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Role of Oligosaccharides in the Pharmacokinetics of Tissue-Derived and Genetically Engineered Cholinesterases

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ABSTRACT

To understand the role of glycosylation in the circulation of cholinesterases, we compared the mean residence time of five tissue-derived and two recombinant cholinesterases (injected intravenously in mice) with their oligosaccharide profiles. Monosaccharide composition analysis revealed differences in the total carbohydrate, galactose, and sialic acid contents. The molar ratio of sialic acid to galactose residues on tetrameric human serum butyrylcholinesterase, recombinant human butyrylcholinesterase, and recombinant mouse acetylcholinesterase was found to be ~1.0. For *Torpedo californica* acetylcholinesterase, monomeric and tetrameric fetal bovine serum acetylcholinesterase, and equine serum butyrylcholinesterase, this ratio was ~0.5. However, the circulatory stability of cholinesterases could not be correlated with the sialic acid-to-galactose ratio. Fractionation of the total pool of oligosaccharides obtained after neuraminidase digestion revealed one major oligosaccharide for human serum butyrylcholinesterase and three or four major oligosaccharides in other cholinesterases. The glycans of tetrameric forms of plasma cholinesterases (human serum butyrylcholinesterase, fetal bovine serum acetylcholinesterase, and equine serum butyrylcholinesterase) clearly demonstrated a reduced heterogeneity and higher maturity compared with glycans of monomeric fetal bovine serum acetylcholinesterase, dimeric tissue-derived *T. californica* acetylcholinesterase, and recombinant cholinesterases. *T. californica* acetylcholinesterase, recombinant cholinesterases, and monomeric fetal bovine serum acetylcholinesterase showed a distinctive shorter mean residence time (44–304 min) compared with tetrameric forms of plasma cholinesterases (1902–3206 min). Differences in the pharmacokinetic parameters of cholinesterases seem to be due to the combined effect of the molecular weight and charge- and size-based heterogeneity in glycans.

ChEs are serine esterases that catalyze the hydrolysis of choline esters. The soluble plasma-derived ChEs from mammalian sources potentially can be used as pretreatment drugs for OP toxicity (Ashani *et al.*, 1991; Brandeis *et al.*, 1993; Broomfield *et al.*, 1991; Maxwell *et al.*, 1992; Raveh *et al.*, 1989, 1993, 1997; Wolfe *et al.*, 1992). Human BChE can alleviate succinylcholine-induced apnea (Lockridge, 1990) and may be used to detoxify ester bond-containing environmental toxins such as cocaine (Lockridge, 1990; Mattes *et al.*, 1996). The successful demonstration of plasma-derived ChEs as OP scavengers is attributed to their ability to sequester rapidly a wide variety of OPs and to their long residence time in circulation compared with ChEs of nonplasma origin (Raveh *et al.*, 1989, 1993, 1997).

It has been suggested that the relatively high stability of the globular tetrameric form of human plasma BChE may be associated with capping of the terminal carbohydrate residues with sialic acid (Douchet *et al.*, 1982). Kronman *et al.* (1995) showed that the macroscopic rate constants for the clearance of various engineered glycoforms of recombinant human AChE from the circulation of mice could be correlated with the number of unoccupied sialylation sites. It was argued further that the enhanced stability of HuS BChE and FBS AChE is due to an almost complete sialylation of the terminal glycan residues on these enzymes. However, a recent report from our laboratory showed that although sialylation was a key factor in maintaining FBS AChE and Eq BChE in the circulation of mice for long periods, complete sialylation of all galactose residues was not essential for extending the circulatory life-time of these glycoproteins. The molar ratio of sialic acid to galactose residues on FBS AChE and Eq BChE suggested that only half of the galactose residues were capped with sialic acid, yet these serum-derived ChEs displayed a mean residence time of ~20 hr in mice (Saxena *et al.*, 1997).

The disposition of glycoproteins in circulation is expected

ABBREVIATIONS: ChE, cholinesterase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; FBS, fetal bovine serum; mFBS AChE, monomeric fetal bovine serum acetylcholinesterase; tFBS AChE, tetrameric fetal bovine serum acetylcholinesterase; Eq, equine (serum); HuS, human serum; rHu, recombinant human; rMo, recombinant mouse; OP, organophosphate; TMS, trimethylsilyl; 2-AB, 2-aminobenzamide; gu, glucose units; MRT, mean residence time; V_p, plasma volume; V_{ss}, volume of distribution at steady state; CL, total body clearance; k_{el}, elimination rate constant.

to be influenced by the size, charge, shape, hydrophobicity, and number and type of carbohydrate chains on the protein. A straightforward correlation between the structures of glycans and pharmacokinetic data is complicated by the facts that the mechanism for the hepatic metabolism and renal clearance of ChEs is not established and there is a substantial microheterogeneity in the oligosaccharides. To date, the correlation between the structure of glycans and MRT has been made with only FBS AChE and Eq BChE. Recombinant ChEs may be used to probe the role of carbohydrates in the circulatory properties of the enzymes (Kronman *et al.*, 1995). However, the glycan structure of recombinant glycoproteins may be affected by the glycosylation site, type of mutation, expression system, and cell culture conditions in use (Goochee, 1992). The number and characteristics of the oligosaccharides on ChEs that are produced by methods of genetic engineering cannot be fully predicted from the sequence of the protein. Therefore, a rigorous structural analysis is required to understand the carbohydrate determinants that control the circulatory stability of ChEs.

In a continued effort to understand the role of glycosylation in the clearance of exogenously administered ChEs, we have examined in mice the dependence of the V_{ss} and MRT of five tissue-derived and two recombinant ChEs on the carbohydrates associated with these enzymes. The results provide for the first time the carbohydrate composition and oligosaccharide profiles of recombinant ChEs. The comparative pharmacokinetic study allowed us to examine the possible relationship among protein size, monosaccharide composition, fraction of acidic oligosaccharides, and circulatory stability of ChEs from diverse sources.

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 National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85–23, 1985).

Electrophoretically pure AChE from FBS was purified as described previously (De la Hoz *et al.*, 1986). The specific activity of this enzyme was 5600 units/mg. The monomeric and tetrameric forms of FBS AChE were resolved by gel permeation chromatography of purified FBS AChE on Biogel A 1.5-m column (1.5 × 170 cm) equilibrated with 50 mM sodium phosphate, pH 8.0. Purified BChE from human serum (specific activity, 750 units/mg) and the dimeric form of AChE from *T. californica* (specific activity, 2500 units/mg) were provided by Dr. Patrick Masson (Center de Recherches du Service de Santé des Armées, La Tronche, France) and Dr. Israel Silman (Weizmann Institute, Rehovot, Israel), respectively. Recombinant Hu BChE expressed in CHO cells was provided by Dr. Oksana Lockridge (University of Nebraska Medical Center, Omaha, NE). A soluble, monomeric form of rMo AChE truncated at its carboxyl-terminal end and expressed in HEK 293 cells was provided by Dr. Palmer Taylor (University of California at San Diego, La Jolla, CA). The specific activity of purified rHu BChE was 600 units/mg and that of rMo AChE was 1850 units/mg.

