyzing 1 μmol of substrate per min.

Heat inactivation studies were performed in citrate buffer, pH 4.4. After determining the total hexosaminidase activity, the enzyme samples were heated for 3 h at 50 °C and the residual activity was then determined [4]. The fraction of residual activity from the total activity is considered to be hexosaminidase B, whereas the heat-inactivated fraction is hexosaminidase A.

Radial Immunodiffusion

Radial immunodiffusion studies were performed in 1.5% agarose gels, in phosphate-buffered saline, following the general procedure of Mancini *et al.* [13]. The various antibody preparations were incorporated in the gels in appropriate concentrations, and 10 ml of antibody-containing agarose were poured onto plastic plates (4.5×9.5 cm). Holes with a diameter of 3 mm were punched, in 10-mm intervals from each other, and 10 μl of enzyme-containing solution were applied to the wells using a precision syringe (Hamilton, 701 N). Diffusion was allowed to proceed for 36 h, following which the plates were rinsed thoroughly with phosphate-buffered saline for 48 h.

The plates were then pre-equilibrated with 0.1 M citrate buffer, pH 4.4, and stained for enzymic activity [12]. The diameter of the stained rings was measured and the net area of the ring (after subtracting the area of the well), was calculated.

Radioimmunoassay

Hexosaminidase A and B were iodinated using the lactoperoxidase method according to Marchalonis [14]. 10 μl of isozyme solution (containing 15 μg protein) were mixed with 10 μl of 0.2 M sodium phos-

phate buffer pH 7.2. Lactoperoxidase (5 µl of 600 µg/ml solution) was added to the mixture, followed by 1 mCi of the radioactive ^{125}I in 10 µl. The iodination was initiated by the addition of 5 µl of H_2O_2 (1:1000 dilution of 30% solution). The reaction proceeded at 37 °C for 30 min with two intermediate additions of H_2O_2. The radioactively labeled enzyme was separated from unbound iodine by gel filtration on Sephadex G-25 (fine). The yield of iodination was 65% for hexosaminidase A and 30% for B. The radioactive enzymes were diluted in phosphate-buffered saline containing 0.1% gelatin and stored at − 20 °C. Binding capacity of ^{125}I-labeled hexosaminidases to antibodies was measured as follows. Serial dilutions of the various antibody preparations were prepared in 1:40 dilution of normal goat serum in phosphate-buffered saline and added in 50-µl amount to plastic tubes (2052, Nunc, Denmark) containing 50 µl of ^{125}I-labeled enzyme. Following 30 min at 37 °C, rabbit anti-goat IgG was added in an amount sufficient for the precipitation of all the goat IgG in the reaction tube. After further incubation for 30 min at 37 °C the tubes were transferred to 4 °C for 16 h. The precipitate was spun down and washed twice with cold phosphate-buffered saline and then dissolved in 1 ml 0.1 M NaOH. The radioactivity was monitored in a Gamma scintillation spectrometer model 3002 (Packard, U.S.A.).

Inhibition studies were carried out in a similar manner, but the labeled isozyme was mixed with 50 µl of unlabeled isozyme solutions, or enzyme-containing biological samples, prior to the addition of the antibodies.

RESULTS

Purified Enzymes and Specific Antisera

The purified hexosaminidase A and B preparations migrated as sharp symmetric peaks in the ultracentrifuge, with an $s_{w,20}$ value of 5.82 and 5.90 S respectively. In polyacrylamide gel electrophoresis each isozyme migrated as a single band, which stained for both protein and enzymic activity (Fig. 1).

Antibodies against both isozymes showed a high extent of cross-reactivity; anti-hexosaminidase B reacted to a similar extent with the two enzymes, whereas anti-hexosaminidase A showed spur formation with hexosaminidase A over hexosaminidase B [12]. The fraction of the antibodies in anti-hexosaminidase A serum, which is reactive exclusively with hexosaminidase A, was isolated by exhaustive absorption of the antiserum with Sepharose-bound crude hexosaminidase B. The specificity of this antiserum was established by double diffusion in agar gel as described previously [12], as well as by its incapacity to bind radioactively labeled hexosaminidase B.

