macol. 10, 78-92.
Tullock, C. W., & Coffman, D. D. (1960) *J. Org. Chem. 25,* 2016-2019.
Vaughn, W. M., & Weber, G. (1970) *Biochemistry 9,* 464-473.
Voss, H. F., Ashani, Y., & Wilson I. B. (1975) *Methods Enzymol. 34,* 581-591.
Weast, R. C., Ed. (1977), in *Handbook of Chemistry and Physics,* 57th ed, p E-221, Chemical Rubber Co., Cleveland, Ohio.
Wustner, D. A., & Fukuto, T. R. (1974) *Pestic. Biochem. Physiol. 4* 365-376.
Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) *J. Mol. Biol. 51,* 573-590.

Biochemical and Immunochemical Characterization of Hexosaminidase P†

Benjamin Geiger, Edna Calef, and Ruth Arnon*

ABSTRACT: Hexosaminidase P, the main isozyme of hexosaminidase in pregnancy serum, was isolated and purified 600-700-fold by a two-step purification procedure—affinity chromatography on Sepharose-bound ϵ-aminocaproyl-*N*-acetylglucosylamine, followed by ion-exchange chromatography on DEAE-cellulose. The purified enzyme was subjected to biochemical and immunochemical analysis. Its catalytic property, namely, kinetic behavior, is similar to that of the major isozymes of hexosaminidase, A and B. However, it differs from these isozymes in its electrophoretic mobility and in its apparent molecular weight which is around 150 000 compared with 100 000 of the A and B isozymes. Immunochemical analysis indicates that the P isozyme is antigenically cross-reactive with both A and B isozymes, but it does not contain the A-specific antigenic determinants, and exhibits identical antigenic specificity to hexosaminidase B. Two possible structures are suggested that are compatible with the experimental data: (a) a hexosaminidase B like structure with higher extent of glycosylation; (b) a hexamer of β chain, possibly arranged as three β_2 subunits.

The enzyme *N*-acetyl-β-D-hexosaminidase (hexosaminidase, EC 3.2.1.30) is present in human tissues as two main isoenzymes designated hexosaminidase A and B (Robinson & Stirling, 1968) which are localized in the lysosomal fraction of the cells. However, analysis of the enzyme obtained from different tissues, cells, or body fluids indicated the existence of some minor hexosaminidase isozymic forms such as hexosaminidase C (Poenaru et al., 1973; Braidman et al., 1974a,b; Penton et al., 1975; Reuser & Goljaard, 1976), I_1 and I_2 (Price & Dance, 1972), etc. Another isoenzyme was detected in sera of pregnant women (Stirling, 1971, 1972) and was denoted hexosaminidase P.

The increase in levels of hexosaminidase activity in serum during pregnancy was first observed by Walker et al. (1960). In later reports O'Brien et al. (1970) and Huddlestone et al. (1971) suggested that this elevation in activity is due to an increased hexosaminidase B level. However, later studies have demonstrated that the increase in enzymatic activity is due to the appearance of a novel enzyme in the serum, namely, hexosaminidase P.

During the last few years remarkable progress has been made in the elucidation of the molecular structure of the two major human isozymes, hexosaminidases A and B. Based on experimental evidence concerning various aspects of the enzyme as derived from biochemical studies, somatic cell hybrids (Lalley et al., 1974; Gilbert et al., 1974; Thomas et al., 1974; Gilbert et al., 1975), conversion experiments (Carmody & Rattazzi, 1974; Beutler et al., 1975) and direct chemical analysis, a molecular model was proposed for hexosaminidases A and B (Geiger & Arnon, 1976; Lee & Yoshida, 1976; Beutler et al., 1976). According to this model, both hexosaminidases are built of two subunits, each subunit composed of two S-S-linked identical polypeptide chains. However, whereas hexosaminidase B is composed of four identical chains ($\beta_2\beta_2$), the A isozyme has one β_2 and one α_2 subunit ($\alpha_2\beta_2$) (Geiger & Arnon, 1976).

In the present study we have investigated some of the biochemical and immunochemical properties of hexosaminidase P, in an attempt to describe its structure in molecular terms.

Materials and Methods

Hexosaminidase A and B were purified from human placentae to an apparent homogeneity by the procedure described previously (Geiger et al., 1975; Geiger & Arnon, 1976). The purity of the preparations was established by analytical ultracentrifugation, electrophoresis on polyacrylamide gel in the presence of $NaDodSO_4$,[1] as well as by gel electrophoresis and isoelectric focusing under nondenaturing conditions.