Release, isolation, labeling, and profile analysis of carbohydrates were performed by Oxford GlycoSciences according to the procedures described below:

Monosaccharide Composition Analysis of ChEs. The oligosaccharides associated with various ChEs were released quantitatively and recovered by automated hydrazinolysis (GlycoPrep 1000; Oxford GlycoSciences) as described previously (Saxena *et al.*, 1997). Samples of ChEs containing 0.15 mg of protein were subjected to exhaustive microflow dialysis against 0.1% (v/v) trifluoroacetic acid using a BRL (Natick, MA) 1200 MA apparatus with 5–10-kKDa cutoff dialysis membrane. Each sample was then transferred to a reaction vessel using 0.1% (v/v) trifluoroacetic acid, followed by lyophilization (<50 mTorr, >24 hr), and the oligosaccharides were released and recovered using the "N + O" program (GlycoPrep; Oxford Glycosciences). As controls, an aliquot of a standard monosaccharide mixture and a reagent "blank" were simultaneously analyzed.

The oligosaccharides were treated with methanolic HCl at 80° for 6 hr to liberate monosaccharides as methyl glycosides, followed by *N*-acetylation of any available primary amino groups and the conversion of individual monosaccharides into TMS-methyl glycosides. The TMS-methyl glycosides were separated on a gas liquid chromatography-mass spectometry system using a CP-SIL8 CB-coated fused silica column (0.32 mm × 25 m) from Chrompack (London, UK). Identification of individual methyl glycosides was by comparison with elution times and mass spectra of standard reference TMS-methyl glycosides. Quantification of the individual TMS-methyl glycosides was by reference to an internal standard (*scyllo*-inositol) added to both the mixture of standard monosaccharides and to each sample before the addition of methanolic HCl (Chaplin, 1982). The molar response factors of the individual TMS-methyl glycosides relative to *scyllo*-inositol were calculated for each monosaccharide standard. These response factors were then used to determine the absolute molar monosaccharide content of the aliquot of the ChEs that were analyzed, and assuming this to represent 0.15 mg, the absolute monosaccharide content/mg of protein was calculated.

Charge-Distribution Analysis of the Total Pool of Oligosaccharides Released from ChEs. The oligosaccharides released from various ChEs (400 μg) by automated hydrazinolysis were labeled with 2-AB according to the standard procedure used by Oxford GlycoSciences (Bigge *et al.*, 1995). The samples were applied to Whatman (Clifton, NJ) 3MM chromatography paper and subjected to ascending paper chromatography at ambient temperature for 30 min using 1-butanol/ethanol/water (4:1:1) as the solvent. The labeled sample remaining at the origin was eluted with water. Carbohydrates of disaccharide or larger size did not move in this solvent system under these conditions. This procedure lead to the quantitative and nonselective recovery of the total pool of oligosaccharides associated with the ChEs as 2-AB-labeled oligosaccharides.

Charge-based separation of the total pool of 2-AB-labeled oligosaccharides was performed using anion exchange high performance liquid chromatography on a GlycoSepC column and acetonitrile and ammonium acetate as eluants according to the standard experimental protocol. To determine the nature of the acidic substituents, an aliquot of the 2-AB-labeled oligosaccharides (20–50 μM) was digested exhaustively with neuraminidase from *Arthrobacter ureafaciens* (1.0 unit/ml) in 0.1 M ammonium acetate, pH 5.0, under a toluene atmosphere at 37° for 18 hr. An aliquot of the neuraminidase-treated oligosaccharides was analyzed by GlycoSep C chromatography, and the two chromatograms were compared. The relative molar content of neutral and acidic oligosaccharides in the total pool was determined by integration of chromatographic peaks.

Size-Distribution Analysis of the Total Pool of Deacidified Oligosaccharides Released from ChEs. An aliquot of the total pool of deacidified 2-AB-labeled oligosaccharides was mixed with an aqueous solution of a partial acid hydrolysate of dextran and subjected to high resolution gel permeation chromatography using the RAAM 2000. Water was used as the eluant at 55° at a constant flow rate of 80 μl/min over 10.6 hr. The eluate from the column was monitored using an in-line fluorescence flow detector to detect fluorescent glycans, as well as an in-line differential refractometer to detect individual glucose oligomers. The hydrodynamic volumes of individual 2-AB- labeled oligosaccharides were determined from their elution position in reference to the glucose oligomers. The conjugation of glycans with 2-AB decreases their hydrodynamic volume by a constant value, and the hydrodynamic volume of the

2-AB-conjugated form of glycan can be correlated with that of the alditol form using the following relationship (Bigge *et al.*, 1995): Hydrodynamic volume of 2-AB form = (1.02 × hydrodynamic volume of alditol) − 2.65.

Sucrose Density Gradient Sedimentation Analysis of the Molecular Forms of ChEs. Aliquots of various ChEs (5 units/100 μl) were mixed with catalase (11.3S, used as a sedimentation marker) and applied to linear 5–20% sucrose gradients prepared in 50 mM sodium phosphate, pH 8.0. The gradients were centrifuged at 75,000 × *g* for 18 hr at 4° in an SW41Ti rotor (Beckman Instruments, Fullerton, CA). Gradients were fractionated from the top using an AutoDensiflow IIC (Buchler Instruments, Lenexa, KS), and fractions were assayed for AChE activity using the micro-Ellman assay (Doctor *et al.*, 1987).

Pharmacokinetic Studies. The experiments were carried out as described previously (Raveh *et al.* 1993). All enzyme samples were exhaustively dialyzed against sterile phosphate-buffered saline, pH 7.4. Each enzyme (39–320 units in a volume of 0.1–0.2 ml) was administered intravenously into the tail vein of Balb/c male mice (22–36 g each). These doses were sufficient to increase the plasma concentration of ChEs well above the level of endogenous ChE. At various time intervals, heparinized blood samples (5–10 μl) were withdrawn from the retro-orbital sinus of mice and diluted 21-fold in distilled water at 4°. ChE activity was determined using acetylthiocholine or butyrylthiocholine as the substrate for AChE or BChE, respectively, using the assay of Ellman *et al.* (1961). Endogenous ChE activity was subtracted from the result. The activity was normalized to plasma volume assuming that it constituted 55% of the whole blood volume.

The MRT, V_{ss}, CL, and terminal-phase rate constant (i.e., k_{el}) for various ChEs were obtained by analyzing the plasma-versus-time (units/ml) data using a Windows-based program for noncompartmental analysis of pharmacokinetic data (Laub and Gallo, 1996).

Curves were fit to the time-course data points using a biexponential decay equation:

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

where C_t is the plasma concentration (units/ml) of the ChE at time t, A_0 and B_0 are the zero time activities 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 fractions of the injected dose of ChEs that cleared at fast and slow rates were obtained by dividing A_0, and B_0, respectively, by the sum $A_0 + B_0$. The parameters A_0, B_0, k_1, and k_2, were obtained by nonlinear least-squares fitting of the data.

***In Vitro* Stability of ChEs in Mouse Blood.** Stock solutions of various ChEs were diluted in mouse blood collected above heparin to a final concentration of 13–27 units/ml and incubated at 37°. Any changes in enzyme activity with time were monitored using the Ellman assay, as described above (Ellman *et al.*, 1961).

