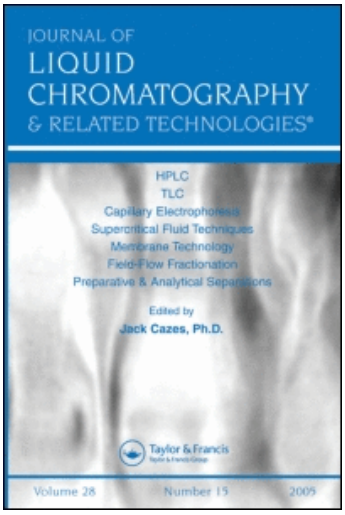

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Competitive Binding of Catecholamines, Indoleamines, Acetylcholine, and Related Metabolites to Various Glassy Carbon Materials

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COMPETITIVE BINDING OF CATECHOLAMINES, INDOLEAMINES, ACETYLCHOLINE, AND RELATED METABOLITES TO VARIOUS GLASSY CARBON MATERIALS

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ABSTRACT

We have previously demonstrated the utility of a pre-column containing glassy carbon particles in the determination of acetylcholine and choline by liquid chromatography with electrochemical detection (Ikarashi et al., 1992). Such a pre-column adsorbs neurochemically related catechol and indole derivatives and, thus, prevents interference in the chromatogram for acetylcholine and choline. In the current studies, we report the results of examining three important and related characteristics of this adsorption process. First, the source materials and conditions used in the preparation of the glassy carbon particles substantially affect adsorption of the 'interfering' catecholamines, indoleamines, and related metabolites. Second, the primary adsorption sites appear to have a substantial amount of graphite-like,

rather than diamond-like, character. Third, the Langmuirian adsorption of the catechol and indole compounds is at least partly competitive, indicating the probable involvement of common adsorption sites.

INTRODUCTION

In the development of methodology for the determination of catecholamines, indoleamines, acetylcholine, and related metabolites in rat and mouse brain tissues using liquid chromatography with electrochemical detection (LCEC), we previously attempted to directly utilize the supernatant of a perchloric acid homogenate to avoid the additional isolation steps normally required for acetylcholine (ACh) and choline (Ch) (1). While the determination of catecholamines, indoleamines, and related metabolites were quite amenable to the direct injection of this homogenate (2), direct injection of the homogenate into the separate LCEC setup normally employed for ACh and Ch, as basically described by Potter et al. (3-4), exhibited both an undesirable large solvent front response and other, unidentified peaks. The unidentified peaks were shown to be catecholamine related species (1). Quite by accident, we discovered that glassy carbon particles (IRICA Type CP-2250) were capable of adsorbing these interfering substances in the LCEC analysis of ACh and Ch, while showing virtually no adsorption of the targeted quaternary amines. A pre-column packed with the glassy carbon particles not only eliminated the interfering peaks, but also substantially decreased the size of the solvent front. Subsequent examination of adsorption by these same particles exhibited a Langmuir type adsorption for virtually all the catecholamines, indoleamines, and related metabolites while not adsorbing any of the quaternary amines; adsorption constants and the number of available adsorption sites were determined for each of the species of concern (6). In general, the adsorption favored the non-ionic form of the compounds of concern over their ionic congeners. Adsorption of the more hydrophobic species was preferred over that of the more hydrophilic species; thus, the indolic compounds were preferentially adsorbed over the catechol species.

Glassy carbon is typically formed in two separable steps (7). In the first, a polymeric precursor is heated with pressure to 300-400°C, under which conditions the material experiences an endothermic thermal reforming. Subsequently, the material is slowly heated to a final curing temperature of 1000-3000°C, with coalescence of stabilized polymer chains occurring between 400 and 1000°C. Between 1000 and 2700°C, local distortions due to internal defects and bonds with

adjacent ribbons are essentially eliminated. During this entire carbonization process, elements other than carbon are expelled from the precursor, and the carbon atoms in materials heated above 2700°C are virtually all in the sp^2, or graphite-like, form as opposed to the sp^3, or diamond-like, form. However, the particular properties of a given glassy carbon material may vary considerably depending on both the polymeric precursor and the exact method of manufacture. Thus, we decided to investigate alternative glassy carbon materials to see if they possessed the same desirable adsorption properties we had previously observed with the IRICA Type CP-2250 (1,6).

