Structure of Glycan Moieties Responsible for the Extended Circulatory Life Time of Fetal Bovine Serum Acetylcholinesterase and Equine Serum Butyrylcholinesterase

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ABSTRACT: Cholinesterases are serine hydrolases that can potentially be used as pretreatment drugs for organophosphate toxicity, as drugs to alleviate succinylcholine-induced apnea, and as detoxification agents for environmental toxins such as heroin and cocaine. The successful application of serum-derived cholinesterases as bioscavengers stems from their relatively long residence time in the circulation. To better understand the relationship between carbohydrate structure and the stability of cholinesterases in circulation, we determined the monosaccharide composition, the distribution of various oligosaccharides, and the structure of the major asparagine-linked oligosaccharides units present in fetal bovine serum acetylcholinesterase and equine serum butyrylcholinesterase. Our findings indicate that 70−80% of the oligosaccharides in both enzymes are negatively charged. This finding together with the molar ratio of galactose to sialic acid clearly suggests that the β-galactose residues are only partially capped with sialic acid, yet they displayed a long duration in circulation. The structures of the two major oligosaccharides from fetal bovine serum acetylcholinesterase and one major oligosaccharide from equine serum butyrylcholinesterase were determined. The three carbohydrate structures were of the biantennary complex type, but only the ones from fetal bovine serum acetylcholinesterase were fucosylated on the innermost *N*-acetylglucosamine residue of the core. Pharmacokinetic studies with native, desialylated, and deglycosylated forms of both enzymes indicate that the microheterogeneity in carbohydrate structure may be responsible, in part, for the multiphasic clearance of cholinesterases from the circulation of mice.

Cholinesterases (ChEs)[1] are serine esterases that hydrolyze choline esters faster than other substrates. In vertebrates, two types of ChEs corresponding to two distinct gene products have been identified: acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C. 3.1.1.8), which can be distinguished by their substrate specificity and sensitivity to various inhibitors (Massoulié et al., 1993). The distribution of the two enzymes in various tissues is also very different. AChE is present predominantly in muscle and in the nervous system, whereas BChE is synthesized predominantly in the liver and secreted into the plasma. Although the role of AChE in cholinergic transmission is well established, the functional significance of BChE is less clear, and it has been suggested that it may play a role in the detoxification of natural as well as synthetic ester bond-containing compounds (Massoulié & Bon, 1982). Molecularly, AChE and BChE consist of monomers or oligomers of similar or identical catalytic subunits (Massoulié et al., 1993). Depending on the type of C-terminal these subunits possess, these enzymes may exist as water-soluble globular forms, membrane-bound globular forms, or collagen-tailed asymmetric forms (Massoulié & Bon, 1982).

ChEs are highly glycosylated proteins (Liao et al., 1991, 1992; Trestakis et al., 1992), with up to 24% of their molecular weight consisting of carbohydrates (Haupt et al., 1966). These carbohydrates are present primarily as asparagine-linked side chains, as suggested by their susceptibility to specific glycohydrolases (Bon et al., 1987; Heider et al., 1991; Liao et al., 1991, 1992; Kronman et al., 1992) and their interaction with specific lectins (Gurd, 1976; Uhlenbruck et al., 1977; Raconczay et al., 1981; Bon et al., 1987; Méflah et al., 1984; Rotundo, 1988; Mutero & Fournier, 1992; Kerem et al., 1993). From the amino acid sequences reported for various ChEs, several putative N-glycosylation sites have been identified for each enzyme. For example, nine sites for human BChE (Lockridge et al., 1987; Prody et al., 1987), four for *Torpedo californica* AChE (Schumacher et al., 1986), five for FBS AChE (Doctor et al., 1990), and three potential sites for mouse (Rachinsky et al., 1990), rat (Legay et al., 1993), and human AChE (Soreq et al., 1990) have been identified. The role of N-glycosylation in ChE function is not fully established. Using recombinant human AChE, it was shown that glycosylation is important for the effective biosynthesis, secretion, and thermal and proteolytic stability of the enzyme but not for its catalytic activity (Velan et al., 1993).

Several studies in the last eight years have successfully demonstrated the potential use of ChEs as pretreatment drugs for organophosphate (OP) toxicity. Protection against OP agents was conferred by pretreatment with human and equine BChE in rats, mice, and rhesus monkeys (Ashani et al., 1991; Broomfield et al., 1991; Wolfe et al., 1987, 1992; Raveh et

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[1] Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; FBS, fetal bovine serum; Eq, equine (serum); OP, organophosphate; TMS, trimethylsilyl; MRT, mean residence time.

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al., 1993; Brandeis et al., 1993; Castro et al., 1994; Genovese & Doctor, 1995) as well as with FBS AChE in mice and rhesus monkeys (Wolfe et al., 1987; Raveh et al., 1989; Maxwell et al., 1992). In addition, human serum BChE is being used as a drug to alleviate succinylcholine-induced apnea (Lockridge, 1990) and is also being considered as a detoxifying candidate drug for environmental toxins such as cocaine (Lockridge, 1990; Mattes et al., 1996). The successful application of serum-derived ChEs as bioscavengers stems from their relatively long residence time in the circulation. Membrane-bound forms of AChE purified from either human erythrocytes or *T. californica* cleared more than 50-fold faster than human serum BChE from the circulation of mice (Raveh et al., 1993). It has been suggested that the relatively high stability of non-membranal soluble forms of either AChE or BChE from serum sources may be attributed to the number and structure of their sialylated carbohydrate residues (Masson, 1982).

In order to shed some light on the relationship between the carbohydrate structure and the stability of ChEs in circulation, we have determined the monosaccharide composition, the distribution of various oligosaccharides, and the structure of the major carbohydrate units present in FBS AChE and Eq BChE. FBS AChE is the only plasma-derived AChE known to date, as most ChEs found in the circulation of mammals exist as BChEs, and the circulatory properties of FBS AChE and Eq BChE are well established. Further, sufficient amounts of both enzymes in purified form were available for this study. Our findings indicate that 70−80% of the oligosaccharides in both enzymes are negatively charged. This finding together with the molar ratio of galactose to sialic acid in both ChEs clearly suggests that the β-galactose residues are only partially capped with sialic acid, yet they display a long duration in circulation. Pharmacokinetic studies with native, desialylated, and deglycosylated forms of both ChEs suggest that the multiphasic clearance of ChEs from the circulation of mice may be attributed to the microheterogeneity in carbohydrate structure.