Results

Monosaccharide Composition Analysis. To determine the type of carbohydrate units that were present in various ChEs, enzymes were subjected to monosaccharide composition analysis. HuS BChE contained the greatest amount of carbohydrate (31% by weight of protein) compared with other plasma-derived ChEs, such as tFBS AChE and Eq BChE, which contained 9% and 23% carbohydrate by weight, respectively (13). *T. californica* AChE, mFBS AChE, rMo AChE, and rHu BChE contained 9%, 9%, 10%, and 13% carbohydrate by weight of protein, respectively (Table 1). Of the total monosaccharides present in all ChEs, 33–40% was present as *N*-acetylglucosamine and 21–31% were present as mannose. All ChEs contained 18–21% carbohydrate in the form of galactose except *T. californica* AChE, which contained only 8% galactose. HuS BChE, rMo AChE, and rHu BChE contained 15–18% of monosaccharides as sialic acid, whereas mFBS AChE (10.8%) and *T. californica* AChE (4.9%) were significantly undersialylated. The molar ratio of sialic acid to galactose residues on HuS BChE, rHu BChE, and rMo AChE was found to be ~1.0. For *T. californica* AChE, mFBS and tFBS AChE, and Eq BChE, this ratio was ~0.5. As shown in Table 2, the total number of complex carbohydrate chains/subunit calculated from their mannose content was 12 for HuS BChE, 4 for *T. californica* AChE, 3 for mFBS AChE, 3 for rMo AChE, and 5 for rHu BChE.

Nature of *N*-Linked Oligosaccharides of ChEs. Charge-based separation of the 2-AB-labeled oligosaccharides associated with various ChEs was performed using anion exchange high performance liquid chromatography on a GlycoSep C column. The resulting chromatograms are shown in Fig. 1, A, C, E, G, and I. The oligosaccharides associated with all ChEs consist of neutral as well as acidic components. The relative content of neutral and acidic oligosaccharides for various ChEs is listed in Table 2. HuS BChE contained 84% acidic oligosaccharides, similar to the value of 81% reported previously for Eq BChE (Saxena *et al.*, 1997). The two recombinant ChEs contained 60–70% acidic oligosaccharides. In contrast, the other two tissue-derived AChEs, *T. californica* AChE and mFBS AChE, contained only 30–40% acidic oligosaccharides. To determine the nature of the acidic substituents, an aliquot of the 2-AB-labeled oligosaccharides (20–50 μM) was digested exhaustively with neuraminidase from *A. ureafaciens*, which cleaves α2–3(6) bonds, and then analyzed by GlycoSep C chromatography. The resulting chromato-

TABLE 1
Monosaccharide composition of various recombinant and tissue-derived ChEs

Monosaccharide	Fucose	Mannose	Galactose	*N*-Acetyl galactosamine	*N*-Acetyl glucosamine	Sialic acid	Total[b]	Carbohydrate
				nmol/mg of enzyme[a]				*% by weight*
Eq BChE[c]	N.D.[d]	383	245	N.D.	524	137	1289	23
HuS BChE	24	428	333	20	613	309	1727	31
tFBS AChE[c]	36	133	102	N.D.	200	49	520	9
T. californica AChE	50	156	40	8	198	23	475	9
mFBS AChE	41	119	104	12	170	54	500	9
rMo AChE	40	124	103	19	193	93	572	10
rHu BChE	58	165	128	19	238	110	718	13

[a] Average of duplicate analyses.
[b] Total carbohydrate calculated from the sum of the residue weights of the monosaccharides/mg of protein.
[c] Data from Saxena *et al.* (1997).
[d] N.D., not detected.

TABLE 2
Number of glycans and content of acidic oligosaccharides in ChEs

Enzyme	Glycans[a]	*N*-Glycosylation sites[b]	*N*-Acetyl glucosamine	Galactose	Sialic acid	Nonsialylated galactose[c]	Fraction of acidic oligosaccharides[d]
	n		*nmol/nmol of subunit*				
Eq BChE[e]	11	8[f]	43.6	20.4	11.4	10.0	0.81
HuS BChE	12	9	51.0	27.8	25.8	2.0	0.84
tFBS AChE[e]	3	5	14.3	7.3	3.5	2.8	0.72
T. californica AChE	4	4	14.1	2.9	1.6	1.3	0.32
mFBS AChE	3	5	12.1	7.4	3.8	2.6	0.43
rMo AChE	3	3	13.8	7.4	6.7	0.7	0.62
rHu BChE	5	9	19.8	10.7	9.1	1.6	0.72

[a] Calculated from mannose content (three residues of this sugar/complex oligosaccharide).
[b] Predicted from published protein/cDNA sequences.
[c] Difference between galactose and sialic acid content.
[d] Calculated from area under the peaks in Fig. 1.
[e] Data from Saxena *et al.* (1997).
[f] Doctor BP, unpublished observation.

grams are shown in Fig. 1, B, D, F, H, and J. No acidic oligosaccharides were detectable after neuraminidase treatment. Therefore, in both cases, the acidic substituent on the oligosaccharide chain was a covalently linked nonreducing, terminal outer-arm sialic acid residue. These results are in agreement with previous findings with FBS AChE and Eq BChE (Saxena *et al.*, 1997). The minor peak in Fig. 1, B and H, is due to the separation of low-molecular-weight glycans from the major oligosaccharide pool.

Fractionation of the total pool of deacidified 2-AB-labeled oligosaccharides obtained after neuraminidase treatment by high resolution gel permeation chromatography provided a basis for identifying the *N*-linked units present in various ChEs. Gel permeation chromatograms for various ChEs are shown in Fig. 2. One major (11.2 gu) and several minor distinct structural components for HuS BChE were identified (Fig. 2A). mFBS AChE contained at least four major components at 14.1, 13.0, 12.2, and 11.2 gu (Fig. 2B); *T. californica* AChE contained at least three major structural components at 11.2, 10.4, and 6.7 gu (Fig. 2C); rMo AChE contained four major structural components at 17.4, 14.6, 12.9, and 12.2 gu (Fig. 2D); and rHu BChE contained four structural components at 17.4, 14.6, 12.2, and 11.1 gu (Fig. 2E).

Sucrose Density Centrifugation Analysis. The molecular forms of various ChEs used in this study were determined by sucrose density gradient centrifugation analysis shown in Fig. 3. As shown in Fig. 3, A and C, HuS BChE and FBS AChE, the two ChEs derived from plasma sources were tetramers, *T. californica* AChE was dimeric in form (Fig. 3E), and mFBS AChE is shown in Fig. 3D. Of the two recombinant ChEs tested, rHu BChE was a mixture of monomers, dimers, and tetramers (Fig. 3B), whereas rMo AChE was in monomeric form only (Fig. 3F).

Pharmacokinetic Studies. After intravenous injection, plasma activity of all ChEs declined in two phases, and curve fitting was carried out in accordance with the equation (Fig. 4). The time course profiles show that tFBS AChE had a longer half-life than mFBS AChE and that tetrameric native HuS BChE had a longer half-life than rHu BChE. Results from seven ChEs examined in Balb/c mice, including tFBS AChE and Eq BChE, did not reveal any clear-cut relationship between the coefficients A_0 and B_0 and the number, composition, or charge of the glycan chains on ChEs (not shown).

To permit a meaningful correlation between the pharmacokinetic characteristics and the structural features of ChEs from various sources, we compared their circulatory properties by determining V_{ss}, MRT, CL, and k_{el}, using a noncompartmental analysis. This approach is independent of both the compartmentalization characteristics of the enzyme and the composite A_0/B_0 ratio (21) and provides a more comprehensive analysis of the time course data. The results are summarized in Table 3. V_{ss} was compared with the initial volume of distribution that is approximated by the plasma volume (V_p). A 1.1–1.4-fold increase in V_{ss} over V_p was observed for plasma-derived tetrameric forms of Eq BChE, HuS BChE, and tFBS AChE. The less stable enzymes, mFBS AChE, rMo AChE, and rHu BChE, clearly equilibrated with a slightly larger volume ($V_{ss}/V_p \sim 2$). The plots of V_{ss} versus the percent acidic fraction of the glycans on each subunit and against the apparent molecular weight of ChEs support such a dependence (Fig. 5, A and C).