Our previous studies also left unanswered two other pertinent questions. These concern the nature of the adsorption site on the glassy carbon and the possible competition between the various neurochemicals studied for a limited total number of available adsorption sites. These issues are also addressed in the current report.

MATERIALS AND METHODS

Reagents

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: acetylcholine (ACh) chloride, choline (Ch) chloride, 5-hydroxytryptamine (5-HT) creatinine sulfate monohydrate, 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA) hydrobromide, norepinephrine (NE) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). Ethylhomocholine (N,N-dimethyl-N-ethyl-3-amino-1-propanol or, simply, EHC) was prepared as described previously (3-4). Chemicals used in the LCEC eluting solvents were obtained from various manufacturers in the highest purity available.

Glassy carbon particles, 100-200 mesh from both the Mitsubishi Pencil Co., Ltd. (Gunma, Japan) and the Tokai Carbon Co., Ltd. (Tokyo, Japan) and derived from phenolic resin fibers, were heated to final curing temperatures of 1000, 1400, 2000, and 3000°C for the initial studies (c.f., Fig. 2). Plastic formed carbon materials (PFC), also 100-200 mesh, were supplied by the Mitsubishi Pencil Co.; these were created by mixing 0, 50, 60, 80, and 100% pure graphite with a vinyl chloride resin and pyrolyzing to form a 'glassy carbon' at a final temperature of 1400°C (c.f., Fig. 3). The investigation of possible competition between different adsorbates employed the 100-200 mesh glassy carbon particles from Tokai Carbon Co. heated to a final temperature of 3000°C (c.f., Figs. 4-8).

LCEC for Catecholamines, Indoleamines, and Related Metabolites

The LCEC system employed for determination of the catecholamines, indoleamines, and related metabolites consisted of a PM60 pump, a CC-4 injector, a Biophase® ODS-IV column (3 μm, 4x110 mm), an LC-4B amperometric detector, a dual glassy carbon electrode (E_{app} = +0.70 volts vs. Ag/AgCl), and an LC-22A temperature controller (35°C), all from Bioanalytical Systems, Inc. (Tokyo, Japan). An SCI Chromatocorder from Yokogawa Co., Ltd. (Tokyo, Japan) was used for data processing. The eluting solvent was a 0.050 M citrate buffer, pH 3.20, containing 0.80 mM sodium 1-octanesulfonate and 0.50 mM disodium ethylenediaminetetraacetic acid; the typical flow rate was 0.8 mL/min. A typical chromatogram for a standard mixture of components is shown in Fig. 1A. Detection limits for the compounds of concern were ≤ 1 pmol.

LCEC for Acetylcholine and Choline

The LCEC system employed for the determination of the acetylcholine, ethylhomocholine, and choline consisted of an LC100P pump, an LC100S injector, and an LC100W/F work station for data processing from Yokogawa Co. Components obtained from Bioanalytical Systems included an LC-4A amperometric detector with dual platinum electrodes (E_{app} = +0.50 volts vs. Ag/AgCl), an LC-22A temperature controller (35°C), an Acetylcholine Separation Column (3 μm, 4x60 mm), and a post-column (4x5 mm) containing immobilized acetylcholinesterase and choline oxidase. The mobile phase was a 0.050 M phosphate buffer, pH 8.40, containing 1.0 mM disodium ethylenediaminetetraacetic acid and 0.40 mM sodium 1-octanesulfonate; typical flow rates were 0.80 mL/min. A chromatogram for a standard mixture of ACh, EHC, and Ch is shown in Fig. 1B. Detection limits for the compounds of concern were 2-5 pmol.