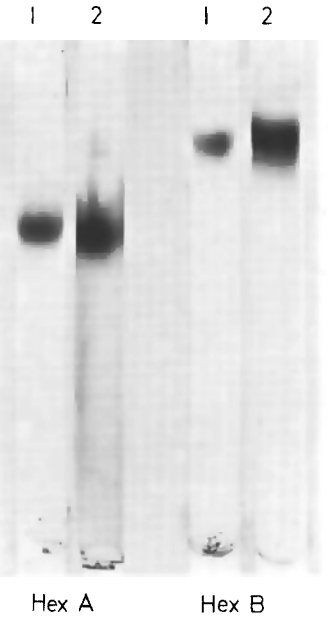

Fig. 1. *Polyacrylamide gel electrophoresis of the purified preparations of hexosaminidases A and B, stained with Coomassie brilliant blue (1), or with the specific substrate naphthol-AS-BI-*N*-acetyl-β-D-glucosaminide and fast garnet GBC salt (2).* The electrophoresis was conducted in 0.09 M Tris-glycine buffer, pH 9.5

Radial Immunodiffusion Assay

The radial immunodiffusion assay was carried out in agarose plates containing either anti-hexosaminidase B serum (1:500), for determination of the accumulated amount of both isozymes, or the IgG fraction (20 mg/ml) of specific anti-hexosaminidase A (1:100), for exclusive determination of hexosaminidase A. Enzymes samples (10 µl) in concentration range of 0.1 − 2 µg/ml were placed in the wells. As seen in Fig. 2, there is a linear relationship between the area of the ring formed and the concentration of the enzyme sample. Substrate-stained rings were obtained with both isozymes in the plates containing anti-hexosaminidase B, whereas in the plates containing specific anti-hexosaminidase A no rings whatsoever were observed with the B isozyme.

Using plates which contain the specific anti-hexosaminidase, it is possible to quantitate hexosaminidase A in the presence of hexosaminidase B. The experiment, shown in Fig. 3, was carried out with enzymes samples containing mixtures of A and B isozymes. The total enzymic activity was identical in all samples, but the fraction of hexosaminidase A in the mixture varied from 0 to 100%. The results were manifested in identical-sized rings for all samples in