MATERIALS AND METHODS

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the NIH *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, 1985).

Materials. Electrophoretically pure AChE from FBS was purified as described (De La Hoz et al., 1986). BChE from equine serum was purified by affinity chromatography using a procedure similar to the one described for FBS AChE. The specific activity of purified FBS AChE and Eq BChE was 5600 and 900 units/mg, respectively. One mg of pure, native AChE and BChE contained approximately 14 and 11 nmol of active sites, respectively.

Methods. Release, isolation, labeling, and structural analysis of carbohydrates were performed by Oxford Glycosystems Ltd., U.K., using the procedures described below.

Release and Recovery of Oligosaccharides Associated with FBS AChE and Eq BChE. The oligosaccharides associated with FBS AChE and Eq BChE were quantitatively released and recovered by automated hydrazinolysis using the GlycoPrep 1000 (Oxford Glycosystems Ltd., U.K.) as previously described (Patel et al., 1993). Samples of FBS AChE and Eq BChE containing 407 μg and 410 μg of protein, respectively, were dialyzed exhaustively against 0.1% (v/v) trifluoroacetic acid (TFA) (microflow dialysis using a BRL 1200 MA apparatus with 5−10 kDa cutoff dialysis membrane). Each sample was transferred to a reaction vessel using 0.1% (v/v) TFA, lyophilized (<50 mTorr, >24 h), and introduced to the GlycoPrep 1000, and the oligosaccharides were released and recovered using the program "N + O". An aliquot of this sample was reduced with a 5-fold molar excess of 6 mM $NaBT[^3H]_4$ (12.8 Ci/mmol) in 50 mM NaOH, adjusted to pH 11.0 with saturated boric acid, and incubated at 30 °C for 4 h. An equivalent volume of 1 M $NaBH_4$ in NaOH/boric acid, pH 11.0, was then added, and the incubation continued for another 2 h. The mixture was acidified to pH 4.5 with 1 M acetic acid and passed through a column of Dowex Ag 50 × 12 (H^+) at room temperature to remove sodium ions. The sample was eluted with water, evaporated to dryness at 27 °C, applied to Whatman 3MM chromatography paper and subjected to descending paper chromatography at 27 °C, 60% relative humidity for 18 h using 1-butanol/ethanol/water (4:1:1) as the solvent. The radiochromatogram was scanned using an LB230 Berthold radiochromatogram scanner, and the radioactivity remaining at the origin was subsequently eluted with water. No carbohydrates of disaccharide or larger size move in this solvent system under these conditions. This eluted material consisted of radiolabeled oligosaccharide alditols.

Monosaccharide Composition Analysis of FBS AChE and Eq BChE. Duplicate samples containing 50 μg of protein were exhaustively dialyzed against 0.1% (v/v) TFA. Each aliquot was then transferred to a reaction vessel and subjected to automated hydrazinolysis using the GlycoPrep 1000 to release intact oligosaccharides from the protein. This was followed by treatment with methanolic−hydrochloric acid at 75 °C for 16 h to liberate monosaccharides as methyl glycosides, followed by N-acetylation of any available primary amino groups, and the conversion of individual monosaccharides into trimethylsilylated O- (TMS) *O*-methyl glycosides. The TMS-methyl glycosides were separated on a GLC-MS system using a CP-SIL8 CB-coated fused silica column (0.32 mm × 25 m) from Chrompack as described (Patel et al., 1993). Identification of individual methyl glycosides was made by comparison with elution times and mass spectra of standard reference TMS methyl glycosides. Quantitation of the individual TMS-methyl glycosides was done by reference to an internal standard (*scyllo*-inositol) added to each sample prior to addition of methanolic HCl (Chaplin, 1982).

Charge-Distribution Analysis of the Oligosaccharide Alditols Released from FBS AChE and Eq BChE. Charge-based separation of the total pool of radiolabeled oligosaccharide alditols was performed using high-voltage paper electrophoresis in pyridine/acetic acid/water (pH 5.4, Whatman 3MM paper, 80 V/cm), before and after exhaustive digestions with neuraminidase from *Arthrobacter ureafaciens*. The oligosaccharide alditols (20−50 μM), were incubated with neuraminidase (1.0 unit/mL) in 0.1 M sodium acetate, pH 5.0, under a toluene atmosphere at 37 °C for 18 h. The reaction mixture was then passed through a column of Dowex AG 50 × 12 (H^+), and the eluant and washings were combined, rotary evaporated to dryness, and analyzed by high-voltage paper electrophoresis. The two radioelec-

trophoretograms were compared, and the relative molar content of neutral and acidic oligosaccharides in the total pool was determined by recovery of these from paper by elution with water and measurement of radioactivity in each pool.

High-pH Anion Exchange Chromatography−Pulsed Amperometric Detection (HPAEC−PAD) Profile of the Total Oligosaccharides Associated with FBS AChE and Eq BChE. The distribution of sialylated oligosaccharides in the total oligosaccharide pools obtained from FBS AChE and Eq BChE was determined by HPAEC−PAD (Lee et al., 1990) using a Dionex BioLC with data collection and analysis by Perkin Elmer Nelson Chromacol 2000 software as described (Patel et al., 1993). Samples were chromatographed on a Carbopac PA1 column (0.4 × 25 cm) at a flow rate of 1 mL/min using the following gradient:

time (min)	[NaOH], mM	[NaOAc], mM
0	100	0
45	100	75
80	100	210

PAD settings: $E_1 = +50$ mV, $E_2 = +600$ mV, $E_3 = -600$ mV, output = 1000 nA, 0.0005-in. gasket.

Size-Distribution Analysis of the Total Pool of Deacidified Alditols Released from FBS AChE and Eq BChE. An aliquot of the total pool of deacidified oligosaccharide alditols was mixed with an aqueous solution of a partial acid hydrolysate of dextran and subjected to high-resolution gel permeation chromatography using a Bio-Gel P4 (400 mesh) column (1.5 × 100 cm). The column was run using water as the eluant at 55 °C at a flow rate of 30 μL/min. The eluate from the column was monitored using an in-line radioactivity detector to detect radiolabeled sample as well as an in-line refractometer to detect individual glucose oligomers. The hydrodynamic volume of individual radiolabeled oligosaccharide alditols was determined from their elution position in reference to the glucose oligomers.