Fig. 5A reveals that the tested ChEs fall into two groups; the first group consists of tetrameric enzymes (Eq BChE, HuS BChE, and tFBS AChE), and the second group consists of monomeric and dimeric enzymes (*T. californica* AChE, mFBS AChE, rHu BChE, and rMo AChE). The V_{ss} value of each group was equally affected by the amount of the acidic component contributed by the carbohydrate chains. Fig. 5C indicates that V_{ss} value approaches a limiting value as the molecular weight increases. rHu BChE was omitted from Fig. 5, C and D, because it is a mixture of monomers, dimers, and tetramers (Fig. 3B), which precluded the assignment of a definite molecular weight. The dimeric form of native *T. californica* AChE displayed a V_{ss}/V_p value similar to that observed for the tetrameric ChEs. In general, it seems that V_{ss} depends on both the molecular weight and the fraction of acidic oligosaccharides of these enzymes.

The parameters for disposition kinetics, MRT, CL, and the elimination half-life for all ChEs essentially followed a similar ranking order (Table 3). This permitted us to separate the enzymes into two major groups according to their total body residence time. As indicated above for V_{ss}, one group contains the tetrameric forms of plasma-derived ChEs, Eq BChE, HuS BChE, and tFBS AChE, which are characterized by extended durations in the body (MRT $\sim$ 1902–3206 min). The second group contains mFBS AChE, rMo AChE, and rHu BChE, with MRT values of 205–304 min, which are 6–15-fold shorter than the tetrameric enzymes. The plots of MRT versus the percent acidic fraction of the oligosaccharides (Fig. 5B) can be drawn two ways. If the recombinant enzymes (rMo AChE and rHu BChE) are omitted, the dependence of MRT on the acidic components of the carbohydrates parallels that

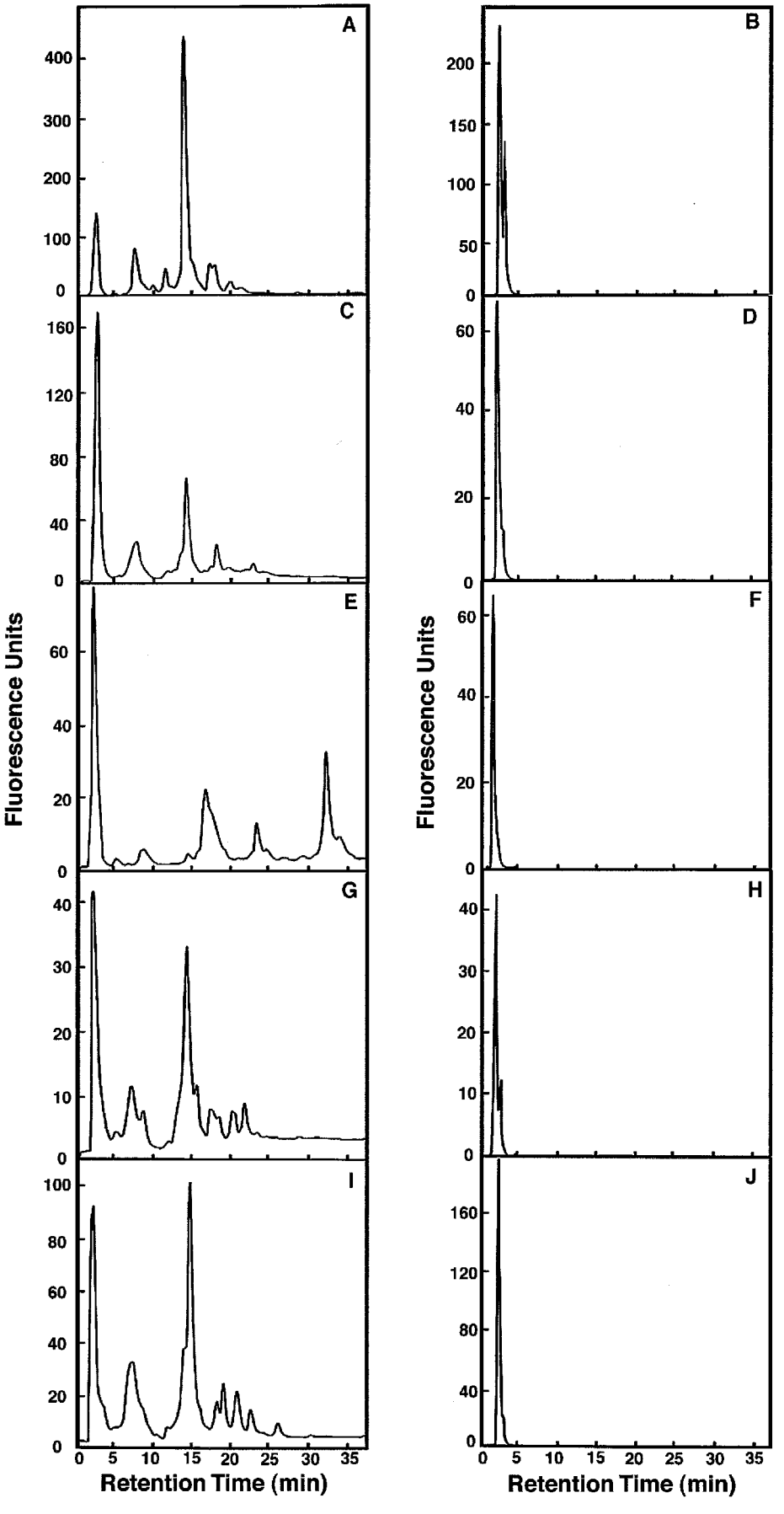
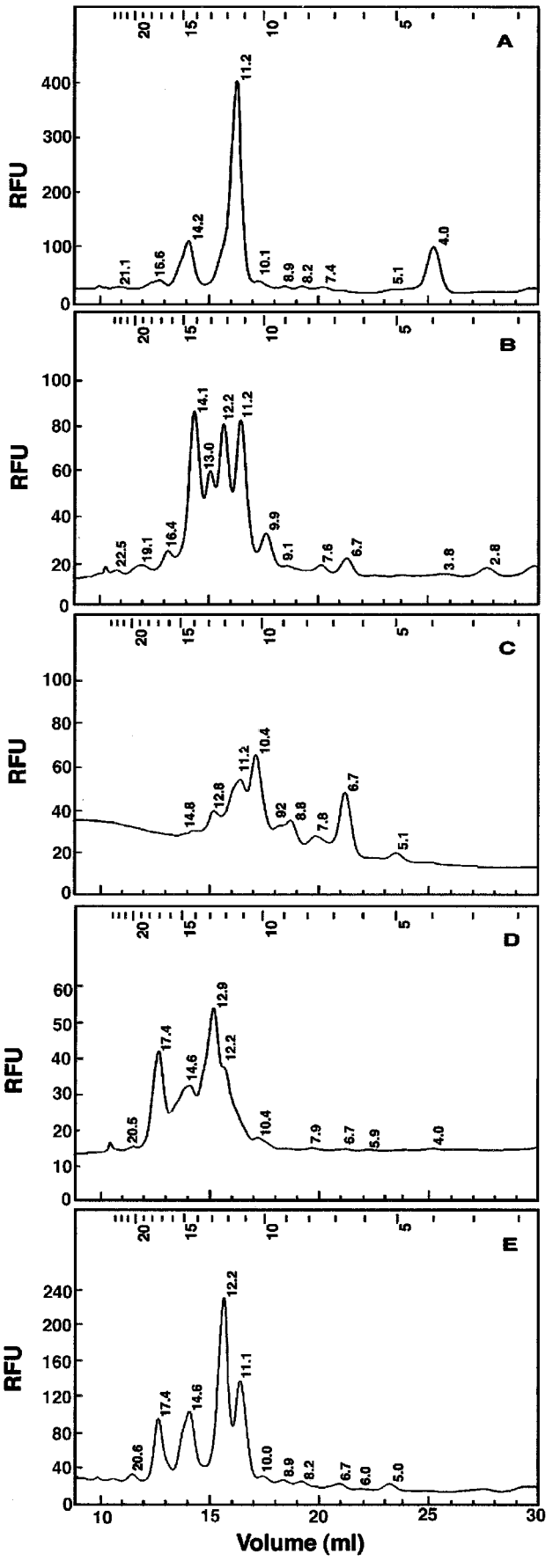


