ANL/PPRNT--90-202

DE90 008705

Reaction of benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide with human hemoglobin and chromatographic resolution of the covalent adducts

by

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ABBREVIATIONS

BP, benzo[a]pyrene; BPDE, (+)anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide; BP tetrols, 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-BP; Hb, hemoglobin; hemoglobin-BPDE and hemoglobin-BP, adducts formed by treatment with BPDE or BP, no structural information implied; HPLC, high-performance liquid chromatography; ISAL, 4-(iodoacetamido)salicylic acid; PAH, polycyclic aromatic hydrocarbon.

SUMMARY

The formation of hemoglobin-carcinogen adducts has been proposed as a measure of human exposure to carcinogens. Hemoglobin-carcinogen adducts have been detected in carcinogen-treated animals and in human populations. Although polycylic aromatic hydrocarbons (PAH) are ubiquitous in the human environment and DNA-PAH adducts have been detected in human tissues, the occurrence of hemoglobin-PAH adducts in humans has not been described. In this study we examined the effects of reaction conditions on the extent of in vitro reaction of human hemoglobin and (+)(anti)-[^{3}H]benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), a metabolite largely responsible for the carcinogenic effect of benzo[a]pyrene. The chromatographic properties of the resulting hemoglobin-BPDE adducts were examined by conventional DEAE-cellulose ion-exchange liquid chromatography and by reversed-phase high-performance liquid chromatography. Several adducts were chromatographically resolved from hemoglobin and from the individual globins. Some adducts were basic and some acidic relative to unaltered hemoglobin, suggesting adduct formation by reaction at carboxyl and basic nitrogen groups, respectively. The alteration of the ion-chromatographic properties of the adducts by an ionic sulfhydryl reagent, and the only moderate effects of pH and state of oxygenation on the extent of adduct formation, indicated that the adducts were not formed via reaction with β93 Cys sulfhydryl groups. The chromatographic techniques employed are potentially applicable to the characterization and analysis of other hemoglobin-carcinogen adducts.

KEY WORDS

adduct, benzo[a]pyrene, benzo[a]pyrene diol epoxide, hemoglobin, high-performance liquid chromatography, ion-exchange chromatography.

INTRODUCTION

Since analysis of covalent adducts formed by reaction of electrophilic carcinogen metabolites with hemoglobin was proposed as a measure of carcinogen exposure [1,2], formation of hemoglobin adducts with several types of carcinogens has been described [3,4], the structures of adducts with several types of carcinogens have been determined [2,5-9], and the dose-response and kinetic relationships for their formation and disappearance in animals have been explored [2,6,7,9-13].

Exposure to ethylene oxide present in occupational environments and in tobacco smoke results in formation of hydroxyethyl-hemoglobin adducts via reaction at N-terminal α-amino groups, imidazole nitrogen, and the β93 Cys sulfhydryl groups [14-16]. Cigarette smoking also results in formation of an adduct between the hemoglobin β93 Cys sulfhydryl groups and 4-nitrosobiphenyl arising from metabolism of the carcinogen 4-aminobiphenyl [17]; homologous adducts with other aryl amines have been observed [18]. Studies with rats have shown that metabolites of nitrosamines present in tobacco smoke also form adducts with hemoglobin [13].

Administration of carcinogenic PAH to rodents results in formation of hemoglobin-PAH adducts [3,11,19,20] for which little structural information is available. Acid treatment (0.1 N HCl, 80°C) of hemoglobin obtained from BP-treated mice released fluorochromatographically detected BP tetrols, indicating that the adducts were at least in part formed by reaction with BP diol epoxides [11], metabolites largely responsible for the carcinogenic and mutagenic activity of BP [21,22]. However, comparative radiometric and immunochemical measurements by Wallin et al. [20] recently showed that BP tetrols released by acid treatment of hemoglobin from BP-treated mice account for only a minor portion of the covalently bound BP.

The purpose of this study was to obtain information about the formation and properties of BPDE-hemoglobin adducts to facilitate their analysis and assess their importance. We also sought to examine separation techniques potentially useful for analysis of hemoglobin-carcinogen adducts in general. Therefore, we examined the effects of reaction conditions on the extent of adduct formation between [^{3}H]BPDE and human hemoglobin in vitro and we explored liquid chromatographic techniques for resolution of intact hemoglobin-BPDE or globin-BPDE adducts from the unmodified polypeptides.

MATERIALS AND METHODS

Chemicals. [1,3-^{3}H]BPDE (1,249 mCi/mmol) and the corresponding nonradioactive compound were obtained from the NCI Radiolabeled Carcinogen Reference Repository. ISAL (Sigma) was recrystallized from ethanol.

Isolation of hemoglobin. Saline-washed, packed human erythrocytes were lysed with two volumes of water. NaCl was added to 0.5 M, and the stroma-free lysate obtained by centrifugation at 40,000 g for 30 min was dialyzed overnight against 0.15 M NaCl to remove 2,3-diphosphoglycerate (23) (all at 0-4°C). Portions of the dialyzed solution were frozen by dropwise addition to liquid nitrogen and stored at -70°C.

Analysis of hemoglobin and globin concentrations. Hemoglobin was determined as cyanmethemoglobin, ε_{540} = 11.0 mM^{-1} [24]. Identical absorbance values were obtained in the presence and absence of 1% Triton X-100. Chromatographic fractions were monitored by measurement of A_{415} (oxyhemoglobin, ε = 125 mM^{-1}) or A_{419} (carboxyhemoglobin, ε = 190 mM^{-1}). Globin was determined from ε_{280} = 12.8 mM^{-1} [25,26]. All concentrations are expressed on the basis of heme or globin monomers. Hemoglobin preparations

contained less than 1% methemoglobin [27]. Carboxyhemoglobin concentrations as low as 0.1 μM were determined by measurement of $\Delta A_{419-500}$.

Reaction of [^{3}H]BPDE with hemoglobin. Hemoglobin solutions were dialyzed (4°C) against 5 mM Tris-Cl buffer, pH 7.7, and concentrated to 10 to 15 mM by surrounding the dialysis bag with dry Sephadex G-100. A solution of [^{3}H]BPDE (0.01 volume, typically 1 mM) in tetrahydrofuran was added to 0.99 vol of hemoglobin in buffer at 37°C. pH values were determined at 37°C.