Structural Analysis of the Major Oligosaccharides Released from FBS AChE and Eq BChE. The structures of the major oligosaccharides released from FBS AChE and Eq BChE were determined by sequential exoglycosidase analysis using the following procedure (Patel et al., 1994). A total aliquot of the oligosaccharide alditol in salt-free aqueous solution was evaporated to dryness and resuspended in an appropriate volume of sterile buffer containing the desired amount of exoglycosidase. The reaction mixture was incubated under toluene at 37 °C for 18 h. At the end of the incubation period, the mixture was desalted and the glycosidase was removed using ion-exchange resins. The sample was evaporated to dryness and resuspended in an aqueous solution of a partial acid hydrolysate of dextran, and the solution was applied to a Bio-Gel P4 (∼400 mesh) gel filtration column. The eluate from the column was monitored using an in-line radioactivity flow detector as well as an in-line differential refractometer. The hydrodynamic volume of the individual radiolabeled oligosaccharide alditols was determined by their elution position in reference to the glucose oligomers. The change, if any, induced in the hydrodynamic volume of an oligosaccharide by a glycosidase of defined specificity allowed us to deduce the number and nature of the monosaccharide(s) at the non-reducing terminus of the oligosaccharide. This process was repeated sequentially using different glycosidases until the oligosaccharide was "sequenced" down to the monosaccharide alditol or a glycosidase-resistant structure was reached. The following exoglycosidases were used in these analyses: β-D-galactosidase (*Streptococcus pneumoniae*), β-*N*-acetyl-D-hexosaminidase (jack bean), β-*N*-acetyl-D-hexosaminidase (*S. pneumoniae*), α-D-mannosidase (jack bean), β-D-mannosidase (*Helix pomatia*), α-D-galactosidase (green coffee bean), and α-L-fucosidase (bovine epididymis). The hydrodynamic volumes in glucose units (gu) of monosaccharides relevant to this study are D-galactose, 1.0 gu; D-mannose, 0.8−1.0 gu; *N*-acetyl-D-glucosamine, 1.8−2.0 gu; *N*-acetylglucosaminitol, 2.5 gu; and L-fucose, 1.0 gu.

Desialylation and Deglycosylation of Native FBS AChE and Eq BChE. Deglycosylation of native FBS AChE and Eq BChE was achieved by incubating the enzymes (1 mg/mL) with a mixture of glycosidases (endo H, endo D, endo F, glycopeptidase F, neuraminidase, α-mannosidase, β-galactosidase, and α-L-fucosidase) from Boehringer Mannheim Biochemicals (Indianapolis, IN) in 0.1 M sodium phosphate, pH 6.5, for 24 h at 37 °C, as described by Hamos et al. (1987). Desialylation of samples was performed by incubating the enzymes (0.5 mg) with 2 units of neuraminidase from *Clostridium perfringens* in 0.1 M sodium phosphate, pH 6.5, for 24 h at 37 °C. Enzyme samples incubated under identical conditions in the absence of glycosidases were used as controls. There was essentially no loss of enzyme activity following these treatments. Aliquots of samples were analyzed by SDS−PAGE and Western blotted onto nitrocellulose membranes. The blots were stained with the digoxigenin glycan/protein double-labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The deglycosylated samples could only be detected by the protein stain (detection limit of 50 ng of protein) and not by the glycan-specific stain (detection limit of 10 ng of glycoprotein), suggesting that most of the carbohydrates had been removed by this treatment. Samples were separated from the glycosidases by sucrose density gradient centrifugation as described (Gentry et al., 1995). Fractions containing ChE activity were pooled and concentrated using Centricon 30 concentrators (Amicon, Beverly, MA).

Pharmacokinetic Studies. The experiments were carried out as described by Raveh et al. (1993). All enzyme samples were exhaustively dialyzed against sterile PBS, pH 7.4. Each enzyme (50−80 units in a volume of 0.1−0.2 mL) was administered intravenously into the tail vein of male albino ICR male mice (20−30 g each). At various time intervals, heparinized blood samples (5−10 μL) were withdrawn from the retro-orbital sinus of mice and diluted 20-fold in distilled water at 4 °C. ChE activity was determined using acetylthiocholine or butyrylthiocholine as the substrate for FBS AChE and Eq BChE, respectively (Ellman et al., 1961).

For native FBS AChE and all preparations of Eq BChE, the curves were fit to the data using a bi-exponential decay equation:

$$C_t = A_0\, e^{-k_1 t} + B_0\, e^{-k_2 t} \qquad (1)$$

where C_t is the blood concentration (units/mL) of the ChE at time t, A_0 and B_0 are the zero time coefficients of the fast and slow components, and k_1 and k_2 are the first-order elimination rate constants for the two phases, respectively. The fraction of the injected dose that cleared at fast and slow

Table 1: Monosaccharide Composition of FBS AChE and Eq BChE

	FBS AChE		Eq BChE	
monosaccharide	nmol/mg of protein[a]	nmol/nmol of subunit	nmol/mg of protein[a]	nmol/nmol of subunit
fucose	36.0	2.6	ND[b]	
mannose	133.0	9.5	383.0	34.8
galactose	102.0	7.3	245.0	22.3
N-acetylglucosamine	200.0	14.3	524.0	47.6
sialic acid	49.0	3.5	137.0	12.5
total[c]	520.0	37.2	1289.0	117.2

[a] Average of duplicate analyses. [b] ND, not detected. [c] Total carbohydrate calculated from the sum of the residue weights of the monosaccharides, per mg of protein.

rates was obtained by dividing A_0 and B_0, respectively, by the sum $A_0 + B_0$. Neuraminidase- and glycosidase-treated FBS AChE were eliminated from the circulation of mice by an apparent first-order kinetics, and eq 1 was reduced to

$$C_t = A_0 e^{-k_1 t} \quad (2)$$

The parameters A_0, B_0, k_1, and k_2, were obtained by nonlinear least-squares fitting of the data. The mean residence time (MRT) of ChE in circulation, a parameter that is independent of the distribution characteristic of the enzyme, was calculated as described (Gibaldi & Perrier, 1982).