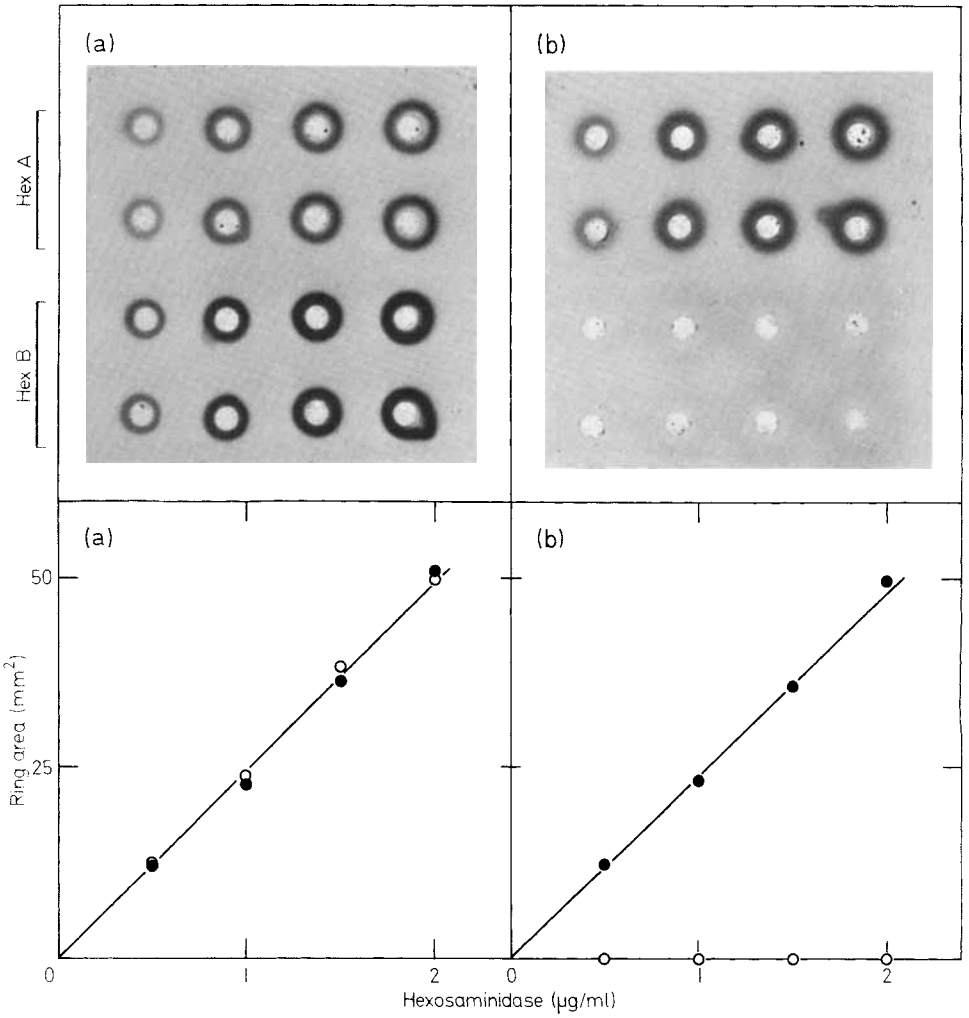

Fig. 2. *Radial immunodiffusion of hexosaminidases A and B, in plates containing, respectively, the cross-reactive anti-hexosaminidase B (a) and the specific anti-hexosaminidase A serum (b).* The plates were stained after washing, with the specific substrate (see text). 10 µl of enzyme solution were placed in each well; the concentration of the enzymes in the different wells (duplicates) is, from left to right: 0.5; 1.0; 1.5 and 2.0 µg/ml respectively. Upper part: Photograph of the plates. Lower part: Correlation between the area of the ring (net area after subtracting the area of the well) and the concentration of hexosaminidases A (●) and B (○)

the plates containing the anti-hexosaminidase B serum, while in the plate containing specific anti-hexosaminidase A a linear relationship was observed between ring area and percentage of A in the mixture.

Radioimmunoassay

The radioimmunoassay was also developed with both whole antiserum to hexosaminidase A, and with specific anti-hexosaminidase A IgG. In both cases the optimal concentration of the antiserum to be used in the assay was determined by antigen binding experiment, using ^{125}I-iodinated hexosaminidase A. The results of the binding assay for both antisera are given in Fig. 4. As emerges from the figure, the optimal dilution of anti-hexosaminidase A serum, that still gave a high extent of binding but was not in antibody excess, was 1:40. In the case of the specific anti-hexosaminidase A, the chosen dilution of the IgG fraction was 1:10.

Using these dilutions of the two sera, respectively, competition was observed between the labeled antigen and known amounts of unlabeled hexosaminidase A and B preparations. The results are depicted in Fig. 5. As seen in Fig. 5a, both A and B competed efficiently and equally with the labeled hexosaminidase A when antiserum to hexosaminidase B was used. On the other hand, when the IgG fraction specific toward the unique specificity determinants of hexosaminidase A was used, no competition was obtained with B, while A was as efficient as in the case when whole anti-hexoaminidase A was used (Fig. 5b). The calibration curves shown in the figure were used for quantitisation of hexosaminidase A and B in various samples.

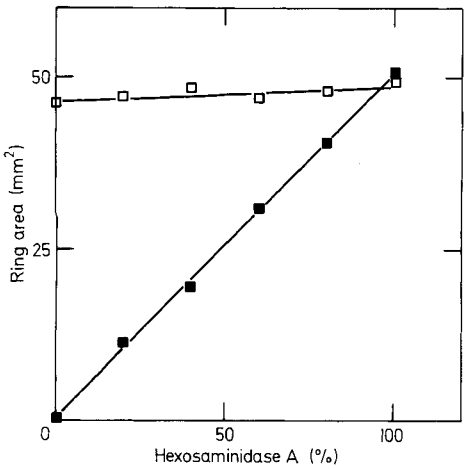

Fig. 3. *Radial immunodiffusion assay for determination of mixtures of hexosaminidases A and B.* All the mixtures contained the same total enzymic activity, but the relative amount of hexosaminidase A in the various mixtures change from 0 to 100%. (□) Rings formed in plate containing the cross-reacting anti-hexosaminidase B; (■) rings formed in the plate containing specific anti-hexosaminidase A