For control reactions a mixture of 0.01 volume of [^{3}H]BPDE solution and 0.89 vol of buffer was incubated at 37°C for 30 min to hydrolyze the [^{3}H]BPDE. Hemoglobin (0.1 vol) was then added and portions were subsequently removed for analysis after incubation for time intervals corresponding to those for complete reactions.

The concentration of [^{3}H]BPDE in each reaction mixture was determined by radiometric measurement using a specific activity obtained by the spectrophotometric determination of BPDE concentration ($\log \epsilon_{344} = 4.69$; ethanol).

Extraction of BPDE-hemoglobin reaction mixtures. Extractions were performed at 0-4°C. Portions of reaction mixtures typically containing 50 nmol hemoglobin (0.02-0.1 mL) were added either to 4 mL of 15 mM HCl in acetone (with vigorous stirring) or to 4 mL of acetone (stirred after 3-5 min). Protein precipitates were recovered by centrifugation at -5°C. Globin precipitates (HCl-acetone) were washed successively with 2 mL each of HCl-acetone, 50 mM imidazole in acetone (to neutralize the acidified globin), acetone, and acetonitrile (to prevent interference by residual acetone). Hemoglobin precipitates (neutral acetone) were washed three times with 2 mL acetone, and then once with 2 mL acetonitrile. The precipitates were dried under nitrogen at room temperature.

Dried globin was dissolved in 1.0 mL water (final pH 5-6), A_{280} was measured, and 0.2 mL was used for liquid scintillation counting (5 mL of Aquasol II; Amersham). Dried hemoglobin was dissolved in 0.5 mL of 1% aqueous Triton X-100; individual 0.2-mL portions were used for cyanmethemoglobin analysis and for liquid scintillation counting. (Heme was decolorized by addition of ethanolic m-chloroperoxybenzoic acid.) The globin and hemoglobin concentrations were used to compute the specific contents of the adduct preparations.

Chromatographic analysis of [^{3}H]BPDE-hemoglobin reaction mixtures. [^{3}H]BPDE-hemoglobin reaction mixtures were applied to columns of Sephadex G-25 (Pharmacia) equilibrated and eluted with water at room temperature.

DEAE-cellulose (DE-52, Whatman) chromatography was performed as previously described [28,29] except that a linear NaCl gradient was used and the buffers were saturated with carbon monoxide to prevent the formation of methemoglobin in fractions containing low concentrations of hemoglobin [30]. A 1- x 25-cm column was equilibrated with glycine-KCN buffer (0.2 M glycine, 0.01% KCN, pH 7.7). The upper 1-2 cm of bed was composed of a 3:1 mixture (v/v, hydrated materials) of Sephadex G-25 and DEAE-cellulose to cause the hemoglobin to bind less densely and more uniformly.

BPDE-treated hemoglobin (20-30 mg) obtained by gel filtration (elution with water) was diluted with an equal volume of glycine-KCN buffer, applied to the DEAE-cellulose column, and hemoglobins were eluted at 36 mL/h with a gradient composed of 600 mL each of 5 mM and 60 mM NaCl in glycine-KCN buffer. In the experiment reported under RESULTS, two columns were operated simultaneously from a common gradient of twice the volume indicated above. NaCl concentrations in the fractions were measured conductometrically.

Reversed-phase HPLC analysis was conducted at 30°C with a 0.4- x 25-cm Vydac C-4 column [31] (1.0 mL/min, 38 to 45% acetonitrile in 52 min).

RESULTS

Effect of pH and extraction procedure on extent of adduct formation. Results of initial experiments using phosphate buffers were complicated by the formation of the BPDE-phosphate ester [32,33] which was removed by extraction of the hemoglobin with HCl-acetone, but not with neutral acetone. Thus, results of experiments presented here were obtained using either Tris-imidazole-Cl (20 mM each) or 20 mM Tris-Cl buffers supplemented with 0.1 M KCl. The low concentration of buffer was selected to minimize Tris-dependent hydrolysis of BPDE [33,34].

The levels of BPDE incorporation depended only moderately on pH between pH 6 and 8, and were similar or identical when measured using the two extraction procedures (Fig. 1). For control reactions (with hemoglobin present for 30 min after a 30-min hemoglobin-free preincubation period), the level of protein-associated radioactivity was 10-15% of that for the complete reactions. (See also gel filtration below.)

Effect of hemoglobin and BPDE concentrations on extent of adduct formation. The level of incorporation was directly dependent on the BPDE concentration for hemoglobin concentrations ranging from 0.1 to 4 mM (Fig. 2A), while for fixed BPDE concentrations, the extent of adduct formation approached maximal values as the hemoglobin concentration was increased (Fig. 2B). From 3 to 27% of the BPDE was incorporated into protein (for 0.1 and 4 mM hemoglobin, respectively). Thus, at the greater hemoglobin

concentrations, reaction of BPDE with protein effectively competed with BPDE hydrolysis.

Kinetics of adduct formation. The pseudo first-order formation of BPDE-hemoglobin adduct (1 mM hemoglobin) was complete in 15-20 min (Fig. 3); the apparent first order rate constant was 0.33 min^{-1} (inset). Under conditions identical except for the absence of hemoglobin and the use of nonradioactive BPDE, the first-order rate constant for BPDE hydrolysis was 0.89 min^{-1} as determined spectrophotometrically [34]. Thus, the presence of hemoglobin may have stabilized BPDE as has been observed for albumin [35], liver microsomes [36], and cellular systems [37].

Effect of carbon monoxide or deoxygenation on the extent of adduct formation. The presence of carbon monoxide had no appreciable effect on the level of BPDE incorporation either in complete or control reactions (data not shown). Thus, because the presence of carbon monoxide essentially prevents oxygen-heme interaction, BPDE incorporation was not appreciably due to heme-dependent oxidation of BPDE or its hydrolysis products.

Under air, hemoglobin is nearly saturated with oxygen. The reactivities of some electrophilic sites in hemoglobin are substantially altered (sterically or electrostatically) by the conformational changes associated with oxygen binding [38-41]. Table 1 shows that the level of adduct formation for deoxyhemoglobin was 88% of the level for oxyhemoglobin.