RESULTS

Carbohydrate Composition of FBS AChE and Eq BChE. To investigate the types of carbohydrate units that were present in FBS AChE and Eq BChE, the two enzymes were subjected to monosaccharide composition analysis. From the saccharide analyses, it was apparent that a substantial portion (8−9% by weight) of FBS AChE and (23−24% by weight) of Eq BChE was in the form of carbohydrate (Table 1). The two enzymes contained mannose, galactose, *N*-acetylglucosamine, and sialic acid; the relative content of these monosaccharides was the same in both enzymes. Although generally similar in composition, Eq BChE differed from FBS AChE in that it did not contain any fucose. These results are different from those reported for *Electrophorus* AChE which did not contain any fucose (Bon et al., 1976) and human serum BChE which contained fucose (Haupt et al., 1966). The relatively high content of mannose suggested the presence of N-linked oligosaccharides, and the absence of *N*-acetylgalactosamine indicated the absence of O-linked oligosaccharides in both enzymes. These results are consistent with those reported earlier for AChE from mammalian brain and erythrocytes (Heider et al., 1991; Liao et al. 1991, 1992, 1993), BChE from human serum (Liao et al., 1992), and chicken brain and serum (Trestakis et al., 1992). Although the presence of N-linked carbohydrates in AChEs is well established, the presence of O-linked carbohydrates has been described only on the globular form of AChE from rat neuromuscular junction (Scott & Sanes, 1984) and AChE from various compartments of bovine chromaffin cells (Bon et al., 1990). The presence of galactose indicated that the glycans were of the complex or hybrid type rather than high-mannose type.

The total number of complex carbohydrate units per subunit, calculated from their mannose content (three residues of this sugar per complex oligosaccharide), was found to be three for FBS AChE and eleven for Eq BChE. The 11 glycans per subunit of Eq BChE is in agreement with the data reported previously (Teng et al., 1976). From the amino acid sequence reported for FBS AChE, five potential N-glycosylation sites have been identified (Doctor et al., 1990). The discrepancy in the number of potential N-glycosylation sites for FBS AChE predicted from its sequence and calculated from its mannose content may be reconciled by the fact that, although the tripeptide Asn-X-Thr/Ser is a consensus sequence for the attachment of N-linked glycans, it is not necessary for this site to be glycosylated (Geisow, 1992). From the amino acid sequence, nine N-glycosylation sites were identified in human serum BChE (Lockridge et al., 1987). Although the sequence of Eq BChE is not known, based on the known sequences of BChEs, it appears that the 11 N-glycosylation sites for Eq BChE, calculated from its mannose content, may be high.

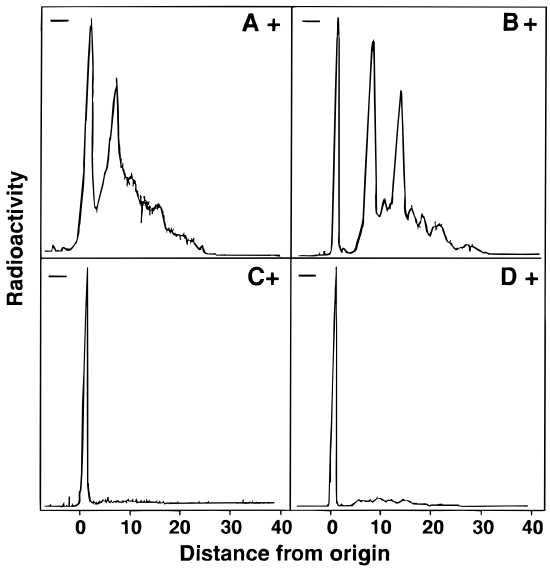

FIGURE 1: High-voltage paper electrophoresis of oligosaccharide alditols released from FBS AChE and Eq BChE. An aliquot of the total pool of radiolabeled oligosaccharides was subjected to high-voltage paper electrophoresis as described in Materials and Methods. The resulting radioelectrophoretograms for FBS AChE and Eq BChE are shown in panels A and B. An aliquot of radiolabeled alditols was treated with neuraminidase from *A. ureafaciens* before being subjected to high-voltage paper electrophoresis. The resulting radioelectrophoretograms for FBS AChE and Eq BChE are shown in panels C and D.

Nature of N-Linked Oligosaccharides of FBS AChE and Eq BChE. Aliquots of radiolabeled oligosaccharide alditols for FBS AChE and Eq BChE were subjected to high-voltage paper electrophoresis, and the resulting radioelectrophoretograms are shown in Figure 1A and 1B. The oligosaccharides associated with both enzymes consist of neutral and negatively charged components. The relative molar content of neutral and acidic oligosaccharides determined by recovery of radioactivity from paper was 28% and 72% for FBS AChE, compared to 19% and 81% for Eq BChE. To determine the nature of the acidic substituents, aliquots of the total pool of radiolabeled oligosaccharide alditols were incubated with neuraminidase from *A. ureafaciens*, which cleaves α2−3(6) bonds, and were then subjected to high-voltage paper electrophoresis. The resulting electrophoretograms are shown in Figure 1C and 1D. As shown, no acidic oligosaccharides were detectable after neuraminidase treatment. For both enzymes, the acidic substituent on the