Adsorption Determinations

All determinations of amount adsorbed and percent adsorbed employed 50 mg of the glassy carbon material tested and 1.00 mL of a 0.10 M phosphate buffer, pH 7.0, containing various amounts of NE, DA, 5-HT, 5-HIAA, HVA, DOPAC, ACh, EHC, and/or Ch. The carbon particles and buffer solution were mixed for a few seconds on a vortex mixer, and the supernatant was collected by passing the

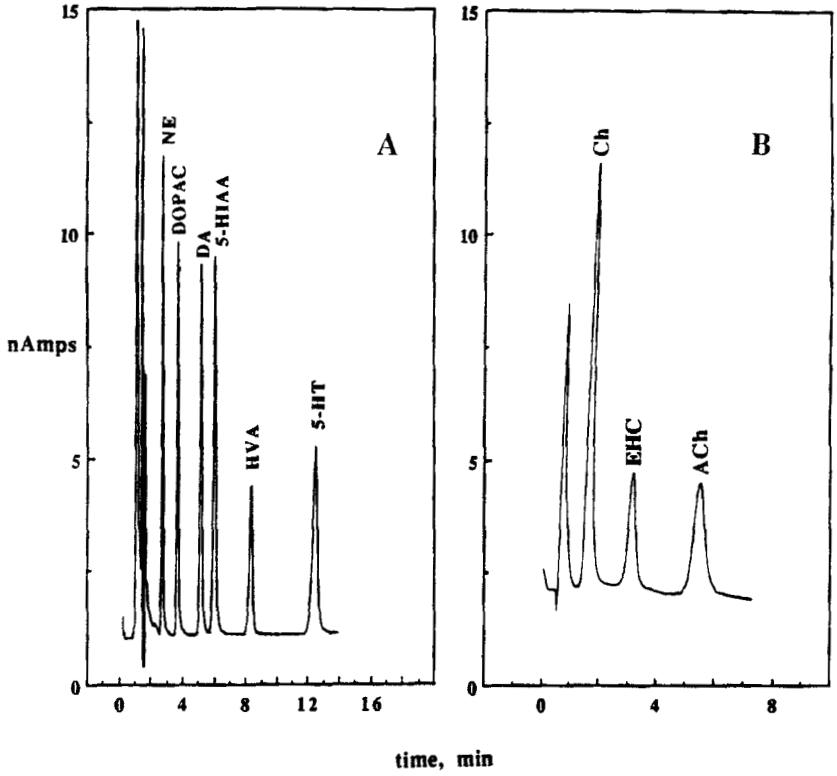

FIGURE 1. Chromatograms obtained from injection of separate mixtures of indoles/catechols and quaternary amines into the LCEC systems designed for the determination of catecholamines, indoleamines, and related metabolites (A) and for the determination of acetylcholine and choline (B). Injections were 4.0 µL aliquots of phosphate buffers containing 2.00 nmol/mL of the substances labeled in each trace.

mixture through a 0.45 µm Millipore® filter; an accurate 4.0 µL aliquot of the supernate was then injected into the LCEC for quantitation of the compound(s) of concern. The amount absorbed for a compound is given as:

$$\text{Amount Absorbed (nmol/g)} = \frac{C(PH - PH_{GC})(1.00\ \text{mL})}{0.050\ \text{g}}$$

where

C = proportionality constant to convert peak height to concentration, µM/nA or [(nmol/mL)/nA],

PH = peak height for compound using the phosphate buffer with no glassy carbon particles, nA,

PH_{GC} = peak height for compound after exposure to the glassy carbon particles, nA,

and the percent adsorbed is given as:

$$\% \text{ Adsorbed} = \frac{(PH - PH_{GC})}{PH} \times 100$$

All determinations are reported as the mean of duplicate measurements.

RESULTS

Effect of Nature of Glassy Carbon Material on Adsorption

In all these experiments, the phosphate buffer aliquots exposed to the glassy carbon materials contained 4.0 nmol of each of the nine neurochemicals listed above under reagents. The percent adsorbed for each of the neurochemicals as a function of the final curing temperature used in the preparation of the glassy carbons, each derived from phenolic resin fibers, is shown in Fig. 2. While no measurable amount of any of the compounds tested was adsorbed onto the 1000 and 1400°C glassy carbons, a small amount of 5-HT (20%) only was adsorbed onto the 2000°C material. The 3000°C material showed adsorption of all the catecholamine and indoleamine related compounds, with maximal adsorption of 5-HT (88%) and minimal adsorption of DOPAC (5%). No adsorption of ACh, EHC, or Ch was observed onto any of the glassy carbon materials tested.