Sera of Pregnant Women. Serum samples were collected from women in the third trimester of pregnancy and 48 h after delivery. Sera used for estimation of enzymatic activity were not pooled and were stored at −20 °C until tested. Sera used for purification of hexosaminidase P were collected, pooled, and stored until used at −20 °C.

Hexosaminidase Assay. Enzyme solution (100 μL, diluted in 0.04 M citrate buffer, pH 4.4) was incubated for 10 min at 37 °C with substrate (200 μL) containing 0.1 mg/mL 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Pierce) and 1 mg/mL bovine serum albumin (Grade A, Calbiochem). The

† From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel. *Received October 26, 1977.*

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; IEF, isolectric focusing.

0006-2960/78/0417-1713$01.00/0

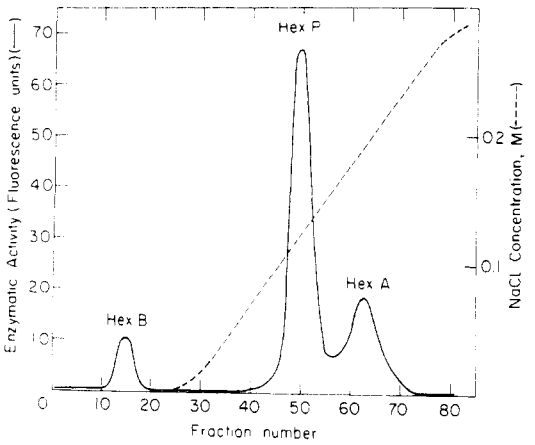

FIGURE 1: Separation of hexosaminidases of pregnancy serum on DEAE-cellulose. Seventy milligrams of Seph-CNAG-purified hexosaminidases was chromatographed on 2.5 × 20 cm DEAE-cellulose column and eluted with NaCl gradient (100 mL of 0.3 M NaCl in 10 mM phosphate buffer into 100 mL of the phosphate buffer).

reaction was stopped by the addition of 3 mL of 0.2 M glycine buffer (pH 10.4) and the fluorescence was measured in a Turner Model 110 filter fluorimeter with filter setting 7-60 (primary) and 48+2A (secondary). One unit of enzymatic activity is defined as the amount of enzyme which hydrolyzes 1 μmol of substrate per min under the specific reaction conditions.

Gel Filtration. Analytical gel filtration of hexosaminidase A and P was performed at 4 °C on a Sephadex G-200 column, equilibrated with phosphate-buffered saline (PBS). The samples (in 1 mL) containing the different hexosaminidases and additional protein markers were loaded on top of the column (1.6 × 87 cm) and eluted with PBS at a rate of 12 mL/h. Fractions of 1.25 mL were collected and both enzymatic activity and absorbance at 280 nm were recorded.

Electrophoresis and Isoelectric Focusing. Starch gel electrophoresis was carried out according to Smithies (1959) in a horizontal starch block in 5 mM phosphate-citrate buffer at pH 6.0. The electrophoretic run proceeded for 12 h at 4 °C at 5 V/cm. The gel was then sliced and the enzyme bands were visualized using the fluorogenic substrate, as described previously (Geiger et al., 1975).

Isoelectric focusing (IEF) was performed in sucrose gradient in a preparative IEF instrument (LKB, 8100, Sweden). The concentration of ampholite (pH range 3.5-10.0) was 1% (v/v) and the IEF was carried out at 4 °C, and 500 V for 72 h. Fractions of 1 mL were collected from the bottom of the column and both pH and enzymatic activity were determined. Analytical IEF in polyacrylamide slabs was carried out according to Williamson (1971).

Antisera. Antisera against placental hexosaminidases A and B as well as specific anti-hexosaminidase A were prepared in goats as detailed elsewhere (Geiger et al., 1975). Antiserum against hexosaminidase B was prepared in a similar manner also in rabbits. Antisera toward hexosaminidase P was prepared by three injections (at 10 day intervals) of partially purified hexosaminidase P (0.5-0.6 unit per animal per injection) emulsified in complete Freund's adjuvant. Blood was collected 1 week after the last injection.