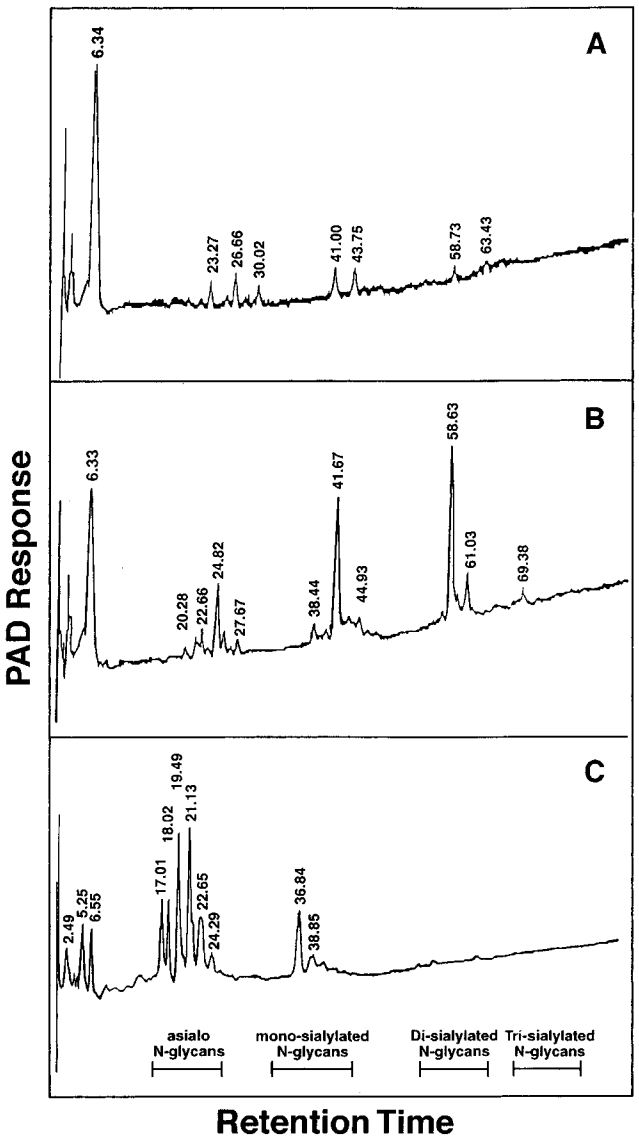

FIGURE 2: HPAEC-PAD profile of the total oligosaccharides associated with FBS AChE and Eq BChE. Aliquots of the total oligosaccharides associated with FBS AChE and Eq BChE were analyzed by HPAEC-PAD on a Carbopac PA1 column (0.4 × 25 cm) as described in Materials and Methods. The profiles are shown in panels A and B. Panel C shows the elution profile of the total oligosaccharide pool from human serum IgG, which was used to identify the positions of neutral, mono-, di-, and trisialyated *N*-glycans.

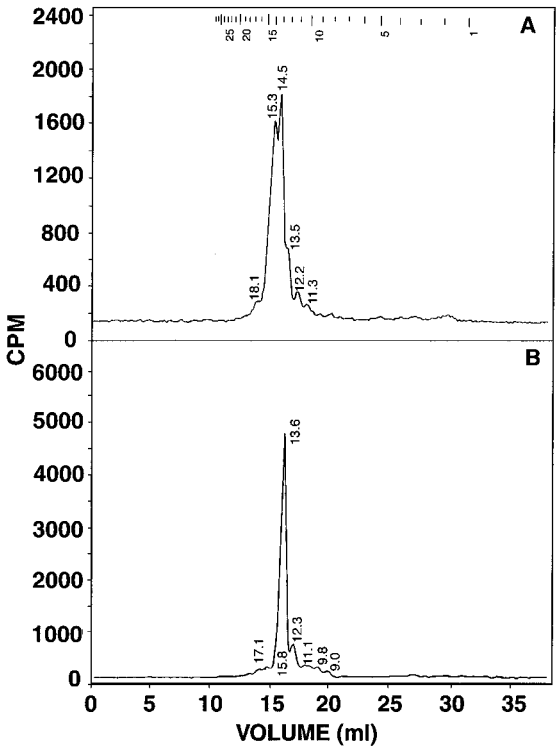

FIGURE 3: Gel permeation chromatography of the total pool of deacidified alditols released from FBS AChE and Eq BChE. An aliquot of the total pool of deacidified oligosaccharide alditols was subjected to high-resolution gel permeation chromatography using a Bio-Gel P4 column (1.5 × 100 cm) as described in Materials and Methods. The resulting chromatograms for FBS AChE and Eq BChE are shown in panels A and B. The eluate was monitored using an in-line radioactivity flow detector to detect radiolabeled sample and an in-line differential refractometer to detect individual glucose oligomers. Numerical superscripts in the figure represent the elution position of the non-radioactive, co-applied, glucose oligomers in glucose units (gu). The hydrodynamic volume of individual radiolabeled oligosaccharide alditols was measured in terms of glucose units, as calculated by cubic spline interpolation between the two glucose oligomers immediately adjacent to the oligosaccharide alditol.

oligosaccharide chain was a non-reducing, terminal sialic acid residue covalently linked to β-galactosyl residues at the C-3 or C-6 position. These results are in agreement with previous findings in which treatment with neuraminidase induced changes in the electrophoretic mobility of AChE from human erythrocytes (Ott et al., 1975) and BChE from human serum (Svensmark & Kristensen, 1963), equine serum (Svensmark & Heibronn, 1964) and human brain (Carlsen & Svensmark, 1970).

The distribution of sialylated oligosaccharides in the total oligosaccharide pools obtained from FBS AChE and Eq BChE was determined by HPAEC-PAD. The HPAEC−PAD profiles for FBS AChE and Eq BChE are shown in Figure 2A and 2B. The elution pattern of the total oligosaccharide pool from human serum IgG shown in Figure 2C was used as the reference to locate the positions of neutral, asialo, mono-, di-, and trisialylated *N*-glycans. The results demonstrate the presence of only mono- and disialylated *N*-glycans in FBS AChE; Eq BChE was found to contain mono-, di-, and possibly trisialylated *N*-glycans.

Fractionation of the total pool of oligosaccharide alditols obtained after neuraminidase digestion (i.e., neutral and desialylated acidic oligosaccharides combined) on the basis of their effective hydrodynamic volume by high-resolution gel permeation chromatography provided a basis for identifying the N-linked units present in FBS AChE and Eq BChE. The gel permeation chromatograms for FBS AChE and Eq BChE are shown in Figure 3A and 3B. At least two major (15.3 and 14.5 gu) and four minor (18.1, 13.5, 12.2, and 11.3 gu) distinct structural components for FBS AChE and one major (13.6 gu) and six minor (17.1, 15.8, 12.3, 11.1, 9.8, and 9.0 gu) distinct structural components for Eq BChE were identified.