Gel filtration of BPDE-treated hemoglobin. The level of radioactivity in hemoglobin obtained by gel filtration of control reaction mixtures (30-min treatment of hemoglobin in preincubated buffer-BPDE mixture; Table 2, Control A) was 10-15% of that for complete reactions (1 mM hemoglobin, 7 μM BPDE, 30 min). This level was diminished to about 2% if the control reaction mixture was applied to the column immediately after addition of hemoglobin rather than

after a 30-min incubation (Table 2, Control B). Presumably the 10-15% incorporation (Control A) was due to reaction with a minor component of the [^{3}H]BPDE preparation--a reaction largely prevented by immediate gel filtration (Control B). Most of the radioactivity not associated with hemoglobin was retained on Sephadex G-25 during extensive elution with water or the glycine-KCN buffer, but was eluted with water-methanol mixtures.

Treatment with acid or base. Ethyl acetate extraction of the hemoglobin fraction obtained by gel filtration removed about 10% of the radioactivity (Table 2). The data in Table 2 also show that of the remaining radioactivity, about 50% was removed by heating in 0.1 N NaOH or HCl, and about 10% by heating at neutral pH. Storage for 3 h at 30°C in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid (conditions used for reversed-phase HPLC) did not increase the amount of ethyl acetate extractable radioactivity.

DEAE-cellulose ion-exchange chromatography of BPDE-treated hemoglobin. Figure 4 (+ISAL, upper panel) shows that two principal types of [^{3}H]BPDE-hemoglobin adduct were resolved by DEAE-cellulose ion-exchange chromatography, one more basic than hemoglobin A (fractions 1 and 2; 28 and 14% of recovered radioactivity, respectively) and one more acidic (fractions 3 and 4; 18 and 8%, respectively). The specific content of fraction 1 (the most highly purified) was at least 100 pmol/nmol hemoglobin (3 pmol/nmol before chromatography).

Some carcinogens and mutagens react with the electrophilic hemoglobin β93 Cys sulfhydryl groups [5,7-9,12,43]. BPDE reacts nonenzymatically with 2-mercaptoethanol, glutathione, and cysteine [44,45]. To investigate the role of the β93 Cys sulfhydryl groups in the formation of BPDE-hemoglobin adducts, we examined the effect of treatment with ISAL (an ionic sulfhydryl reagent) on their ion-chromatographic behavior. BPDE-hemoglobin (1 mM) obtained by gel

filtration was treated for 6 h at 5°C in a mixture containing 0.1 M sodium phosphate buffer (pH 7.0), 0.2 M KCl, and 10 mM ISAL, followed by addition of dithiothreitol (20 mM) and gel filtration on a column equilibrated with glycine-KCN buffer. ISAL reacted stoichiometrically with the β93 Cys sulfhydryl groups as determined by colorimetric analysis of sulfhydryl content [46,47] and spectrophotometric analysis [48,49] of the covalently bound salicylyl moiety in globin and hemoglobin (data not shown).

Comparison of the two chromatograms in Fig. 4 shows that treatment with ISAL, increased the retention volumes for the all of the major and minor optically detected hemoglobins as well as for all of the [^{3}H]BPDE-hemoglobin adducts. ISAL-modified adduct fractions 1 and 2 were better resolved after ISAL treatment (Fig. 4, lower panel), presumably because of the greater elution volume.

Resolution of globins by reversed-phase HPLC. In the ion-exchange DEAE-cellulose chromatography system described above, hemoglobin migrates as $\alpha_2\beta_2$ tetramers in equilibrium with $\alpha\beta$ dimers [50,51]. In contrast, a reversed-phase HPLC system, Fig. 5, allows resolution of the individual α and β globins (31).

Consistent with the results from preparative ion-exchange chromatography (Fig. 4) the results in Fig. 5 show that several protein-BPDE adducts were detected by HPLC. The widths for peaks 5 and 6 suggest that they represent predominant single components. The increased hydrophobicity of components 5 and 6 (greater retention times relative to native globins) is consistent with incorporation of the aromatic moiety. Some of the less abundant adducts with retention times similar to or less than that for α globin may have arisen from homologous reaction of [^{3}H]BPDE with minor β globin variants, [e.g. δ globin

(present in hemoglobin A_2 tetramers) and glycated α and β globins (present in hemoglobin A_1 tetramers)], while other adducts may be structurally unique.

DISCUSSION

Metabolic activation of PAH in human populations is evident from the detection of DNA adducts with BP metabolites in human lung [52] and white blood cells [53]. BP-Hemoglobin and BP-DNA adducts are formed in BP-treated rats and mice [3,11,20]. Thus, BP-hemoglobin adducts are probably present in BP-exposed humans.

This study was designed to characterize adducts formed between BPDE and human hemoglobin as the basis for analysis of hemoglobin-PAH adducts in humans. Our results provide evidence regarding the structure of the adduct formed between human hemoglobin and BPDE, a BP metabolite largely responsible for the carcinogenic activity of BP. Studies are in progress to evaluate the relative importance of BPDE in the formation of adducts in a system that includes human hemoglobin and the products of metabolic activation of BP.

The ISAL-dependent alteration of the ion-chromatographic behavior of all [^{3}H]BPDE-hemoglobin adducts demonstrated that adduct formation does not involve the β93 Cys sulfhydryl groups. This conclusion is consistent with the relatively small effect of pH on adduct formation; the reactivity of the β93 Cys sulfhydryl groups of oxyhemoglobin is strongly dependent on pH in the neutral range [40,54].

Ion-exchange chromatography of BPDE-treated hemoglobin revealed the presence of at least two types of adducts: those apparently more basic (fractions 1 and 2) than hemoglobin A and those apparently more acidic (fractions 3 and 4) than hemoglobin A. Alteration of the ion-chromatographic

mobility or the isoelectric point of hemoglobin resulting from reaction with a neutral reagent can occur either because of reaction at an ionizable site or less directly by alteration of local electrostatic interactions [55,56].

If we assume that the BPDE-dependent alterations of chromatographic behavior were due primarily to reactions at ionizable sites, then the predominant basic adducts (fractions 1 and 2, Fig. 4) may have arisen via reaction of BPDE at carboxyl groups, yielding the corresponding esters. If this were true, the level of adduct formation would depend relatively little on pH, as we observed, because the concentration of nucleophilic carboxylate anion would not vary significantly between pH 6 and 8. However, the effect of pH must be interpreted cautiously because the rate of BPDE hydrolysis increases with decreasing pH [33,57].