Fig. 1. GlycoSepC anion exchange chromatography of oligosaccharides released from ChEs. An aliquot of the total pool of 2-AB-labeled oligosaccharides was subjected to GlycoSepC anion exchange chromatography as described in Materials and Methods. A, C, E, G, and I, Resulting chromatograms for HuS BChE, mFBS AChE, *T. californica* AChE, rMo AChE, and rHu BChE. An aliquot of 2-AB-labeled oligosaccharides was treated with neuraminidase from *A ureafaciens* before being subjected to anion exchange chromatography; the resulting chromatograms are shown (B, D, F, H, and J, respectively). The peaks eluting at 1–5 min were neutral oligosaccharides, and the peaks eluting at 6–34 min were acidic oligosaccharides.



of the MRT versus molecular weight plot (Fig. 5D). When the low-molecular-weight ChEs are connected (*dotted line*), the enzymes are separated into the same two groups shown in Fig. 5A. In general, MRT and V_{ss} were reasonably correlated with the molecular weight of the proteins that reflect the assembly of the ChE subunits and with the fraction of sialylated oligosaccharides. It should be pointed out that a 2-fold difference in the MRT of tFBS AChE and Eq BChE was observed between ICR (Saxena *et al.*, 1997) and Balb/c mice, suggesting species-related differences in the clearance of ChEs.

The CL is the proportionality factor for the dependence of the rate of elimination on the concentration of ChE (rate of elimination = CL × [ChE]) and was obtained by dividing the injected dose by the area under the concentration-time curve (CL = dose/area under the concentration-time curve) (Gibaldi and Perrier, 1982). It is independent of the concentration of ChE and therefore provides a useful parameter for a comparison of the clearance rate of various ChEs that possess the same plasma concentration. For example, according to Table 3, if the concentration of ChE is 1 μg/ml, the rate of elimination for the tetrameric enzyme is 1.3 μg/hr/kg, and the rates for mFBS AChE, rMo AChE, rHu BChE, and *T. californica* AChE are 16.2, 21.6, 33.0, and 72.0 μg/hr/kg, respectively. The half-life of the terminal phase reflects the combinatorial contribution of CL and V_{ss} to the residence time of the ChE. For example, the CL of tFBS AChE was found to be 12.5-fold slower than that of mFBS AChE, whereas the elimination half-life ratio viewed from the plasma terminal phase shows that the clearance of tFBS AChE was slowed by only 5.6-fold. This observation is consistent with the fact that tFBS AChE is distributed in a smaller volume compared with mFBS AChE, and an increase in elimination of an enzyme that is restricted to a smaller volume is to be expected. Similarly, the CL ratio of rHu BChE to HuS BChE is 25.4 compared with the elimination half-life increase of 11.7. Thus, as observed with mFBS AChE, the higher volume of distribution of rHu BChE partially compensates for the large differences in CL.

***In Vitro* Stability of ChEs in Mouse Blood.** No significant inactivation of Eq BChE, HuS BChE, tFBS AChE, and rMo AChE was detected after a 24-hr incubation of these enzymes in mouse blood at 37°. mFBS AChE, *T. californica* AChE, and rHu BChE were less stable and lost 30–50% of the original activity within 24 hr. Due to the rapid clearance of these enzymes, the apparent blood-induced inactivation did not significantly affect interpretation of the pharmacokinetic data. However, it suggests that the carbohydrates may control in part the susceptibility of ChEs to proteolytic degradation.

Fig. 2. Gel permeation chromatography of the total pool of deacidified alditols released from ChEs. An aliquot of the total pool of deacidified oligosaccharides was subjected to high resolution gel permeation chromatography using a BioGel P4 column (1.5 × 100 cm) as described in Materials and Methods. A–E, Resulting chromatograms for HuS BChE, mFBS AChE, *T. californica* AChE, rMo AChE, and rHu BChE. The eluate was monitored using an in-line fluorescence flow detector to detect 2-AB-labeled sample and an in-line differential refractometer to detect individual glucose oligomers. *Superscripted numbers*, elution position of the 2-AB-labeled, coapplied glucose oligomers (gu). The hydrodynamic volume of individual 2-AB-labeled oligosaccharides was measured in terms of glucose units, as calculated by cubic spline interpolation between the two glucose oligomers immediately adjacent to the oligosaccharide. RFU, relative fluorescence units.