Partial Structure of the N-Linked Complex Oligosaccharides of FBS AChE and Eq BChE. Individual fractions containing the two major oligosaccharides released from FBS AChE (15.3 and 14.5 gu, Figure 3A) and one major oligosaccharide from Eq BChE (13.6 gu, Figure 3B) were pooled, and the structures present within each fraction were obtained by sequential glycosidase digestions. The sensitivity of an oligosaccharide fraction to exoglycosidases (as

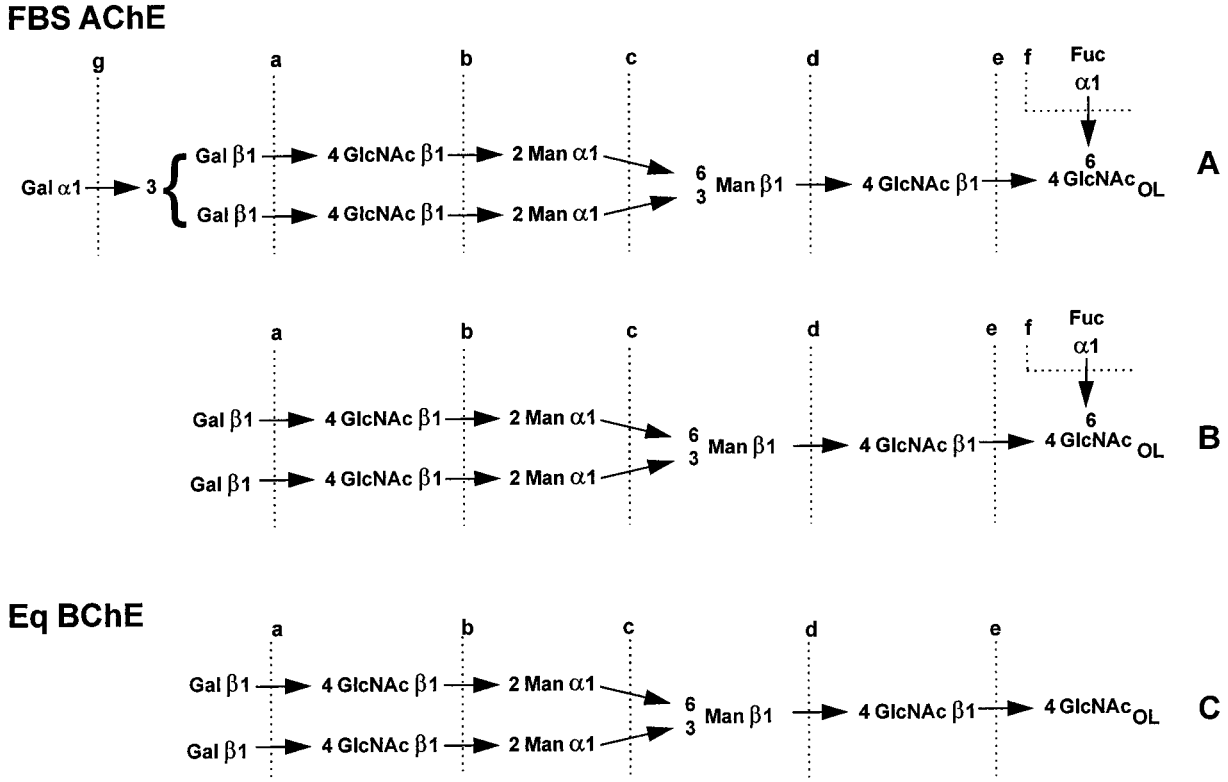

FIGURE 4: Structures of the major oligosaccharides present on FBS AChE and Eq BChE. Structural analysis was performed on individual fractions containing the two major oligosaccharides released from FBS AChE [15.3 gu (A) and 14.5 gu (B); see peaks in Figure 3A] and one major oligosaccharide from Eq BChE [13.6 gu (C); see peak in Figure 3B] by sequential exoglycosidase digestions. The following exoglycosidases were used: (a) *β*-D-galactosidase *(S. pneumoniae*), (b) *β-N*-acetyl-D-hexosaminidase (*S. pneumoniae*), (c) α-D-mannosidase (jack bean), (d) *β*-D-mannosidase (*H. pomatia*), (e) *β-N*-acetyl-D-hexosaminidase (jack bean), (f) α-L-fucosidase (bovine epididymis), and (g) α-D-galactosidase (green coffee bean). Changes in the hydrodynamic volume of oligosaccharide structures were affected by exoglycosidases when used in the following order: (A) a-b-g-a-b-c-d-f-e; (B) a-b-c-d-f-e; (C) a-b-c-d-e. The points of hydrolysis of each structure by individual exoglycosidases are indicated by dashed lines.

judged by a decrease in effective hydrodynamic volume), when these were used in the order summarized in Figure 4, allowed the glycosyl residue sequence and anomeric configuration of individual glycosidic linkages to be determined. The structural pattern of the complex oligosaccharides shown in Figure 4 was notable for its relative simplicity. The three carbohydrate structures were of the biantennary complex type, but only the ones from FBS AChE were fucosylated on the innermost *N*-acetylglucosamine residue of the core. This result is consistent with the previously noted absence of fucose in Eq BChE (Table 1). In addition to fucose, the oligosaccharides from FBS AChE also contained capping sugars in the form of sialyl- and α-galactosyl residues. In contrast, sialic acid was the only capping sugar present in oligosaccharides from Eq BChE. Recently the structure of the major oligosaccharide from human serum BChE, which had a hydrodynamic volume of 13.5 gu, was identified to be of the biantennary complex type (Okhura et al, 1994).

Pharmacokinetic Studies of FBS AChE and Eq BChE. The *in vivo* time course of FBS AChE and Eq BChE were determined in mice following an iv injection of 50−80 units of the native enzymes and their modified forms after treatment with neuraminidase or glycosidases. The decline in blood concentration of native FBS AChE and the three Eq BChE preparations (Figure 5) required more than one exponential term to characterize the time course, therefore the data were fit to eq 1. Neuraminidase- and glycosidase-treated FBS AChE were eliminated from the circulation of mice by apparent first-order kinetics, and data were analyzed according to eq 2. The parameters A_0, B_0, k_1, and k_2 are summarized in Table 2.

The macroscopic constants, k_1 and k_2, are actually composites of the true rate constants that characterize the individual elimination processes. Since the mechanism of clearance of ChEs from the circulation of animals is not established, the three main two-compartment pharmokinetic models are kinetically indistinguishable, and there was a difference in A_0/B_0 for various preparations, we compared the circulatory properties of the enzymes by using a noncompartmental analysis (Gibaldi & Perrier, 1982). The MRT of ChE in circulation, a parameter that is independent of the distribution characteristics of the enzyme, was calculated as described by Gibaldi & Perrier (1982) and summarized in Table 2. The areas under the concentration *versus* time curves (Figure 5) and under the product of concentration−time *versus* time curves (not shown) were computed using the parameters A_0, B_0, k_1, and k_2.