Using the same nine component phosphate buffer mixtures, the effect of adding various amounts of graphite to the material used in the preparation of the glassy carbon particles was examined. In these studies, the glassy carbon precursor was composed of a vinyl chloride resin with 0, 50, 60, 80, or 100% graphite. The carbon mixture was heated to a final curing temperature of 1400°C. The results, shown in Fig. 3, clearly indicate a general trend toward increased adsorption of all of the individual compounds at increased graphite content of the carbon mixture. While the exact order of maximal to minimal adsorption for the catechols and indoles was not identical to that seen in the previous Fig. 2, the same general trend prevailed, with 5-HT and 5-HIAA showing the strongest interaction with the glassy carbon. Additionally, the quaternary amines were not immune to adsorption with this graphite-containing glassy carbon. Some of the EHC (15%)

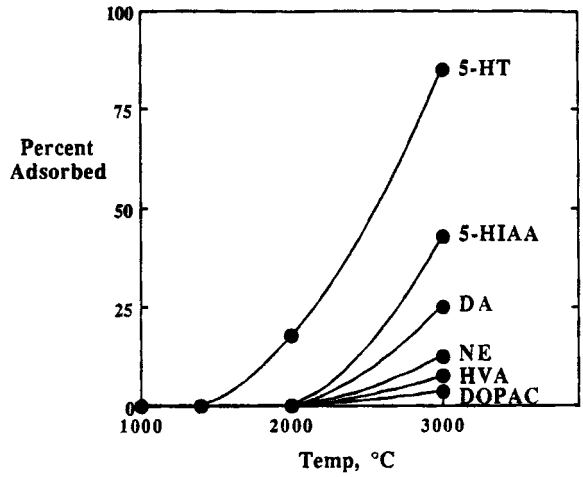

FIGURE 2. Adsorption of various neurochemicals onto glassy carbon derived from phenolic resin fibers as a function of the temperature of the final heat treatment. The glassy carbon particles were exposed to 1.00 mL of a phosphate buffer containing 4.0 nmol/mL of the chemicals shown as well as the same concentration of ACh, EHC, and Ch. No adsorption of the latter three quaternary amines was observed.

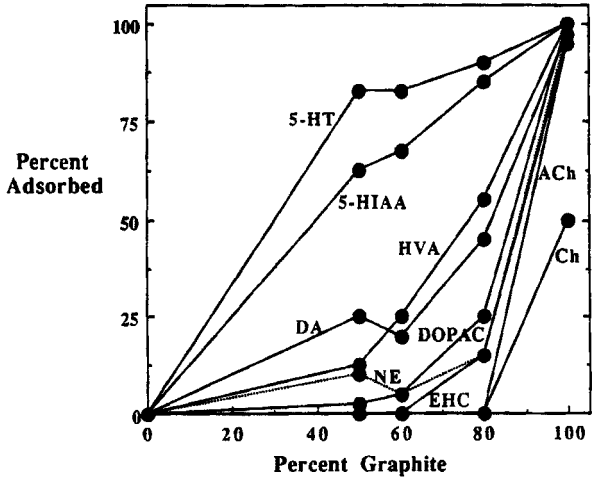

FIGURE 3. Adsorption of various neurochemicals onto glassy carbon derived from a mixture of graphite and polyvinyl chloride as a function of the original graphite content. The glassy carbon particles were exposed to 1.00 mL of a phosphate buffer containing 4.0 nmol/mL of the nine chemicals shown.

was adsorbed on the 80% graphite material, and all three quaternary amines (ACh, EHC, and Ch) were clearly and substantially adsorbed on the pure graphite heated to 1400°C.

Competitive Adsorption of Neurochemicals onto Glassy Carbon

In a preliminary investigation of possible competition between neurochemicals for a limited number of adsorption sites on the glassy carbon particles, we measured the amount of individual components adsorbed from a six component catechol/indole mixture. The individual phosphate solutions tested contained 2.0, 4.0, and 10.0 nmol/mL of each of the compounds. The glassy carbon employed in this and all subsequent competitive tests was the 3000°C material derived from the phenolic resin fibers. As seen in Fig. 4, as the amount of the individual components in the mixture increased, an increased amount of 5-HT and, to a lesser extent, 5-HIAA were adsorbed; however, the amounts of DA, NE, HVA, and DOPAC adsorbed clearly decreased as the amount of all the components increased. These results suggest competition between at least some of the compounds for shared adsorption sites on the carbon.