Reaction of BPDE with a globin carboxyl group would be consistent with its reaction with acetate, observed by Yagi et al. [58], and also with the release during mild acid hydrolysis (0.12 N HCl, 80°C) of 7,8,9,10-tetrahydroxy-BP from globin of BP-treated mice [11] and from BPDE-treated mouse globin [20]. Evidence has been reported for esterification of hemoglobin by metabolites of dimethylnitrosamine [42] and a tobacco-specific nitrosamine [13].

Glucose-dependent modification of N-terminal valine in $^{\beta}$ globin is readily detected by conventional ion-exchange chromatography (hemoglobin A_{1c} in Fig. 4A), while modification of N-terminal valine in $^{\alpha}$ globin has only a minor effect on ion-chromatographic properties [59,60]. Thus, the acidic BPDE-hemoglobin adducts (fractions 3 and 4, Fig. 4) may have arisen via reaction of BPDE with basic groups (e.g. histidine or N-terminal valine) presumably decreasing their pK values (as for benzyl amine). Alkene oxides and metabolites of alkyl nitrosamines form adducts with basic nitrogens in

hemoglobin [2,8,14,16,42,43,61]. Reaction of BPDE at sites other than carboxyl, amino, or imidazolyl groups cannot be ruled out. The negligible effect of deoxygenation on the extent of adduct formation (Table 1) suggests that the sites of adduct formation are unlikely to include those whose reactivity depends on the hemoglobin conformation.

The recognition of two classes of adducts according to their ion-chromatographic properties (Fig. 4) may be related to our observation of differential sensitivity to hydrolysis and the in vitro observation of Wallin et al. [20] that heating [^{3}H]BPDE-treated mouse globin in 0.1 N HCl (80°C) released about 40% of the radioactivity, primarily as BP tetrols.

The small difference we observed between the levels of adduct formation for oxy- and deoxyhemoglobin suggests that sites of adduct formation do not include those whose reactivity is substantially different for oxy- and deoxyhemoglobin (including some carboxyl groups and amino groups) [38,39]. We cannot rule out the explanation that qualitatively different adducts are formed by reaction with deoxyhemoglobin and oxyhemoglobin. An understanding of the significance of the results will require further structural analysis.

In addition to providing specific information about the reaction of BPDE with hemoglobin and the isolation of the adducts, our results suggest alternate approaches for characterization and analysis of hemoglobin-carcinogen adducts. The chromatographic approach we used for evaluating the role of the β93 Cys sulfhydryl groups in adduct formation is applicable to the study of adducts with other electrophiles. Analysis of the relative levels of adduct formation for oxy- and deoxyhemoglobin can also provide indirect information about sites of adduct formation.

Furthermore, the results suggest that it may be generally possible, either with or without prechromatographic chemical modification of the native

hemoglobin, to use ion-chromatographic or electrophoretic techniques to isolate fractions enriched in hemoglobin- or globin-carcinogen adducts formed by reaction with the $^{\beta}93$ Cys sulfhydryl groups, basic nitrogen groups, or carboxyl groups of the protein.

ACKNOWLEGEMENTS

We appreciate the expert technical assistance provided by Vernon Pahnke and Suzanne Dornfeld. This research was supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-Eng-38.

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TABLE 1. Effect of deoxygenation on the extent of [^{3}H]BPDE-hemoglobin adduct formation[a]

Reaction no.	Oxygen	BPDE concentration (μM)	Specific content (pmol BPDE/nmol globin)
1	-	9.30	2.59 ± 0.14
2	-	9.55	2.57 ± 0.04
3	+	9.38	2.98 ± 0.13
4	+	9.46	2.87 ± 0.12

[a]The gas phases above hemoglobin solutions (0.6mL; 1 mM hemoglobin in 40 mM Tris-Cl, 100 mM KCl, pH 7.4) in cylindrical vessels sealed with a septum were purged with humidified nitrogen at 15-20°C with magnetic stirring. Conversion to deoxyhemoglobin was complete after 80-90 min as indicated by periodic measurement of $\Delta A_{758-845}$, $\Delta\varepsilon$ ~0.2 mM^{-1}. After the solutions were warmed to 37°C, BPDE was added to vessels that had either remained sealed (reactions 1 and 2) or had been opened to allow reconversion to oxyhemoglobin (reactions 3 and 4). After 20-25 min, the absence of oxyhemoglobin in the sealed vessels was confirmed, and three 0.05-mL portions of each reaction mixture were extracted with neutral acetone. The values for the specific content are the mean ± SD (N = 3).

TABLE 2. Gel filtration and treatment with acid and base

Treatment	Ethyl acetate-[a] extractable radioactivity %	Specific[b] content (pmol BPDE/nmol hemoglobin)	
		As obtained	Acetone precipitation
EXPERIMENT 1: Covalent Binding[c]			
Gel filtration			
Complete	11.3 ± 0.2	1.03	1.09 ± 0.03
Control A	52.3 ± 2.8	0.12	0.079 ± 0.004
Control B	70.7 ± 7.4	0.02	0.008 ± 0.0004
EXPERIMENT 2: Stability[d]			
0.2 M phosphate, pH 7.4			
Room temperature			
Complete	10 (0)	--	--
Control A	54	--	--
100°C, 10 min			
Complete	18 (8)	--	--
Control A	30	--	--
0.1 N NaOH, 37°C, 2 h			
Complete	65 (55)	--	--
Control A	39	--	--
0.1 N HCl, 80°C, 3 h			
Complete	57 (47)	--	--
Control A	38	--	--

[a]Aqueous solutions were extracted three times with two volumes of ethyl acetate to remove noncovalently bound radioactivity either before (EXPERIMENT 1) or after the treatments described under EXPERIMENT 2. Conditions for Controls A and B are described in the text. The same Complete and Control A fractions were used for experiments 1 and 2.

[b]Specific contents of the untreated gel filtration fractions were determined before (as obtained) and after acetone precipitation.

[c]Values represent the mean ± SD (N = 3) or mean of duplicates (as obtained).

[d]The treatments with phosphate buffer, NaOH, and HCl were as described [9,11,13,20,42]. Values are means for duplicate analyses. Values in parentheses are corrected for noncovalently bound radioactivity (10%).