Double immunodiffision was performed in 1.5% agarose gels in PBS. The gels were rinsed and the enzyme-containing precipitin arcs visualized using the chromogenic substrate Naphthol AS-BI-*N*-acetyl-β-D-glucosaminide and Fast Garnet GBC salt.

TABLE I: Serum Hexosaminidase in Pregnant Women.[a]

Serum tested		Hexosaminidase act. (munits/mL) Third trimester	48 h after delivery
Nonpregnant	Controls	4.6 (3.4-5.6)	
Pregnancy serum	S.S.	23.6 (22.0-25.7)	6.2
	A.K.	16.7 (16.3-17.5)	6.9
	Z.L.	15.9 (15.1-16.3)	10.9

[a] The results are based on the mean values of total hexosaminidase of 12 nonpregnant women, and on at least four serum samples taken from the same individuals during the third trimester of pregnancy. The numbers in parentheses indicate the complete range of activities obtained.

Enzyme binding assay was performed as described elsewhere (Geiger et al., in press). The various enzyme samples (50 μL) containing identical activities were incubated with 50 μL of antiserum, diluted 1:20 in PBS. Control samples consisted of 5% normal serum. Second antibody (goat anti-rabbit, or rabbit anti-goat, respectively) was added in an amount sufficient to precipitate completely the antibody of the anti-hexosaminidase antiserum and the enzymatic activity associated with the precipitate was determined.

Immunoadsorbent hexosaminidase B (partially purified) was covalently bound to CNBr-activated Sepharose 4B as described previously (Geiger et al., 1975).

Results

Purification of Hexosaminidase P. Hexosaminidase P was purified from pooled sera of women in the third trimester of pregnancy. The serum (in 200-mL portions) was dialyzed three times against 10 L of 10 mM sodium phosphate buffer, pH 6.0, and the formed precipitate was removed by centrifugation (17 600*g*, 30 min). The supernatant (19 munits/mL) was applied to a Sepharose-bound ε-aminocaproyl-*N*-acetylglucosylamine column (Lis et al., 1974; Geiger et al., 1975). The column (25 × 2 cm) was washed consecutively with 10 mM phosphate buffers of pHs 6.0 and 7.0, and the peak of hexosaminidase was then eluted by the same buffer at pH 8.3. The fractions under the activity peak were pooled, concentrated to 10 mL (7 mg of protein/mL, 175 munits/mL), and dialyzed against 10 mM phosphate buffer (pH 7.0). The sample was applied to a DEAE-cellulose column (2.5 × 20 cm) equilibrated with the same buffer. Hexosaminidase B was eluted with the buffer in the void volume, whereas hexosaminidases P and A were subsequently eluted with a linear gradient of NaCl (0-0.3 M) in a volume of 200 mL. Each fraction (3 mL) was monitored for enzymatic activity. Figure 1 depicts the elution pattern of the three isozymes from the column. It can be seen that most of the activity is in the intermediate peak between the A and the B isozymes, corresponding to hexosaminidase P. The material under the peak of hexosaminidase P was pooled and concentrated to 10 mL (2.2 mg/mL, 161 munits/mL) and stored in several vials at −20 °C until used.

Determination of Hexosaminidase in Maternal Serum. Samples of serum were obtained from 12 nonpregnant as well as from women in the third trimester of pregnancy (four samples from each individual), and the total hexosaminidase activity in them was determined. The results summarized in Table I show that an average increase of fourfold in serum hexosaminidase takes place during pregnancy. A short time after delivery, a sharp drop in the activity of hexosaminidase

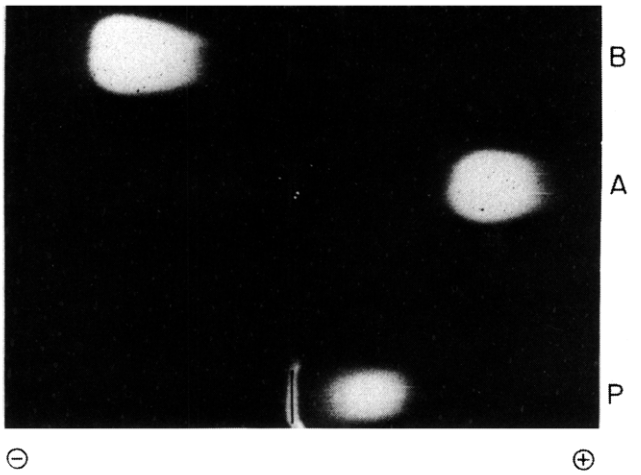

FIGURE 2: Starch gel electrophoresis of hexosaminidases A, B, and P. The electrophoresis was carried out at 180 V, for 17 h. Staining for activity was performed using 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide.