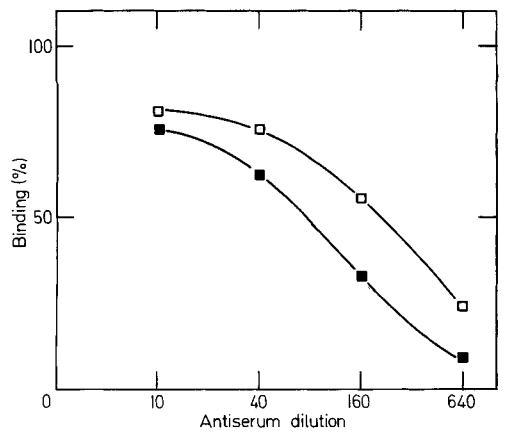

Fig. 4. *Binding capacity of total anti-hexosaminidase (□) and specific anti-hexosaminidase A (■), for ^{125}I-labeled hexosaminidase A, after subtraction of the nonspecific binding (15%)*

The standard deviation values for the inhibition, along the linear region of the standard curves were the following: for A, when tested with whole and specific anti-hexosaminidase A, 12.3% and 14.2%, respectively; for B, when tested with whole anti-hexosaminidase A, 9.8%.

A parallel radioimmunoassay was carried out using labeled hexosaminidase B. This assay gave identical results to the one described above when whole anti-hexosaminidase A was used, allowing the quantitisation of both A and B. Specific anti-hexosaminidase A IgG fraction gave no binding whatsoever with this antigen.

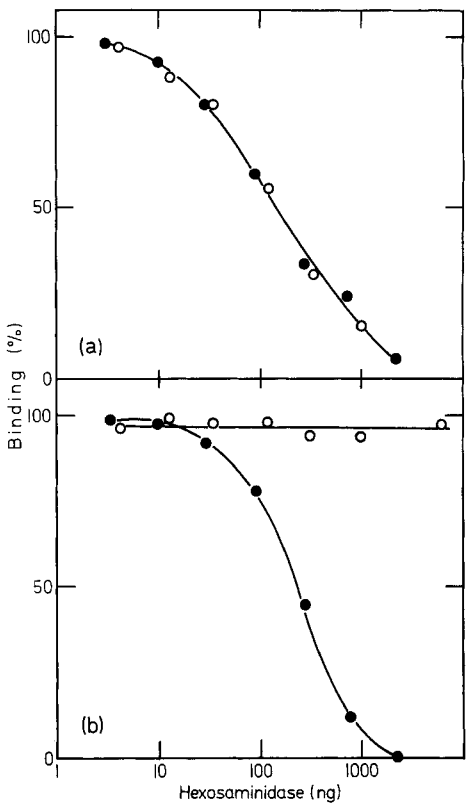

Fig. 5. *Radioimmunoassay of hexosaminidases A (●) and B (○) with the cross-reactive antiserum, total anti-A (a) and the specific anti-A serum (b)*

Determination of Hexosaminidases A and B in Tissues

The two assays described above were used for determination of hexosaminidase A and B in tissues obtained from normal individuals (adults and fetuses), and Tay-Sachs patients (children and suspected fetuses).

The results obtained in the radial diffusion method for the various specimens are shown in Fig. 6. Each sample was tested in two different dilutions. As can be seen in the figure, normal tissues gave precipitin rings with both the cross-reactive anti-hexosaminidase A and with the anti-hexosaminidase A-specific serum, whereas the Tay-Sachs tissues reacted with the cross-reactive serum but gave no ring at all with the A-specific antiserum. The results are expressed in a quantitative manner in Fig. 7, which depicts the correlation between the ring area and the enzyme concentration in the samples, in their various dilutions, as measured by enzymic activity.

As shown, normal and Tay-Sachs tissues gave identical values with the cross-reactive antiserum, both obeying a linear relationship, whereas when the specific anti-hexosaminidase A serum was used, only normal tissues reacted, giving a similar linear curve. It is pertinent to remark that the slope of the curve