FIGURE LEGENDS

Figure 1. Effect of pH and extraction procedure on extent of adduct formation. Reaction mixtures (0.3 mL; 1 mM hemoglobin; 10 μM BPDE) were incubated for 30 min in Tris-imidazole-KCl buffer. Duplicate 0.05-mL portions were extracted with either acidic (open symbols) or neutral acetone (closed symbols). The mean values for complete reactions (two upper curves) are corrected for the level of binding for the corresponding control reactions (two lower curves).

Figure 2. Effect of BPDE and hemoglobin concentrations on extent of adduct formation. After incubation of hemoglobin:BPDE mixtures at 37°C in Tris-imidazole-KCl buffer (pH 7.4), for 30 min, a 10- to 50-nmol portion of hemoglobin was extracted with neutral acetone. Control reactions were conducted only for 1 mM hemoglobin; data are uncorrected for the control level of incorporation. Identical data are shown in Panels A and B, respectively illustrating the effects of BPDE and hemoglobin concentrations. The concentrations of hemoglobin (mM, panel A) and BPDE (μM, panel B) are shown at the ends of the curves.

Figure 3. Kinetics of adduct formation. Reaction mixtures (0.7 mL) contained Tris-imidazole-KCl buffer (pH 7.4), 1 mM Hb, and 10 μM BPDE. Reaction was stopped by addition of a 0.05-mL portion to HCl-acetone (to rapidly hydrolyze BPDE). The complete reaction (UPPER CURVE and INSET) was initiated by addition of BPDE to the hemoglobin solution. For the control reaction (LOWER CURVE) BPDE was preincubated for 30 min before addition of hemoglobin at t = 0.

Figure 4. DEAE-cellulose ion-exchange chromatography and the effect of treatment with ISAL. Hemoglobin (2.0 mL, 2 mM) in Tris-Cl (20 mM, pH 7.4) and KCl (100 mM) was treated with [^{3}H]BPDE (13 μM), and equal portions (20 mg) obtained by gel filtration were analyzed before (-ISAL) or after (+ISAL) treatment with ISAL.

Figure 5. Reversed-phase HPLC. BPDE-treated hemoglobin (54 μg in 10 μL) obtained by gel filtration was analyzed, and 0.4-min fractions were collected.

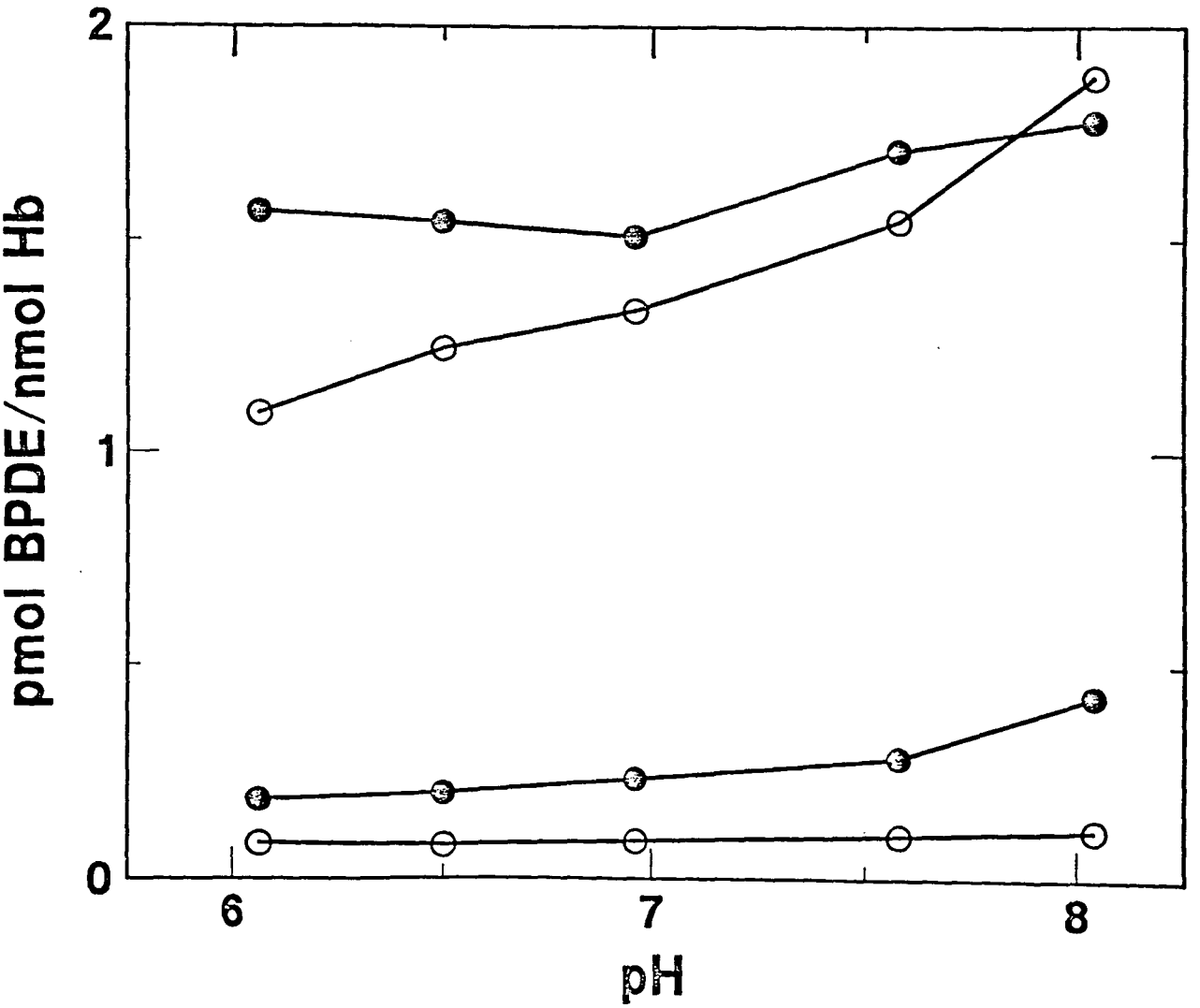


Figure 1.