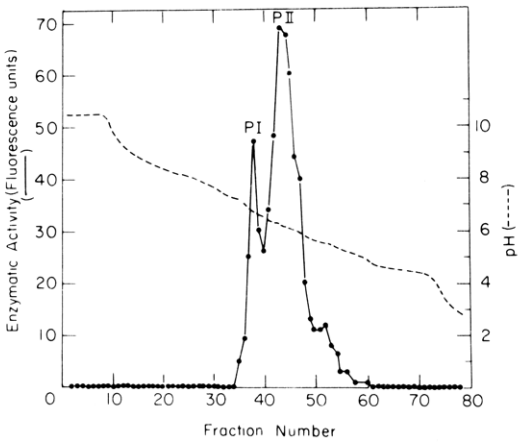

FIGURE 3: Isoelectric focusing of hexosaminidase P in sucrose gradient.

is observed and normal enzyme levels were found 2–3 days after delivery.

Biochemical Properties of Hexosaminidase P. Starch gel electrophoresis of purified hexosaminidase P (Figure 2) demonstrated that this enzyme indeed differs from the A and the B isozymes. Most of the enzymatic activity was associated with a band slowly migrating to the anode, whereas part of the enzyme remained in the origin. By preparative IEF it has been possible to separate the P isozyme into two main peaks, designated P_I and P_{II}, having isoelectric points of 6.3 and 6.7, respectively (Figure 3). The microheterogeneity of the P isozyme was demonstrated further by analytical IEF in a polyacrylamide gel stained with a chromogenic substrate (Figure 4). Hexosaminidase B was focused as one major band at pH 7.6–7.7; the A isozyme appeared as a twin band at pH 5.2–5.3 with two very minor acidic and two more basic bands. Traces of B-like hexosaminidase activity were found in the preparation of the A isozyme, probably due to interconversion during the IEF. The microheterogeneity of hexosaminidase P was found to be remarkably higher and 5–6 major bands and some minor bands of activity could be resolved in the pH range of 5.5–7.0.

The validity of the separation profile was verified by a reproducible refocusing of single bands excised from the gel on a second IEF slab. Analysis of unfractionated individual ma-

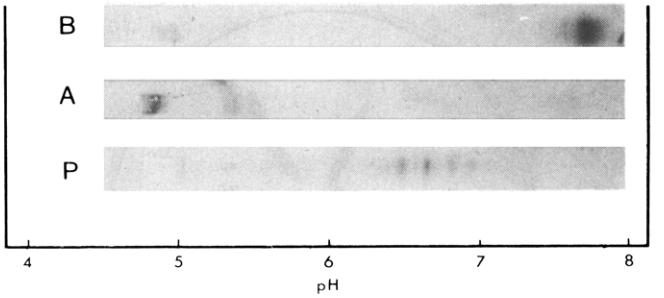

FIGURE 4: Analytical isoelectric focusing of hexosaminidases A, B, and P. The gel was stained with the chromogenic substrate Naphthol AS-B1–*N*-acetyl-β-D-glucosaminide and Fast Garnet GBC salt.