To further investigate this phenomena, we selected three compounds (5-HT, 5-HIAA, and DA), representing strongly, moderately, and weakly adsorbed substrates, respectively. The adsorption of each of these three species was tested at concentrations varying between 2 and 100 nmol/mL individually, i.e., with no other compounds in the buffer solution exposed to the carbon particles. As seen in Fig. 5, in the absence of other components, 5-HT is, indeed, the most strongly adsorbed; on the other hand, in the absence of other components, the adsorption of both 5-HIAA and DA are quite similar. Employing a Langmuir framework and least squares fitting routine along with the data for Fig. 5, we obtained values for the adsorption constants and the number of adsorption sites for the compounds shown. The pertinent adsorption equation is

$$K_{ads} = \frac{[SA]}{[S]\ [A]}$$

where K_{ads} is the adsorption constant (μM^{-1}) for the particular form (neutral or ionic) of the compound adsorbed, [SA] is the concentration of adsorption sites in the mixture of 1.00 mL of phosphate buffer and 50 mg of glassy carbon which are

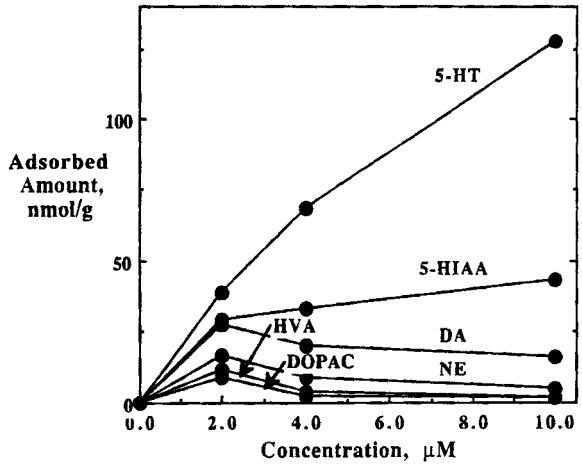

FIGURE 4. Adsorption of six catechols/indoles from a mixture containing increasing concentrations of each. The phosphate solution contained 2.0, 4.0, or 10.0 μM of each of the species shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

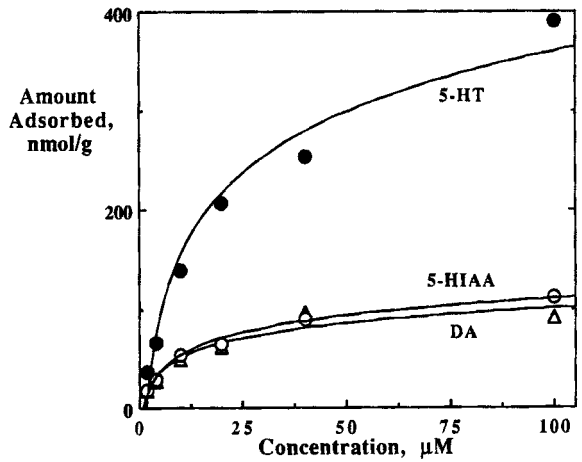

FIGURE 5. Adsorption of 5-HT, 5-HIAA, or DA alone with increasing concentrations of the substrate. The phosphate solutions used in this case contained only one of 5-HT, 5-HIAA, or DA at the concentration shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

occupied by the adsorbate, [A] is the concentration of unoccupied adsorption sites, and [S] is the concentration of the adsorbable form of the component in the phosphate solution (respectively, 5-HT free base, 5-HIAA anion, and DA free base), where all concentrations are expressed as μM or nmol/mL. The total number of available adsorption sites for a particular species, C*, is expressed on a per gram of glassy carbon basis and is defined for these experiments, each using 50 mg of glassy carbon, as

$$C^* = \frac{(\text{nmol}_{SA} + \text{nmol}_A)}{0.050\ \text{g}}$$

where

$$\text{nmol}_{SA} = [SA]\ (1.00\ \text{mL})$$

and

$$\text{nmol}_A = [A]\ (1.00\ \text{mL})$$

Using this formulation, the least squares fitted K_{ads} and C* values (± s.d.) for 5-HT, 5-HIAA, and DA, respectively, were: 781±267 and 188±37; 0.189±0.018 and 93±6; and, 21±6 and 71±11. It is also pertinent to note that the fraction of the adsorbable form of the compound relative to the total amount of free, unbound compound in solution at pH 7.0 are, respectively, 0.141%, 99.9%, and 1.30%; these values were obtained using the pK_a values of 9.85, 4.14, and 8.88, respectively, for deprotonation (8-10). The derived C* values for these three compounds were, notably, approximately the same within a factor of ca. 2; this would indicate that the number of available adsorption sites is approximately the same for all three compounds thus examined.