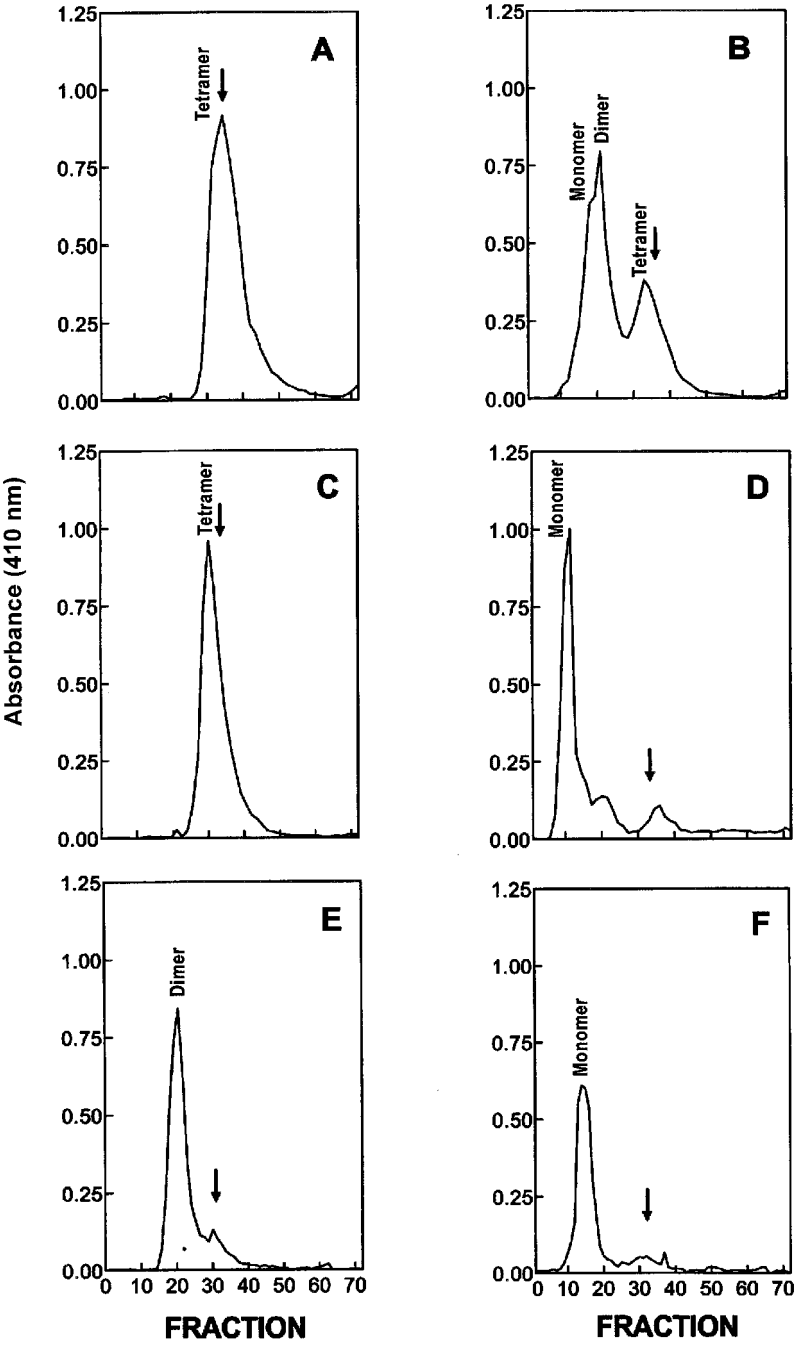


Fig. 3. Sucrose density gradient sedimentation analysis of the molecular forms of ChEs. Aliquots of various ChEs (5 units/100 μl) were mixed with catalase (11.3S, used as a sedimentation marker) and applied to linear 5–20% sucrose gradients prepared in 50 mM sodium phosphate, pH 8.0. The gradients were centrifuged at 75,000 · *g* for 18 hr at 4° in an SW41Ti rotor. Gradients were fractionated from the top, and the fractions were assayed for ChE activity using the micro-Ellman assay (Doctor *et al*, 1987). The molecular forms of various ChEs are shown as follows: (A) HuS BChE; (B) rHu BChE; (C) tFBS AChE; (D) mFBS AChE; (E) *T. californica* AChE, and (F) rMo AChE. *Left*, top of the gradient. *Arrow*, position of catalase.

Discussion

The successful application of native and recombinant ChEs as detoxifying drugs largely depends on their ability to remain at therapeutic plasma levels for prolonged periods. The aim of this study was to highlight the structural features of ChEs that may determine the circulatory fate of these enzymes. The integrity of the oligosaccharide chains on native ChEs has been demonstrated to be essential for maintaining enzyme activity in circulation (Douchet *et al.*, 1982; Saxena *et al.*, 1997). Therefore, we attempted to elucidate the structural/functional relationship between the carbohydrates and the pharmacokinetic behavior of ChEs. Two major questions were addressed: Does the ratio of sialic acid to galactose correlate with the pharmacokinetics of ChEs? To what extent can the major differences in the glycan composition be correlated with the volume of distribution and the total body MRT of ChEs in mice? The availability of a variety of ChEs also permitted an analysis of the relationship between subunit organization and the disposition behavior of the enzyme.

Monosaccharide composition analysis revealed differences in the total carbohydrate, galactose, and sialic acid contents of various ChEs. The relatively high content of mannose suggested the presence of *N*-linked oligosaccharides, and the presence of *N*-acetylgalactosamine indicated the presence of *O*-linked oligosaccharides in the ChEs examined. It seems that recombinant ChEs possess a higher amount of *O*-linked oligosaccharides than tissue-derived ChEs. Although the presence of *N*-linked carbohydrates in AChEs is well estab-

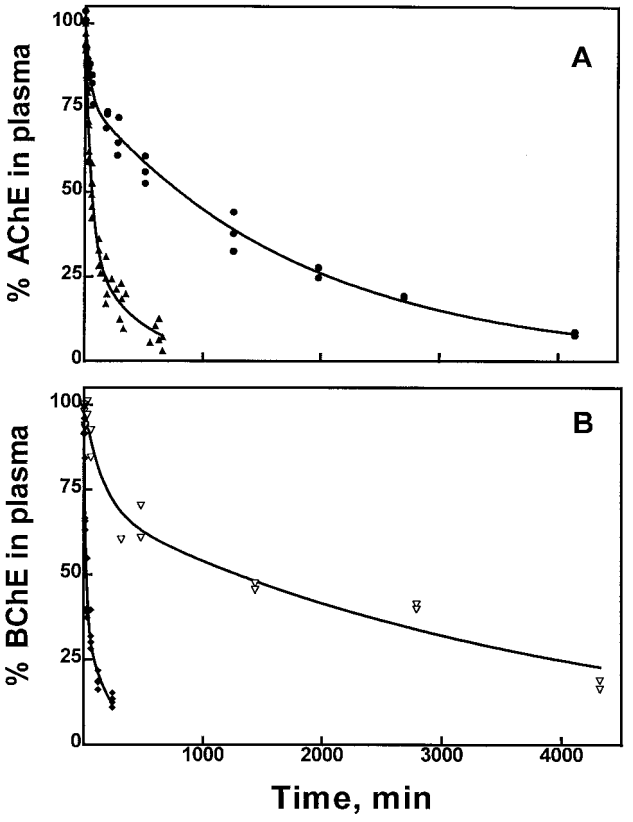


Fig. 4. Time course of ChEs in the circulation of mice. Individual time courses of ChEs following their intravenous injection into the tail vein of Balb/c mice are shown. A, tFBS AChE (●, 100 units/animal; three experiments) and mFBS AChE (△, 100 units/animal; six experiments). B, HuS BChE (▽, 39 units/animal; two experiments) and rHu BChE (♦, 60 units/animal; four experiments). Curve fitting was carried out in accordance with the equation. Percent ChE activity was calculated by dividing the plasma activity at time = t by the activity at $t = 0$ (obtained by extrapolating the curve to $t = 0$).

lished (Heider *et al.*, 1991; Liao *et al.*, 1992, 1993), the presence of *O*-linked carbohydrates has been described only on the globular form of AChE from rat neuromuscular junction (Scott and Sanes, 1984) and AChE from various compartments of bovine chromaffin cells (Bon *et al.*, 1990). The presence of galactose indicated that the majority of glycans in all ChEs except *T. californica* AChE were of the complex or hybrid type rather than the high-mannose type. In addition, substantial amounts of the oligosaccharides in all ChEs except Eq BChE were fucosylated (Saxena *et al.*, 1997).

On the basis of the amino acid sequences reported for various ChEs, nine *N*-glycosylation sites for human BChE (Lockridge *et al.*, 1987), four potential *N*-glycosylation sites for *T. californica* AChE (Schumacher *et al.*, 1986), five sites for FBS AChE (Doctor *et al.*, 1990), and three sites for rMo AChE (Rachinsky *et al.*, 1990) have been identified. The number of *N*-glycosylation sites for HuS BChE (12) and Eq BChE (11), as calculated on the basis of mannose content, is greater than the number predicted from the sequence. This result suggests that in addition to complex oligosaccharides, these enzymes contain high-mannose oligosaccharides. The number of *N*-glycosylation sites of three for mFBS AChE is in agreement with the number calculated on the basis of the mannose content for tFBS AChE (Saxena *et al.*, 1997).