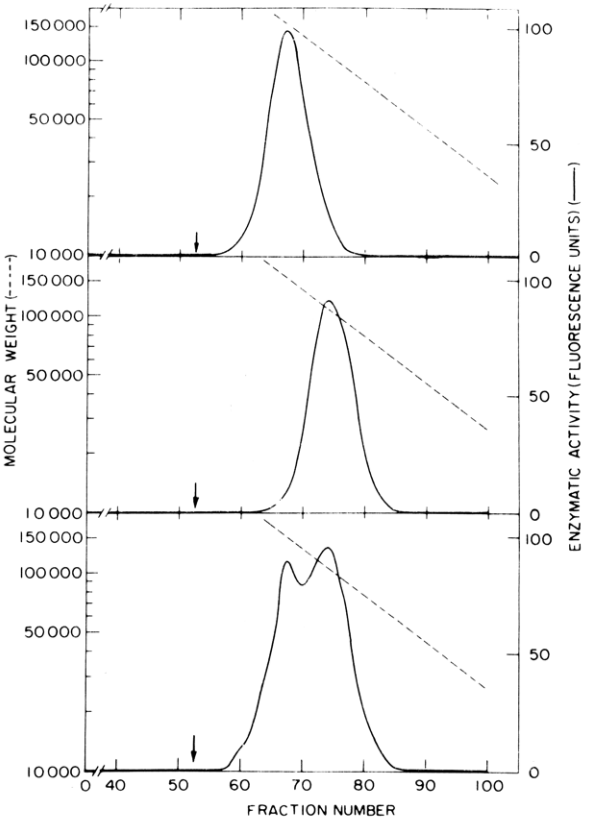

FIGURE 5: Gel filtration of hexosaminidase P (top), hexosaminidase A (middle), or mixture of the two (bottom) on Sephadex G-200 column.

ternal serum samples has shown that slightly different degrees and patterns of microheterogeneity may exist between different samples, but the overall pattern is very similar to that obtained with the purified isozyme.

The apparent molecular weight of hexosaminidase P, determined by gel filtration on Sephadex G-200 column, was 155 000–160 000 (Figure 5). Hexosaminidase A migrated in the same column as a protein with molecular weight of 100 000–103 000. The significant difference between the molecular weights of hexosaminidases A and P was confirmed by cochromatography of a mixture of these two isozymes on the same column (Figure 5, bottom).

Analysis of the kinetic parameters of the hydrolysis of *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl substrates by hexosaminidase P indicated that its K_m values toward the two substrates were 6.0×10^{-4} and 1.0×10^{-4} M, respectively, values which are almost identical with those obtained previously for hexosaminidase A and B (Geiger et al., 1974). Moreover, the maximal velocity obtained for the *N*-acetyl-

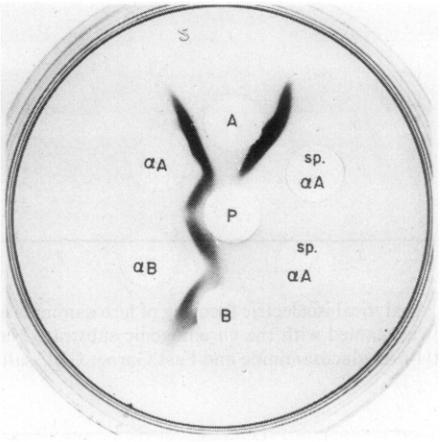

FIGURE 6: Double immunodiffusion in agarose gels. Solutions of hexosaminidases A, P, and B were applied to the three central wells (from top to bottom) and goat antisera to hexosaminidase A (αA), to hexosaminidase B (αB), or specific antibodies to hexosaminidase A (SpαA) were added to the neighboring wells.

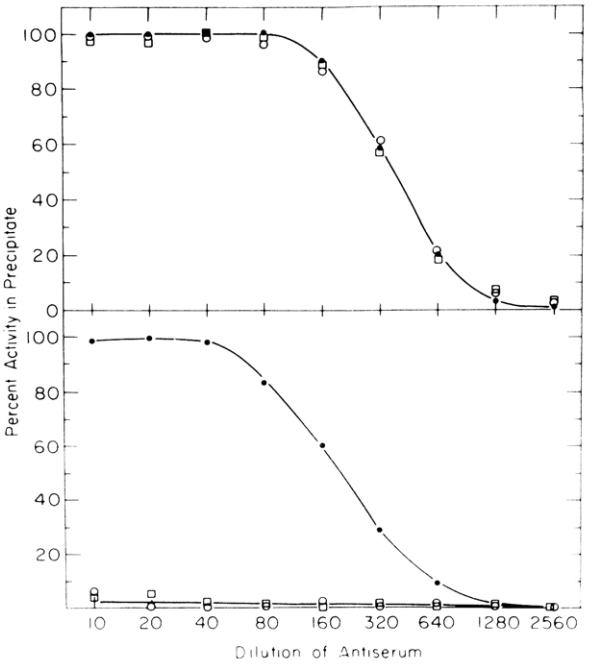

FIGURE 7: Antigen binding assay of hexosaminidases A (●), B (○), and P (□) with different concentrations of anti-hexosaminidase B (top) or specific anti-hexosaminidase A (bottom).

glucosaminyl substrates is about 5.5 times higher than that obtained with the *N*-acetylgalactosaminyl derivative, a value which is similar to the value obtained previously with the placental hexosaminidases A and B.