Employing a fixed, initial concentration of 5-HT of 10 nmol/mL, we examined the adsorption of both 5-HT and 5-HIAA while varying the initial concentration of the latter between 2 and 100 nmol/mL. As seen in Fig. 6, increasing concentrations of 5-HIAA provided increased amounts of adsorbed 5-HIAA at the expense of the adsorption of the 5-HT. In a comparable experiment using fixed concentrations of 5-HT and varying concentrations of DA, as shown in Fig. 7, a similar result was obtained; the amount of adsorbed DA increased with increasing initial concentrations of DA, while the amount of 5-HT adsorbed from the fixed concentration of 5-HT declined correspondingly.

In a final experiment, a fixed concentration of 10 nmol/mL of 5-HT was combined with concomitantly increasing concentrations of both 5-HIAA and DA. As seen in Fig. 8, the increasing concentrations of 5-HIAA and DA provided, as

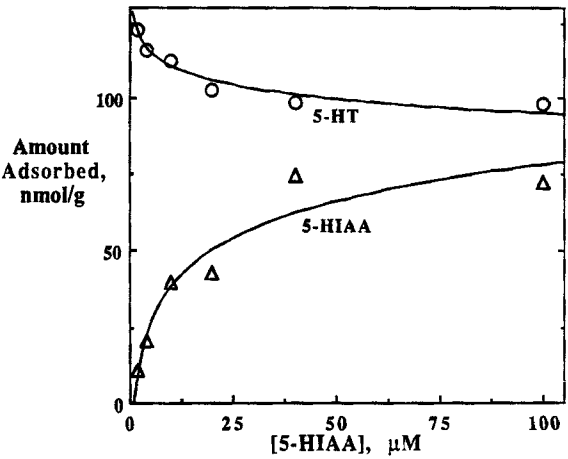

FIGURE 6. Competitive adsorption of 5-HT and 5-HIAA. The phosphate solution contained a fixed, 10 μM 5-HT, while the concentration of 5-HIAA was varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

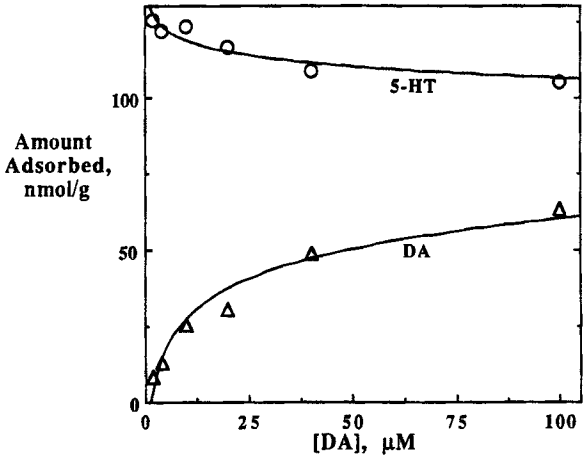

FIGURE 7. Competitive adsorption of 5-HT and DA. The phosphate solution contained a fixed, 10 μM 5-HT, while the amount of DA was varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

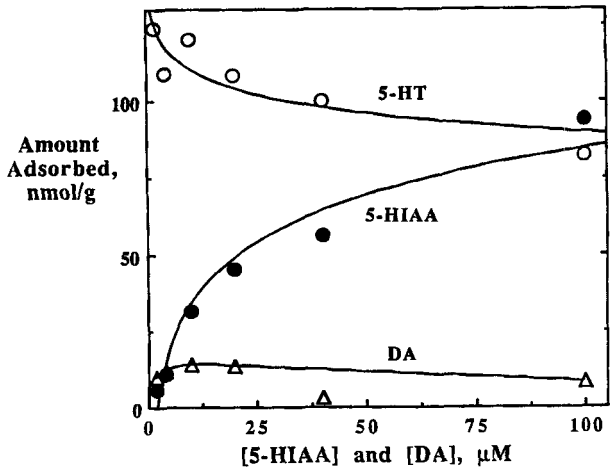

FIGURE 8. Competitive adsorption of 5-HT, 5-HIAA and DA. The phosphate solution contained a fixed, 10 μM 5-HT, while the concentrations of both 5-HIAA and DA were simultaneously varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