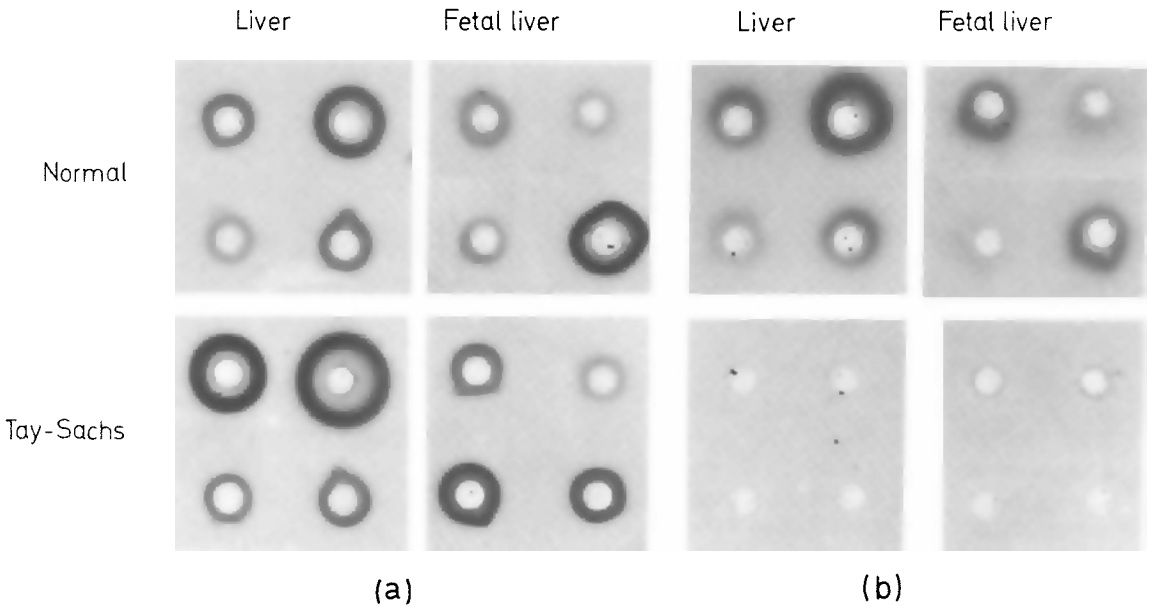

Fig. 6. *Determination of hexosaminidases A and B in tissue extracts by radial immunodiffusion.* The plates contained, respectively, anti-hexosaminidase B (a) and specific anti-hexosaminidase A (b). Identical samples of the various tissues were assayed in the two plates. Quantitative presentation of these results is depicted in Fig. 7

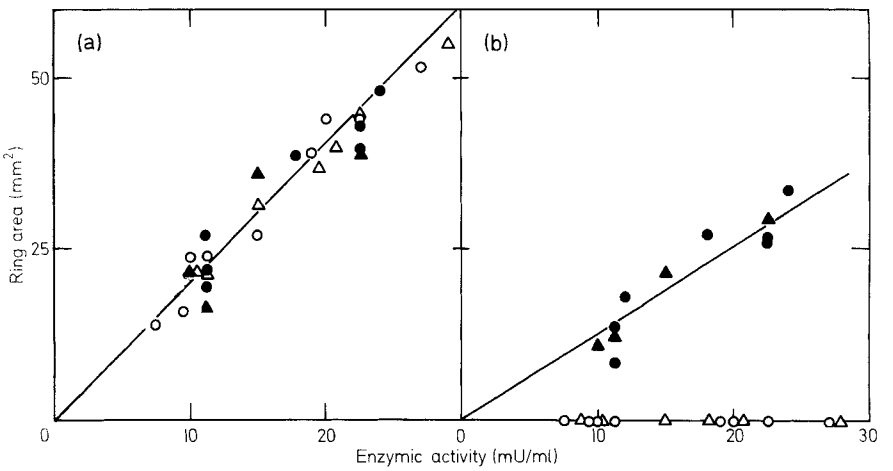

Fig. 7. *Quantitative presentation of the results of radial immunodiffusion assay of liver homogenates of healthy individuals (solid symbols), or Tay-Sachs patients (empty symbols).* Liver samples were obtained from adults (circles) or fetuses (triangles). The samples were tested in 1.5% agarose containing anti-hexosaminidase B (a) or specific anti-hexosaminidase A (b). The plates containing some of these samples are shown in Fig. 6

obtained by the reaction of such samples with the A-specific serum is about 60% of that obtained with the cross-reactive serum, indicating that only 60% of the enzyme in the normal tissues is hexosaminidase A.