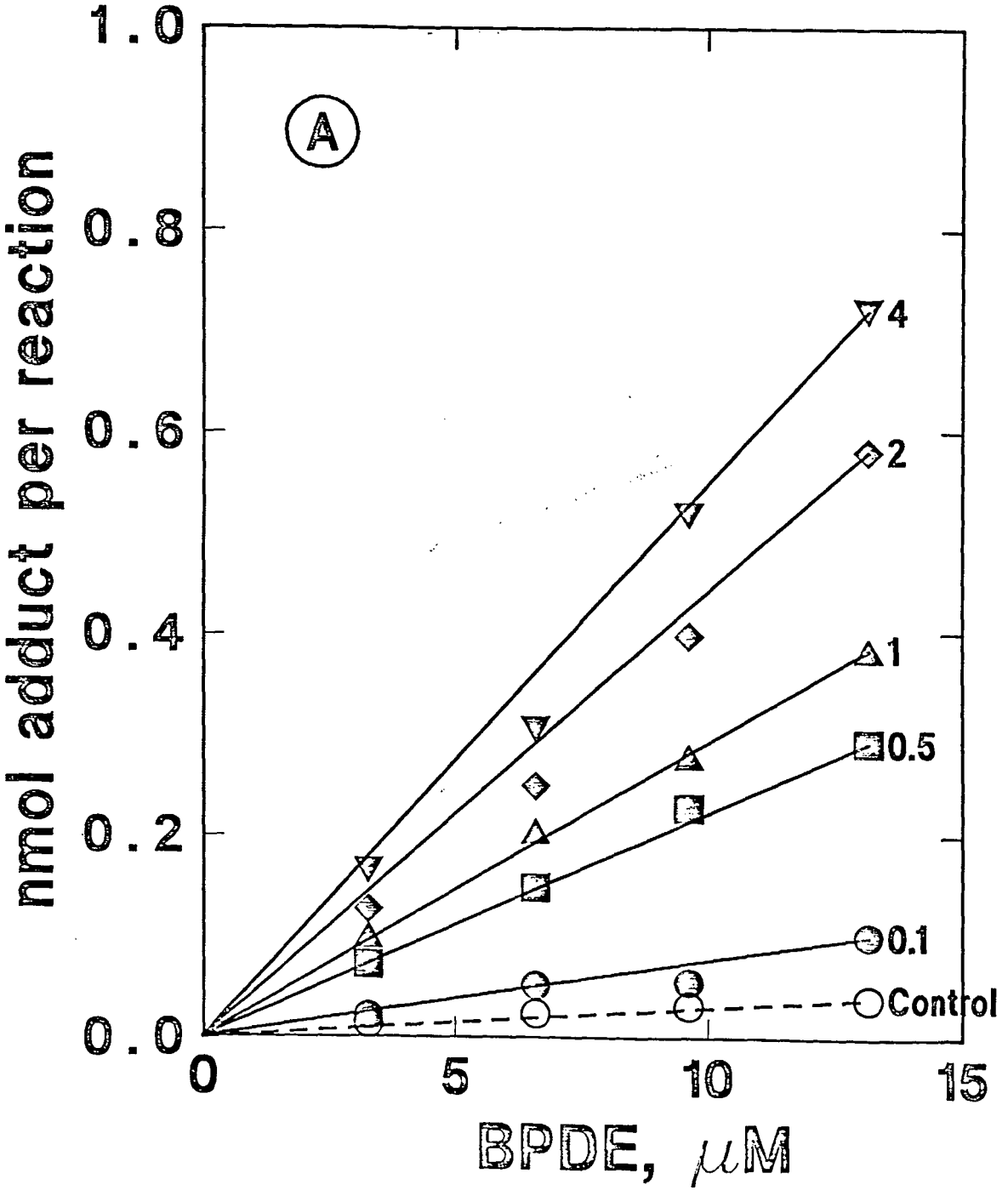

A
nmol adduct per reaction
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
BPDE, μM
4
2
1
0.5
0.1
Control

Figure 2A.