Depending on the ChE, treatment with neuraminidase or glycosidases resulted in approximately 10−40-fold decrease in MRT of the enzyme. The enhancement of clearance was significantly more pronounced with FBS AChE (MRT decreased from 1046 min to 26 min) compared to Eq BChE (MRT decreased from 1437 min to 150 min). These treatments did not change the multiphasic time course of Eq BChE in mice. The fraction of the fast component of native Eq BChE (A_0) increased from 0.27 to 0.55 and that of the slow component (B_0) decreased from 0.73 to 0.44 following treatment with neuraminidase. After treatment with gly-

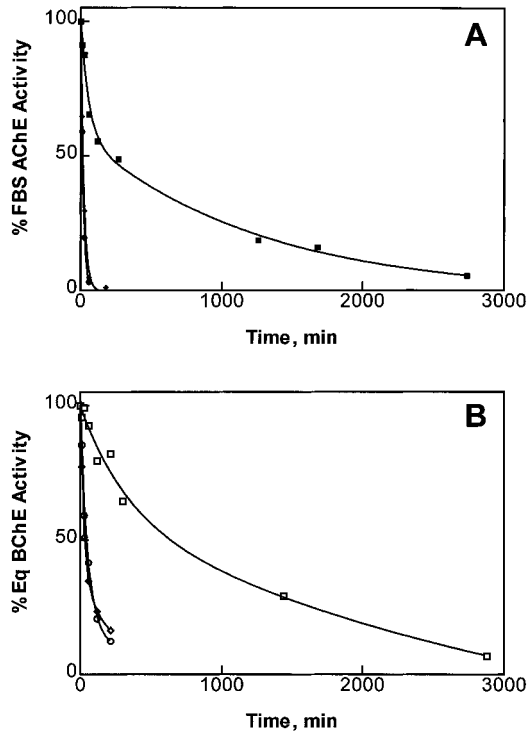

FIGURE 5: Time course of FBS AChE and Eq BChE in the circulation of mice. Stability of FBS AChE (panel A) and Eq BChE (panel B) in the circulation of mice following an iv injection of 50−80 units of ChE/animal are shown. Curves depict the time course of ChEs in individual mice and were generated in accordance with monoexponential (● and ◆) or biexponential decay equations (remaining curves). Each data point is an average of two measurements. Symbols represent native (■ and □), desialylated (● and ○), and deglycosylated (◆ and ◇) ChEs.

cosidases, A_0 increased to 0.68 and B_0 decreased to 0.32. Both modified enzymes cleared at a similar rate. In contrast, FBS AChE, after incubation with neuraminidase or glycosidases, showed an apparent homogenous clearance from the circulation that could be reasonably described by a single exponent. In general, these results are consistent with a substantial microheterogeneity in the carbohydrate of serum ChEs and confirm the importance of sialic acid in maintaining the long duration of FBS AChE and Eq BChE in blood. The difference in the time course profiles of deglycosylated FBS AChE and Eq BChE raise the possibility that, although the carbohydrate residues distinctively extend the biological half-lives of ChEs, they may contribute only in part to the longevity of ChEs in circulation.

DISCUSSION

Although the association of carbohydrates with ChEs is well documented, their role in enzyme function is not well established. Using site-directed mutagenesis studies, several laboratories have demonstrated that glycosylation is not necessary for the catalytic activity of these enzymes (Mutero & Fournier, 1992; Velan et al., 1993). This conclusion is also supported by the fact that binding to lectins did not affect the catalytic activity of soluble or membrane-bound forms of AChE from rat brain (Gurd, 1976) and that recombinant human AChE expressed in *Escherichia coli* was catalytically active (Fischer et al., 1993).

In a recent study the possible role of N-glycosylation and sialylation on the circulatory life-time of ChEs was investigated using recombinant human AChE (Kronman et al., 1995). The rate of clearance of recombinant human AChE was compared with that of plasma-derived ChEs such as FBS AChE and human serum BChE. All three enzymes cleared from the circulation of mice with biphasic kinetics consisting of an initial (fast) phase and a second (slow) phase. However the distribution of recombinant human AChE between the two phases was different from that of plasma-derived ChEs. Almost 75% of the wild-type recombinant AChE cleared in the first (fast) phase compared to 40% of the plasma-derived ChEs. The authors speculated that the rapid clearance of recombinant human AChE, which has three glycosylation sites, was due to the fewer number of N-glycans associated with this enzyme compared to FBS AChE. Comparison of the half-lives of circulation for various engineered forms of recombinant human AChE containing three to five N-glycosylation sites showed no correlation between the number of *N*-glycan moieties and the half-life in circulation. However, the rate of clearance of these glycoforms from the circulation of mice could be correlated with the number of unoccupied sialylation sites. These conclusions were arrived at without any knowledge of carbohydrate structures and their distribution in recombinant human AChE or human serum BChE and with no consideration of the preliminary data on the carbohydrate structure of FBS AChE (Saxena & Doctor, 1995).

It is well-known that the structure of the oligosaccharide chains on a glycoprotein is species- and cell type-specific. For example, studies with recombinant forms of tissue plasminogen activator and erythropoietin expressed in different cell lines suggest that although the type of oligosaccharide carried at each site is determined by the polypeptide itself, the precise structure of the oligosaccharide is determined by the cell type (Sasaki et al., 1987; Rademacher et al., 1988; Takeuchi et al., 1989; Parekh et al., 1989). Therefore, a polypeptide expressed in cell types other than that in which it is normally expressed will differ from the native glycoprotein with respect to the structure of certain oligosaccharides as well as the relative amounts of common oligosaccharides. These differences can have profound effects on the biological properties of the glycoprotein (Sasaki et al., 1987; Parekh et al., 1989).