The radioimmunoassay was carried out with samples at the enzyme concentration which caused inhibition at the linear range of the curves, in which the assessment of enzyme amounts is most sensitive. In each case the same sample at identical concentrations was used in the assay performed with either the cross-reactive anti-hexosaminidase A or with the specific anti-hexosaminidase A. As seen in Table 1, normal tissues caused inhibition in the assay performed with both types of antisera, but gave lower inhibition values with the A-specific antiserum. According to the relative inhibitions observed with the two antisera the percentage of isozyme A in the total amount of hexosaminidase could be calculated, and gave a mean value of 62.0 ± 10.4%. These results are comparable with the values obtained for normal tissues in the heat-inactivation method (61.1 ± 2.9%, Table 1), and also with the results calculated for the radial diffusion method (62.2 ± 4.1%), as depicted in Fig. 7. On the other hand, Tay-Sachs tissues gave inhibition only in the system using the whole anti-hexosaminidase A

Table 1. *Determination of hexosaminidase A and hexosaminidase B in normal and Tay-Sachs tissues by radioimmunoassay*
Hex = hexosaminidase

Sample	Sample number	Heat inactivation	Radioimmunoassay		
			inhibition		amount Hex A
			with anti-Hex A	with specific anti-Hex A	
		%			
Normal liver	22	60	46.2	29.1	62.9
	23	55	65.1	38.2	58.0
	24	62	62.9	30.2	48.0
	25	64	73.9	60.1	81.3
	26	59	60.0	32.0	53.3
	27	64	51.2	28.0	54.7
	28	62	50.0	36.5	73.0
Tay-Sachs liver	1	10	61.2	3.5[a]	0
	2	8	77.7	1.5	0
	31	9	59.3	4.3	0
	32	6	74.2	− 1.1	0
	33	12	59.6	− 5.0	0
	34	10	58.1	1.8	0
Normal fetal liver	8	62	62.1	43.1	69.4
	38	62	63.4	38.4	60.6
Tay-Sachs suspected fetal liver	10	9	67.5	2.1[a]	0
	11	7	75.1	− 3.2	0
	12	12	50.0	0	0
	13	6	62.0	2.6	0

[a] The same degree of inhibition was observed also when 10-fold concentrated tissue homogenates were tested, thus indicating that the inhibition values obtained merely represent fluctuations in the nonspecific binding of ^{125}I-labeled hexosaminidase A, but not the presence of any hexosaminidase A in the preparation.

serum, whereas the values of inhibition obtained in the system of the specific anti-hexosaminidase A serum were below the linear range of the curves, even when used in 10-fold higher concentrations of homogenates. Thus, the inhibition figures in the table are not indicative of actual inhibition but rather stem from fluctuations in the binding values. The same phenomenon was observed with fetal liver of normal and Tay-Sachs suspected individuals. It can, therefore, be concluded that the Tay-Sachs tissues do not contain hexosaminidase A at all. These results are comparable with the low values obtained in the heat-inactivation method.

DISCUSSION

In this presentation we report on the development of two sensitive and reliable assays for quantitative determination of hexosaminidases A and B, separated or in the presence of each other. Using these two methods it was demonstrated that whereas normal tissues contain both hexosaminidases A and B, in comparable amounts, Tay-Sachs tissues do not contain hexosaminidase A, or any material which carries the specific antigenic determinants of this isozyme. It was possible to draw these conclusions due to the fact that the assays were performed with two types of antisera, the one reacting identically with both A and B, whereas the second reacts specifically and exclusively with A. The important feature of these assays is that they enable the direct positive determination of hexosaminidase A in the presence of hexosaminidase B.

The methods available today for the detection and the determination of hexosaminidases A and B are based on the catalytic activity of the enzymes. Since the two isozymes are essentially identical in their specificity and kinetic parameters [12,15,16], the distinction between them was made usually using the heat-inactivation method, which monitors the disappearance of activity after heating, and attributes it to the presence of hexosaminidase A. The assays developed in the present study are based on the antigenic properties of the enzymes, and on the existence of unique antigenic determinants present on hexosaminidase A, which enable the direct demonstration of this isozyme. In this manner hexosaminidase A is quantitised independently of its enzymic activity, in the presence of hexosaminidase B.

The sensitivity of the two assays is rather high, and allows for the determination of hexosaminidase at a concentration level of 200 ng/ml. In the radioimmunoassays 10 − 50 ng, namely 0.05 − 0.25 ml, of a solution with this concentration are required per assay in order to reach the linear part of the inhibition curve. The radial diffusion assay is even more sensitive, allowing the determination of amounts as low as 2 ng (10 µl of a 200 ng/ml solution). In both assays the sensitivity is somewhat lower when the specific anti-hexosaminidase A serum is used.

In the radial immunodiffusion assay, although the quantitative reaction is based on the antigenic properties, advantage is taken of the enzymic activity of the preparation in the final step of ring development, in order to achieve the high sensitivity. The radioimmunoassay is based solely on the antigenic properties, and is independent of the catalytic activity of either isoenzyme.