The molar ratio of sialic acid to galactose residues on HuS BChE, rMo AChE, and rHu BChE was ~1.0, suggesting that all the terminal galactose residues were capped with sialic acid. However, the MRT of HuS BChE was 9- and 14-fold greater than that of rMo AChE and rHu BChE, suggesting that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability to ChEs. For *T. californica* AChE (MRT, 44 min) and mFBS AChE (MRT, 304 min), this ratio was ~0.5, suggesting that only half of the terminal galactose residues were capped with sialic acid, yet these enzymes differed greatly in their circulatory stability. In contrast, a molar ratio of 0.5 for sialic acid to galactose was previously observed for the highly stable tFBS AChE and Eq BChE (Saxena *et al.*, 1997). The lack of a correlation between the sialic acid-to-galactose ratio and MRT also was reported for human IgM monoclonal antibodies (Maiorella *et al.*, 1993). These observations substantiate the previous suggestion that although the presence of sialic acid seems to be essential for maintaining ChEs in circulation, the location rather than the number of the nonsialylated galactose residues may be affecting circulatory stability (Saxena *et al.*, 1997).

Differences in oligosaccharides of ChEs from various sources and the microheterogeneity in glycans on each ChE were elucidated by charge- and size-based separation analyses. Anion exchange chromatography of the oligosaccharide pools suggested that these ChEs differ substantially in the amount of negatively charged glycans they carry. Fractionation of the total pool of desialylated oligosaccharides on the basis of their effective hydrodynamic volume revealed only one major oligosaccharide for HuS BChE. This result is in agreement with previous studies in which one major component for HuS BChE at 11.2 gu (13.5 gu for the alditol) and for Eq BChE at 11.3 gu (13.6 gu for the alditol) was identified. The structure of this glycan was determined to be of the complex biantennary type (Ohkura *et al.*, 1994; Saxena *et al.*, 1997). The elution position of the oligosaccharide for HuS BChE suggests that its structure may be of the complex biantennary type. The two major oligosaccharides for tFBS AChE also were found to be of the complex biantennary type (Saxena *et al.*, 1997).

In contrast, size-based fractionation of the desialylated oligosaccharide pools yielded three or four major oligosaccharides for *T. californica* AChE, mFBS AChE, rMo AChE, and rHu BChE. The glycans eluting at 11.2 and 12.2 gu are most likely of the complex biantennary type (Saxena *et al.*, 1997), and the other peaks probably correspond to high-mannose, hybrid triantennary and tetra-antennary structures. This conclusion was arrived at by combining the information obtained from the elution profiles with the galactose-to-mannose and *N*-acetylglucosamine-to-mannose ratios for various ChEs. For example, the galactose-to-mannose (0.64) and *N*-acetylglucosamine-to-mannose (1.37) ratios observed for Eq BChE are consistent with the presence of predominantly complex biantennary type of structures in this enzyme (Saxena *et al.*, 1997). An increase in the galactose-to-mannose (0.78–0.83) and *N*-acetylglucosamine-to-mannose (1.43–1.56) ratios suggest that in addition to complex biantennary complex type of structures, HuS BChE, mFBS AChE, rMo AChE, and rHu BChE contain triantennary and tetra-antennary structures. The galactose-to-mannose ratio is even higher for mFBS AChE (0.87), probably due to the presence of glycans containing the galactose α1–3 galactose β1–4 determinant

TABLE 3
Noncompartmental analysis of time course of various recombinant and tissue-derived ChEs in plasma of mice

Enzyme	MRT	CL	Elimination half-life	V_p[a,b]	V_{ss}[a]
	min	*ml/hr/kg*	*min*		*ml/kg*
Eq BChE	3206 ± 317	1.2	2379 ± 230	41.6 ± 1.9	57.7 ± 2.5
HuS BChE[c]	2791	1.3	1683	51.0	58.3
tFBS AChE	1902 ± 129	1.3	1210 ± 84	30.8 ± 2.4	40.9 ± 2.8
T. californica AChE	44 ± 2	72	40 ± 7	41.2 ± 0.9	51.8 ± 1.4
mFBS AChE	304 ± 99	16.2	215 ± 14	39.5 ± 4.8	81.5 ± 8.2
rMo AChE	301 ± 40	21.6	230 ± 31	49.8 ± 4.2	107.8 ± 10.9
rHu BChE	205 ± 62	33.0	144 ± 36	54.6 ± 4.0	114 ± 20

[a] Normalized to body weight.
[b] Calculated by dividing the administered dose by ChE plasma concentration at t = 0, estimated by log-linearized extrapolation of the initial time course data.
[c] Data are average from two mice.
Values are mean ± standard deviation for three to six experiments.

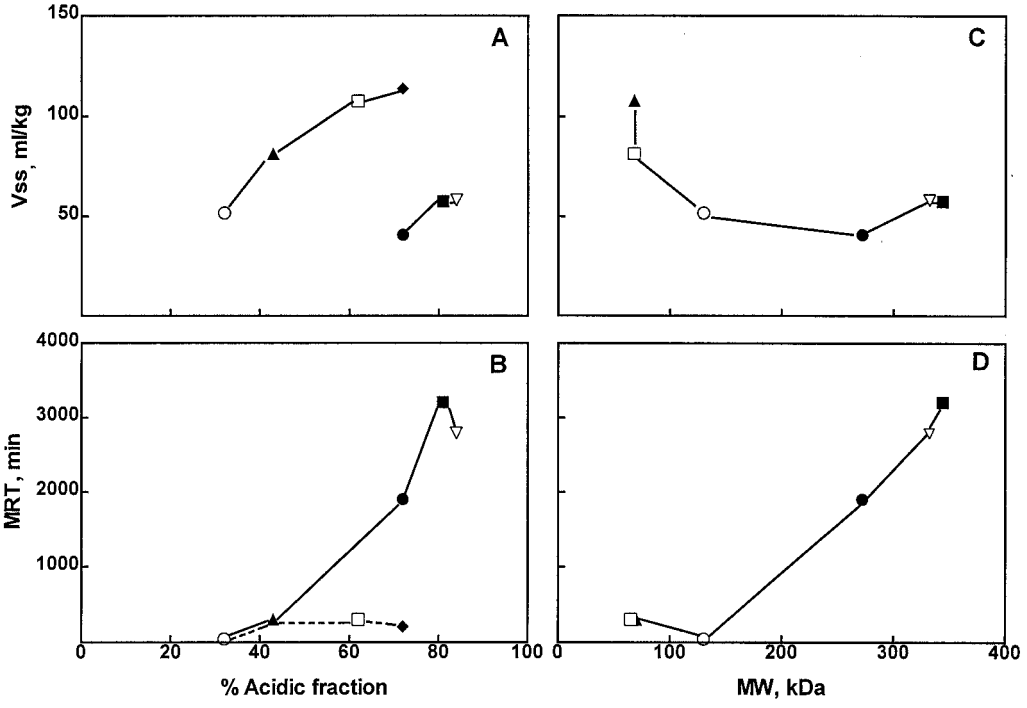


Fig. 5. Correlation of V_{ss} and MRT in mice with the acidic oligosaccharide content and molecular weight of ChEs. A, V_{ss} versus percent acidic fraction of glycans on ChEs. B, V_{ss} versus the molecular weight of ChEs. C, MRT versus the percent acidic fraction of glycans on ChEs. D, MRT versus the molecular weight of ChEs. Enzymes shown are HuS BChE (▽), Eq BChE (■), tFBS AChE (●), *T. californica* AChE (○), mFBS AChE (△), rMo AChE (□), and rHu BChE (♦). B, *Dotted line*, connects the monomeric and dimeric forms of ChEs.

that was recently identified in tFBS AChE (Saxena *et al.*, 1997). The galactose-to-mannose (0.26) and *N*-acetylglucosamine-to-mannose (1.27) ratios for *T. californica* AChE suggest the presence of high-mannose glycans in this enzyme.