An indirect approach for the study of possible differences in the sugar moiety of hexosaminidases A, B, and P was by analytical affinity chromatography on Sepharose–concanavalin A. Solutions containing equal enzymatic activity of the three hexosaminidase isozymes, A, B, and P (50 munits in 0.5 mL), were each applied to a 5-mL column of Sepharose–Con A. The column was rinsed with PBS and the enzyme which adhered to the column was eluted with a linear gradient of methyl α-glucopyranoside (0–10%, 30 mL). Fractions of 1 mL were collected and both enzymatic activity and sugar concentration (measured by polarimetry) were determined. The results indicated that all three isozymes exhibit an identical pattern, yielding a peak of enzyme activity eluted in concentration of 1.8–2.0% methyl α-glucopyranoside.

Immunochemical Analysis. A qualitative comparative characterization of hexosaminidases A, B, and P with respect to the presence or absence of hexosaminidase A-specific antigenic determinants (which are most probably associated with the α_2 subunit) was performed by double immunodiffusion in agarose gels. The results, presented in Figure 6, indicate that hexosaminidase P does not contain the A-specific antigenic determinants. In this respect the P enzyme resembles hexosaminidase B. This conclusion was corroborated by a more quantitative test, namely, enzyme binding assay. Figure 7 shows the results of a binding experiment performed with anti-hexosaminidase B (top) and with specific anti-hexosaminidase A (bottom). All three enzymes were equally bound by the cross reactive anti-B serum, whereas no binding whatsoever of either P or B isozyme was achieved by the specific anti-A, even when testing a high serum excess.

The apparent antigenic similarity between hexosaminidases B and P was further investigated using antisera against each of the two isozymes. Antibodies to hexosaminidase P were found to bind equally well the homologous enzymes and the B isozyme. Moreover, it was possible to absorb totally the anti-hexosaminidase P activity by hexosaminidase B immunoadsorbent.

Discussion

In view of the availability of a structural model for the two major isozymes A and B of the human hexosaminidase, it seemed of importance to investigate whether a unified model could be suggested that will account also for the minor isozymes which are found in tissues and body fluids under various physiological and pathological conditions.

One step in this direction was the finding that hexosaminidase S, the residual enzyme found in tissues of individuals with the O-variant form of GM_2 gangliosidoses, is a dimer of the α_2 subunit with a proposed structure of $\alpha_2\alpha_2$ (Geiger et al., 1977a).

The results presented above indicate that hexosaminidase P, an isozyme which appears typically during pregnancy, is closely related in its enzymatic properties to the A and the B isozymes. Similarly, the immunochemical data point to very close antigenic relationships between hexosaminidase P and the placental isozymes. In the latter respect, hexosaminidase P was shown to be indistinguishable from hexosaminidase B and not to contain the antigenic determinants specific to hexosaminidase A, which are probably associated with the α_2 subunit of the A enzyme.

However, notable physicochemical differences were observed between hexosaminidases A and B on the one hand, and hexosaminidase P on the other. The main differences are its considerably higher apparent molecular weight as compared with the A and B isozymes (about 155 000 in contrast to about 100 000) (Figure 5) and its electrophoretic behavior (Figures 2 and 3). The hexosaminidase P exhibits also a remarkable microheterogeneity in contrast to the limited heterogeneity of either hexosaminidases A or B (Figure 4).

A more detailed direct biochemical analysis of the structure of hexosaminidases P was hampered by the unavailability of large enough quantities of pregnancy serum to allow purification of the enzyme in sufficient amounts. The data accumulated in this study are derived, therefore, only from biological assays and they point to two alternative suggestions for hexosaminidase P structure. The first is that hexosaminidase P is composed of 6 β or β-like chains arranged in three β_2 subunits. The reason for such molecular assembly is not clear,

but it may result from various posttranslational modifications which may also be manifested in the wide microheterogeneity of the P isozyme. This possible model is supported on the one hand by the molecular weight of hexosaminidase P which is higher by a factor of 1.5 than that of the A and B isozymes, and on the other hand by the lack of any A-specific antigenic determinants on this isozyme.

An alternative possibility is that the P enzyme shares the same protein moiety with hexosaminidase B but differs from this isozyme in its degree of glycosylation, a difference that may strongly affect the hydrodynamic and electrophoretic properties of the molecule. The possibility that hexosaminidase P contains another, yet uncharacterized subunit, seems unlikely since anti-hexosaminidase P antibodies can be completely adsorbed on immobilized hexosaminidase B.