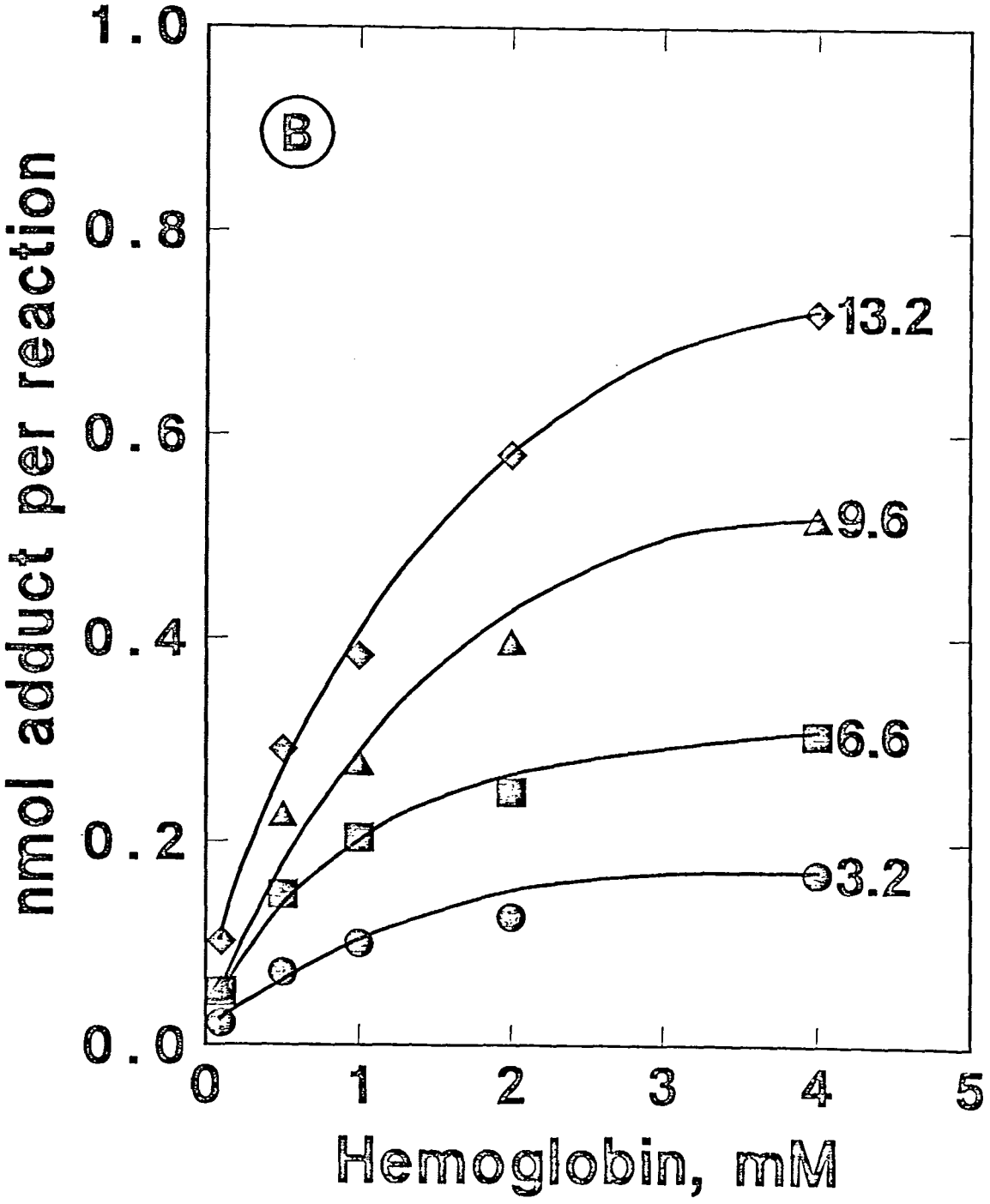

B
nmol adduct per reaction
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Hemoglobin, mM
13.2
9.6
6.6
3.2

Figure 2B.

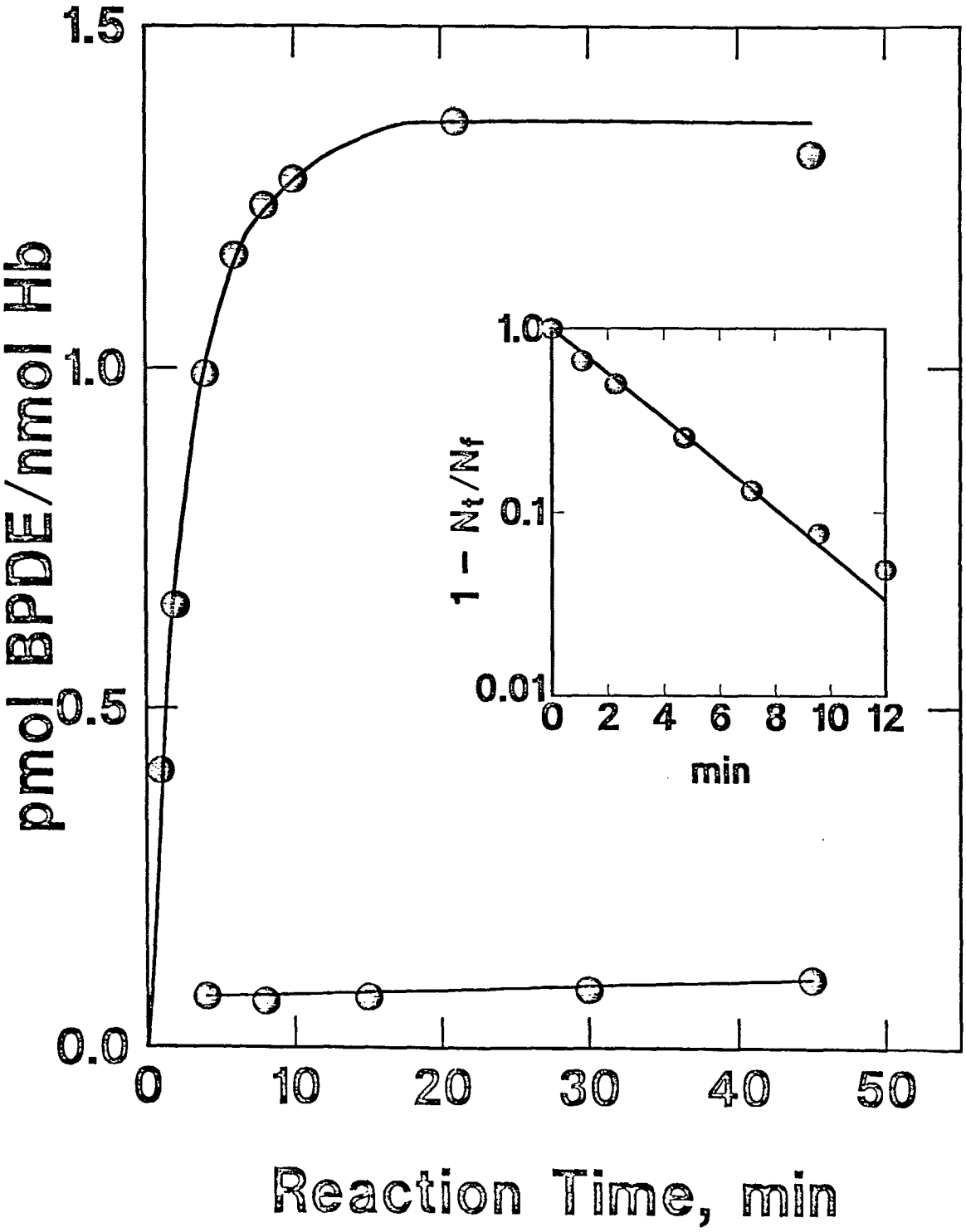
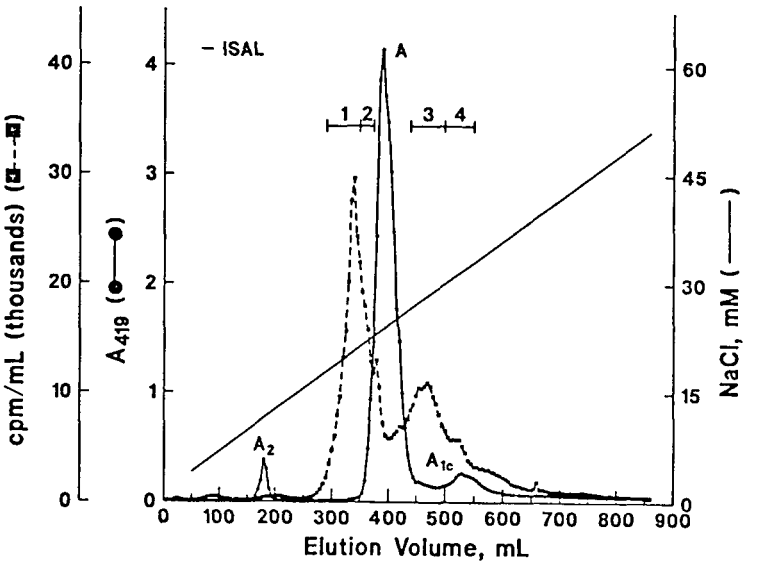

1.5
1.0
0.5
0.0
pmol BPDE/nmol Hb
0
10
20
30
40
50
Reaction Time, min
1.0
0.1
0.01
1 - Nt/Nf
0
2
4
6
8
10
12
min

Figure 3.