Differences in the oligosaccharide profiles of HuS BChE and rHu BChE are consistent with observations made with the recombinant forms of tissue plasminogen activator and erythropoietin expressed in different cell lines, which showed that a polypeptide expressed in cell types other than that in which it is normally expressed differs from the native glycoprotein with respect to the structure of certain oligosaccharides, as well as the relative amounts of common oligosaccharides (Parekh *et al.*, 1989; Takeuchi *et al.*, 1989). In another study, a human monoclonal IgM antibody produced by ascites culture possessed a 80–100-fold greater MRT in rats compared with that produced by *in vitro* culture methods (Gauny *et al.*, 1991; Maiorella *et al.*, 1993). Therefore, it seems that the structure and microheterogeneity in the oligosaccharide chains on ChEs may be species and cell type specific and may depend on the culture conditions being used for expressing recombinant ChEs.

No unambiguous correlation could be made between pharmacokinetic parameters and monosaccharide composition or the amount of nonsialylated galactose residues. The apparent V_{ss} after equilibrium was achieved, and the total body MRT seemed to depend on subunit organization and the negative charge donated by the sialic acid residues. For high-molecular-weight proteins, the extravascular distribution is expected to be very low. Indeed, variations in V_{ss} were relatively small, compared with V_p, and rapidly reached a limiting value with the dimeric form of *T. californica* AChE. The increase in V_{ss} could be a result of the binding of ChEs to the endothelial capillary walls, due to the charged oligosaccharides, and/or their distribution in the extravascular spaces. Regardless of the rate of elimination, the larger V_{ss} for mFBS AChE, rMo AChE, and rHu BChE compared with the tetrameric forms of plasma ChEs requires a higher dose of these enzymes to achieve the same plasma concentration of all ChEs. On the whole, V_{ss} did not increase >2-fold over the V_p value, and it seemed to be enhanced by the presence of negatively charged glycans.

To examine possible combinatorial influences of the molecular size and charge on the circulatory stability of ChEs, a three-dimensional graph was constructed from the plot of MRT on a molecular weight/percent acidic fraction grid (Fig.

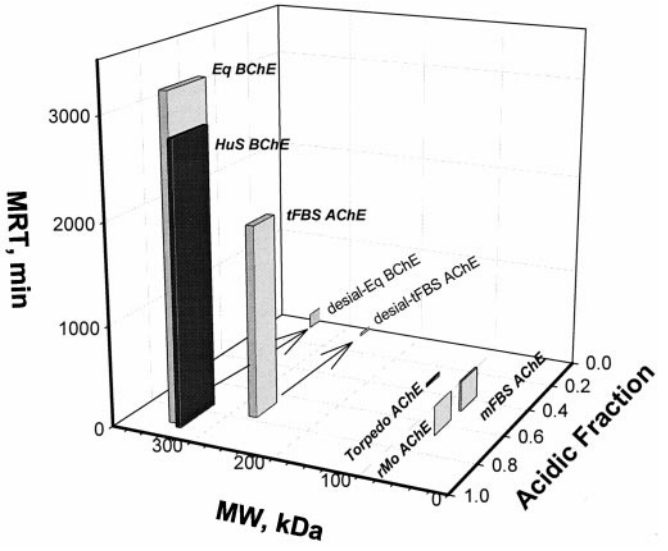


Fig. 6. A three-dimensional plot of MRT on a molecular weight/percent acidic fraction grid. Data for desialylated Eq BChE and tFBS AChE were taken from Saxena *et al* (1997).

6). The graph includes data for desialylated tFBS AChE and Eq BChE, which maintained their catalytic activity and subunit assembly intact (Saxena *et al.*, 1997). Inspection of the graph suggests the following: (1) the removal of sialic acid was accompanied with a substantial decrease in MRT of tFBS AChE and Eq BChE; (2) because the subunit organization remained unchanged, the enhanced clearance does not seem to be caused by renal excretion but seems to be due to accelerated hepatic metabolism; and (3) mFBS AChE possesses a much shorter MRT than tFBS AChE. Because the two enzymes carry the same number of charged oligosaccharides and a similar monosaccharide composition, the presence of size heterogeneity in glycans (Fig. 2) did not permit a clear-cut conclusion regarding the effect of subunit assembly on the circulatory life-time of FBS AChE. Similarly, comparison of HuS BChE and rHu BChE shows that the former is tetrameric in form, whereas the latter consists of predominantly monomers and dimers and some tetramers. In both enzymes, the galactose residues seem to be completely capped with sialic acid, and they contain mostly acidic oligosaccharides. Although the MRT of HuS BChE is 14-fold greater than that of rHu BChE, the observation that the former enzyme contains one major oligosaccharide and rHu BChE contains at least four major oligosaccharides precludes definite conclusions regarding the contribution of the quaternary structure to the circulatory stability of ChEs.

The *in vitro* data on the stability of ChEs in mouse blood raise the possibility that the variability in glycan chains may influence the resistance of these enzymes to proteolytic degradation. It has been suggested that large *N*-glycans may prevent the proteolysis of the extracellular domain of human erythrocyte CD59 (Rudd *et al.*, 1997). It is possible that the mature *N*-glycans of plasma ChEs at specific sites may be protecting them from proteases *in vivo*, contributing to their circulatory stability.

In conclusion, the results presented here reveal differences in the oligosaccharides of native and recombinant ChEs with regard to the total carbohydrate content and charge- and size-based oligosaccharide profiles. However, neither the carbohydrate composition nor the oligosaccharide profile could be completely correlated with the pharmacokinetic parameters of these enzymes. Although the correlation between glycan characteristics and pharmacokinetic parameters is not fully understood, it is noteworthy that the glycans of recombinant ChEs and mFBS AChE displayed a remarkable heterogeneity in size and consist of hybrid and complex biantennary, triantennary, and tetra-antennary structures. *T. californica* AChE also contains high-mannose structures. The three plasma ChEs, on the other hand, contain mature glycans that are predominantly of the complex biantennary type, confirming that these structures are responsible for the extended MRTs of the enzymes. The possible clearance of ChEs from the circulation of animals via galactose receptors (Ashwell and Morell, 1974; Ashford and Harford, 1982), fucose/*N*-acetylglucosamine receptors (Lehrman *et al.*, 1986), and/or mannose receptors (Day *et al.*, 1980) emphasizes the need for determining the structures of the individual glycans. Thus, the site-specific analysis of glycan structures may elucidate the structures responsible for the rapid clearance of nonplasma ChEs and clarify the mechanism for the uptake of ChEs from the circulation of animals.

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