This feature is of importance in the application of these assays in the investigation of genetic disorders. In those cases, *e.g.* Tay-Sachs or Sandhoff's diseases, the phenomenological observation is the absence of enzymic activity corresponding to one isozyme (A) or to both. This could theoretically stem

either from the absence of the respective isozyme, or from a molecular defect in it which impairs its activity, without affecting its antigenic properties. A distinction between these two possibilities could be made only by the availability of an assay which detects the presence of both isozymes, in an immunoassay which is independent of their enzymic activity. Our findings, demonstrating that in Tay-Sachs-affected children no hexosaminidase A, or any cross-reactive material which carries its specific antigenic determinants, can be detected, support the view that in this disease the enzyme is indeed missing, in accordance with reports from other laboratories [7,8]. However, we cannot rule out the remote possibility that a defective enzyme is present, in which the distinctive A-determinants are specifically missing.

The two assays developed herewith could be useful not only in the study of several disorders belonging to the family of GM_2 gangliosides, but could also be applied to variants related to these disorders, which do not exhibit any pathological symptoms, but manifest apparent deficiency of hexosaminidase A [17]. Another potential application of these sensitive assays is the direct detection of hexosaminidase A in cell-free amniotic fluids. If successful, this could serve for prenatal diagnosis of Tay-Sachs. The main method presently used for prenatal diagnosis necessitates the culturing of the amniotic cells *in vitro* and the assay of hexosaminidase A in them by the heat-inactivation procedure [18]. This time-consuming procedure (approximately 1 month) of culturing *in vitro*, is essential, in view of the fact that the cell-free amniotic fluid contains relatively low contents of hexosaminidase A (probably due to its low stability). Consequently, any hexosaminidase A determination in the fluid itself by the heat-inactivation method, which rely on the subtraction of the residual activity from the original value before heating [19,20], is subjective to high errors, and is hence inaccurate.

The immunoassays presently described, which detect the hexosaminidase A directly, even in large excess of hexosaminidase B may serve for testing of amniotic fluid immediately following amniocentesis.

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REFERENCES

1. Robinson, D. & Stirling, J. L. (1968) *Biochem. J. 107*, 321–327.
2. Sandhoff, K., Andreae, V. & Jatzkewitz, H. (1968). *Life Sci. 7*, 283–288.
3. Okada, S. & O'Brien, J. S. (1969) *Science (Wash. D.C.) 165*, 698–700.
4. O'Brien, J. S., Okada, S., Chen, A. & Fillerup, D. L. (1970) *New Eng. J. Med. 283*, 15–20.
5. Padeh, B. & Navon, R. (1971) *Israel J. Med. Sci. 7*, 259–263.
6. Navon, R. & Padeh, B. (1972) *J. Pediatr. 80*, 1026–1030.
7. Srivastava, S. K. & Beutler, E. (1973) *Nature (Lond.) 241*, 463.
8. Bartholomew, W. R. & Rattazzi, M. C. (1974) *Int. Arch. Allergy, 46*, 512–524.
9. Geiger, B., Ben Yoseph, Y. & Arnon, R. (1974) *FEBS Lett. 45*, 276–281.
10. Reference deleted.
11. Hayase, K., Reisher, S. R. & Kritchevsky, D. (1973) *Proc. Soc. Exp. Biol. Med. 142*, 466–470.
12. Ben Yoseph, Y., Geiger, B. & Arnon, R. (1975) *Immunochemistry, 12*, 221–226.
13. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry, 2*, 235–254.
14. Marchalonis, J. J. (1969) *Biochem. J. 113*, 299–305.
15. Wenger, D. A., Okada, S. & O'Brien, J. S. (1972) *Arch. Biochem. Biophys. 153*, 116–129.
16. Sandhoff, K. & Wässle, W. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1119–1133.
17. Navon, R., Padeh, B. & Adam, A. (1973) *Am. J. Hum. Genet. 25*, 287–293.
18. Navon, R. & Padeh, B. (1971) *Brit. Med. J. 4*, 17–20.
19. Friedland, J., Perle, G., Saifer, A., Schneck, L. & Volk, B. W. (1971) *Proc. Soc. Exp. Biol. Med. 136*, 1297–1298.
20. Murphy, J. V. (1972) *Life Sci. 11*, 309–318.

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