− ISAL
A
1 2
3 4
A2
A1c
cpm/mL (thousands) (■--■)
A419 (●—●)
NaCl, mM (——)
Elution Volume, mL
0
100
200
300
400
500
600
700
800
900
10
20
30
40
1
2
3
4
15
45
60

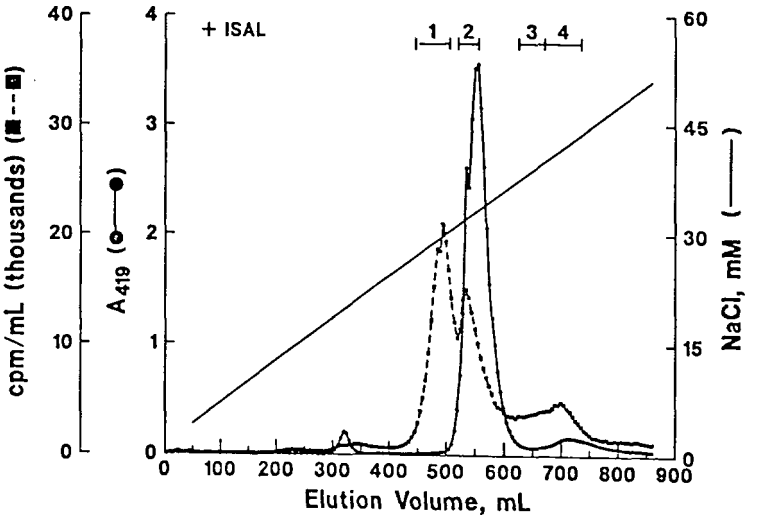

+ ISAL
1 2
3 4
cpm/mL (thousands) (■--■)
A419 (●—●)
NaCl, mM (——)
Elution Volume, mL
0
100
200
300
400
500
600
700
800
900
10
20
30
40
1
2
3
4
15
45
60

Figure 4.

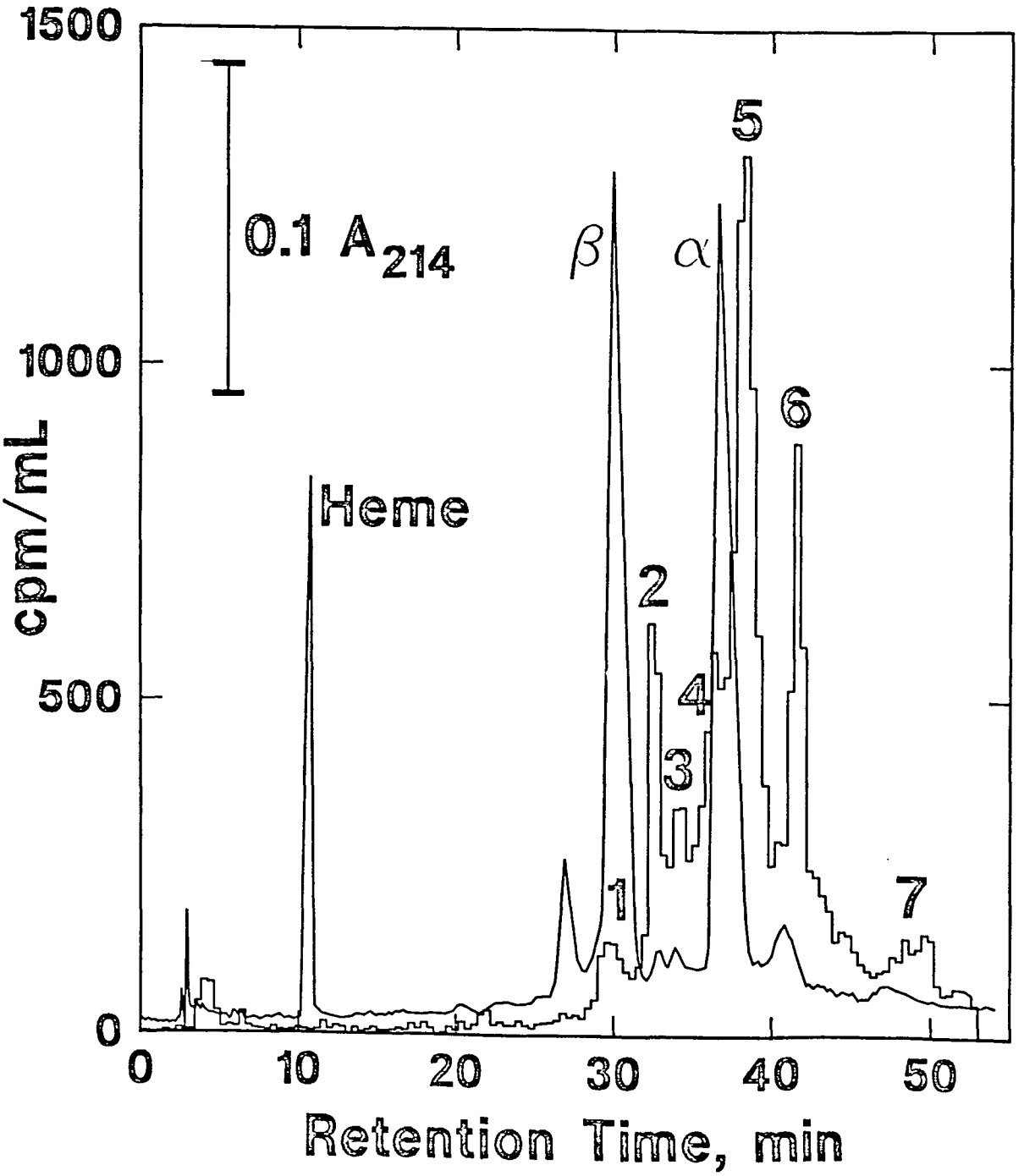

1500
1000
500
0
cpm/mL
0.1 A214
β
α
5
6
Heme
2
4
3
1
7
0
10
20
30
40
50
Retention Time, min

Figure 5.