The physiological mechanism which is involved in the production of hexosaminidase P and its consequent appearance in maternal serum in increased level is still obscure. It should, however, be mentioned that the P enzyme is probably not unique for pregnancy and may be detected, though in low concentration, also in nonmaternal blood (Price & Dance, 1972). Thus, the elevation of this enzyme in the blood may result not only from enhanced synthesis or release, but may rather be due to impaired clearance from circulation. Recent studies on the fate of hexosaminidase A injected to the circulation of rabbits have shown that a rapid clearance of the enzyme takes place (complete disappearance of hexosaminidase A was obtained within 20–40 min; Geiger et al., 1977b). Thus, even a small modification in structure, which will result in hindered clearance, may cause a progressive increase in the level of this isozyme in the blood.

References

Beutler, E., Villacorte, D., Kuhl, W., Guinto, E., & Srivastava, S. (1975) *J. Lab. Clin. Med. 86,* 195.

Beutler, E., Yoshida, A., Kuhl, W., & Lee, J. E. S. (1976) *Biochem. J. 159,* 541.

Braidman, I., Carrol, M., Dance, N., Robinson, D., Poenaru, L., Weber, A., Dreyfus, J. C., Overdijk, B., & Hooghwinkel, G. J. M. (1974a) *FEBS Lett. 41,* 181.

Braidman, I., Carrol, M., Dance, N., & Robinson, D. (1974b) *Biochem. J. 143,* 295.

Carmody, P. J., & Rattazzi, M. C. (1974) *Biochim. Biophys. Acta 371,* 117.

Geiger, B., & Arnon, R. (1976) *Biochemistry 15,* 3489.

Geiger, B., Ben Yoseph, Y., & Arnon, R. (1974) *Isr. J. Med. Sci. 10,* 117.

Geiger, B., Navon, R., Ben-Yoseph, Y., & Arnon, R. (1975) *Eur. J. Biochem. 56,* 311.

Geiger, B., Arnon, R., & Sandhoff, K. (1977a) *Am. J. Hum. Genet. 29,* 508.

Geiger, B., v. Specht, B. V., & Arnon, R. (1977b) *Eur. J. Biochem. 73,* 141.

Gilbert, F., Kucherlapati, R., Creagan, R., Murnane, M. J., Darlington, G. J., & Ruddle, F. H. (1974) *Cytogenet. Cell. Genet. 13,* 93.

Gilbert, F., Kucherlapati, R., Creagan, R. P., Murnane, M. J., Darlington, G. J., & Ruddle, F. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 263.

Huddlestone, J. F., Cefalo, R. C., Lee, G., & Robinson, J. C. (1971) *Am. J. Obstet. Gynecol. 111,* 804.

Lalley, P. A., Rattazzi, M. C., & Shows, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 1569.

Lee, J. E. S., & Yoshida, A. (1976) *Biochem. J. 159,* 535.

Lis, H., Lotan, R., & Sharon, N. (1974) *Methods Enzymol. 34,* 341.

O'Brien, J. S., Okada, S., Chen, A., & Fillerup, D. L. (1970) *N. Engl. J. Med. 283,* 15.

Penton, E., Poenaru, L., & Dreyfus, J. C. (1975) *Biochim. Biophys. Acta 391,* 162.

Poenaru, L., Weber, A., Vibert, M., & Dreyfus, J. C. (1973) *Biomedicine 19,* 538.

Price, R. G., & Dance, N. (1972) *Biochim. Biophys. Acta 271,* 145.

Reuser, A. J. J., & Goljaard, H. (1976) *FEBS Lett. 71,* 1.

Robinson, D., & Stirling, J. L. (1968) *Biochem. J. 107,* 321.

Smithies, O. (1959) *Biochem. J. 71,* 585.

Stirling, J. L. (1971) *Biochem. J. 123,* 11P.

Stirling, J. L. (1972) *Biochim. Biophys. Acta 271,* 154.

Thomas, G. H., Taylor, H. A., Miller, C. S., Axelman, J., & Migeon, N. R. (1974) *Nature (London) 250,* 580.

Walker, P. G., Woolen, M. G., & Pugh, D. J. (1960) *J. Clin. Pathol. 13,* 353.

Williamson, A. R. (1971) *Eur. J. Immunol. 5,* 390.