To better understand the dependency of circulatory residence of plasma ChEs *in vivo* on the structure of the glycan moieties they carry, we determined the monosaccharide composition, oligosaccharide profile, and structures of the major glycans of FBS AChE and Eq BChE. The aim of this study was to identify specific types of oligosaccharide structures common to two different forms of ChEs from two different species that may be contributing to the extended mean residence time of these enzymes in circulation. Monosaccharide composition analysis revealed similarities as well species-related differences in the carbohydrate structures of the two ChEs. The absence of *N*-acetylgalactosamine suggested that both enzymes were associated with N-linked oligosaccharides only, and the presence of galactose indicated that these oligosaccharides were of the complex or hybrid type. In addition, substantial amounts of the oligosaccharides in FBS AChE were fucosylated. Charge-distribution analysis of the oligosaccharide pool suggested that 70−80% of these oligosaccharides in both enzymes were acidic in nature due to the presence of terminal outer-arm sialic acid residues. Fractionation of the total pool of oligosaccharide alditols obtained after neuraminidase diges-

Table 2: Pharmacokinetic Parameters[a] for the Clearance of FBS AChE and Eq BChE from the Circulation of Mice

		fast component[c]		slow component[c]		
enzyme[b]	treatment	A_0	$k_1 \times 10^2$ (min^{-1})	B_0	$k_2 \times 10^2$ (min^{-1})	MRT (min)
FBS AChE	none	0.32±0.08	3.07 ± 1.13	0.68 ± 0.08	0.096 ± 0.014	1046 ± 85
	neuraminidase	1.0[d]	4.52 ± 0.99			28.2 ± 12
	glycosidases	1.0[d]	4.20 ± 1.40			23.3 ± 5
Eq BChE	none	0.27 ± 0.08	0.73 ± 0.25	0.73 ± 0.08	0.082 ± 0.004	1437 ± 323
	neuraminidase	0.55 ± 0.06	6.30 ± 3.70	0.44 ± 0.06	0.67 ± 0.21	164 ± 64
	glycosidases	0.68 ± 0.01	5.05 ± 2.70	0.32 ± 0.09	0.64 ± 0.17	134 ± 27

[a] Values are mean ± SD ($n = 3$). [b] 50−80 units/mouse were injected iv. [c] Data were fit to eq 1. [d] Data were fit to eq 2.

tion (i.e., neutral and desialylated acidic oligosaccharides combined) on the basis of their effective hydrodynamic volume revealed two major (15.3 and 14.5 gu) oligosaccharides for FBS AChE and one major (13.6 gu) oligosaccharide for Eq BChE. The three carbohydrate structures determined by sequential glycosidase digestions were of the biantennary complex type, but only the ones from FBS AChE were fucosylated on the innermost N-acetylglucosamine residue of the core. In addition to fucose, the oligosaccharides from FBS AChE also contained capping sugars in the form of sialyl- and α-galactosyl residues. In contrast, sialic acid was the only capping sugar present on the oligosaccharides from Eq BChE. The structural characterization of glycans associated with human serum BChE published recently showed that 73% of the glycans were present as sialylated biantennary oligosaccharides and the remainder as tri- and tetraantennary oligosaccharides (Okhura et al., 1994).

The molar ratio of sialic acid to galactose residues on FBS AChE and Eq BChE was surprisingly found to be ∼0.5, suggesting that only half of the terminal galactose residues were capped with sialic acid. However, in the case of FBS AChE the presence of a galactose α1-3 galactose β1-4-determinant was detected in one of the major oligosaccharides indicating that α-galactose can be effectively utilized to cap the β-galactose residue. It is interesting to note that this disaccharide sequence has been identified as a potential immunogenic oligosaccharide determinant in secreted and cell surface glycoproteins of various non-primate mammalian species, prosimians, and New World monkeys but is absent in glycoproteins from man, apes, and Old World monkeys (Thall & Galili, 1990). This species-related difference in α-galactosylation has been attributed to the supression of specific α1→3 galactosyl transferase activity as a result of evolutionary events which occurred 20−30 million years ago (Galili et al., 1988).

In summary, structural studies on the carbohydrate moieties of FBS AChE and Eq BChE (this study) and human serum BChE (Okhura et al., 1994), the three plasma-derived ChEs with extended circulatory properties, showed that the major oligosaccharides present on these enzymes were of a sialylated biantennary type. However, 20−30% of the total oligosaccharide pools present on these enzymes were not capped with sialic acid. In the case of FBS AChE these oligosaccharides could be capped with α-galactose. The observation that only half of the galactose residues on both FBS AChE and Eq BChE are capped with sialic acid, and yet these serum-derived ChEs displayed a long duration in circulation, suggests that while sialylation was essential for the extended circulatory properties of these ChEs, capping was probably required only at certain glycosylation sites on the glycoprotein molecule. This conclusion is at odds with a report that attributed the long life time of FBS AChE in blood to an almost complete sialylation of an assumed five biantennary *N*-glycan chains (Kronman et al., 1995). These authors reported nine sialic acid residues per subunit of FBS AChE; our data showed an average of 3.5 sialic acid residues and 7.3 galactose residues per subunit which utilized only three of its five putative glycosylation sites. The observation that asialo FBS AChE cleared rapidly from the circulation of mice indicates that capping alone with α-galactose did not protect the enzyme from rapid elimination. The extended circulatory life time of Eq BChE lacking the α-galactose was remarkably high. Further, the MRT of asialo Eq BChE was significantly higher than that of asialo FBS AChE. We propose that sialylation is a key factor in maintaining FBS AChE and Eq BChE in circulation for long periods; however, complete sialylation of all galactose residues on serum-derived enzymes is not a prerequisite for extending the circulatory life time of these glycoproteins.

The detachment of carbohydrate constituents of FBS AChE and Eq BChE by treatment with neuraminidase or glycosidases resulted in sharp decreases in MRT as well as in significant shifts in the relative distribution of the fast and slow eliminated components. Most drugs exhibit multicompartment pharmacokinetics that are reflected in a multiexponential decline (Gibaldi & Perrier, 1982). The generally accepted explanation is that the multiphasic behavior is due to a rapid distributive phase that equilibrates a homogenous drug between the central compartment (i.e., blood) and other compartment(s). Such profiles reported previously for ChEs (Raveh et al., 1993; Kronman et al., 1995) were also observed here. In view of the substantial microheterogeneity of the oligosaccharides on FBS AChE and Eq BChE, shown here for the first time, it is tempting to speculate that the observed heterogenous kinetics of the clearance of ChEs from the circulation may be related, in part, to multiple glycoforms that are cleared at different rates. Although the mechanism for the uptake of ChEs by liver cells has not been established, the observation that the presence of 20−30% neutral oligosaccharides on the two enzymes is similar to the fractional size of the fast component (27−32%) and provides a clue to the heterogenous clearance of ChEs. The increase in the fast component fraction following induced desialylation is consistent with this observation. The multiphasic clearance of deglycosylated ChEs and the fact that native ChEs in plasma may be desialylated by endogenous sialidases complicate this explanation. It will be interesting to compare the A_0/B_0 ratio of tissue-derived ChEs from other sources, as well as recombinant ChEs, with the ratio of neutral to negatively charged oligosaccharide species present in these enzymes.

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