might have been expected, correspondingly increasing amounts of adsorbed 5-HIAA and decreased amounts of adsorbed 5-HT. However, the amount of adsorbed DA decreased as its concentration and that of 5-HIAA increased above 20 nmol/mL; this indicates fairly substantial competition between DA and 5-HIAA for binding sites on the glassy carbon, with DA losing the battle.

DISCUSSION

Glassy carbon is a categorization for carbon materials which includes a considerably wide range of substances. The nature of the starting material(s) used in preparation, the initial modest heat treatment with or without high pressure, the final carbonization step at high temperature, the rate(s) of heating in the treatment step(s), and possible mixing or mechanical manipulations between the treatments inevitably produces carbon materials which exhibit a variety of distinctive physical properties (7,11-16). However, the details for preparation of the many different available glassy carbons are frequently only available within a given manufacturing organization, where they are carefully maintained as trade secrets. Our previous

study (1,6) demonstrated the utility of IRICA Type CP-2250 glassy carbon, used as a pre-column in the determination of ACh and Ch, in the elimination of interfering catechols and indoles via effective adsorption processes. The current results with the glassy carbon derived from phenolic resin fibers prepared by either Mitsubishi Pencil Co. or Tokai Carbon show that these materials are also suitable for the same application. However, substantial adsorption of the interferents without concomitant adsorption of the quaternary amines for these latter materials was only achieved with the materials treated at a final carbonization temperature of 3000°C as opposed to the 2250°C of the previously employed IRICA material; thus, we can only recommend the 3000°C material from these two manufacturers for use in pre-columns in ACh and Ch determinations.

The chemical nature of the adsorption site for catechols and indoles on glassy carbon is at least partly addressed by the studies using varying amounts of graphite in conjunction with a vinyl chloride resin as the starting materials for a glassy carbon which received final heat treatment at 1400°C. Increasingly larger amounts of catechols and indoles were adsorbed on the final material as the fraction of graphite was increased, implicating graphite-like domains as the site(s) of adsorption. At the highest fractions of graphite tested (80-100%), even the quaternary amines were adsorbed; thus, too much graphite-like character in the glassy carbon could be deleterious for the utilization of such materials in the determination of ACh and Ch. The investigation discussed in the previous paragraph also supported the involvement of graphite-like domains in the adsorption process. It is well known that the graphite-like character of glassy carbons increases as the final heating temperature increases, with the carbon atoms in materials heated to $\geq$ 2700°C existing almost completely, if not exclusively, in an sp^2 hybridization. Adsorption of the catechols and indoles in this investigation did, indeed, increase as the final temperature of heat treatment was increased. Further, since the 50% graphite material in the second case exhibited roughly comparable adsorption properties for the catechols and indoles to the phenolic resin derived material heated to a final temperature of 3000°C in the first case, one might conclude that approximately one-half of the latter material is composed of graphitic domains.

Investigation of the adsorption of individual components from a six component mixture of catechols and indoles revealed possible competitive binding between these compounds. Further examination with 1, 2, and 3 component mixtures of 5-HT, 5-HIAA, and DA confirmed this competition. While 5-HT was most strongly

attracted to the adsorption sites, at least some of the adsorbed 5-HT could be displaced by either 5-HIAA or DA. In the 3 component mixture, the attraction of the 5-HT and, particularly, 5-HIAA for the binding sites was relatively so strong that adsorption of DA was suppressed. Adsorption binding constants were derived for the one component mixtures, and their magnitudes were found to fall in the order of 5-HT(free base) >> 5-HIAA(anion) ≈ DA(free base). The number of available adsorption sites for all three species was within a relatively small factor of ca. 2, supporting the concept of sharing of the same adsorption sites by the different compounds. The competitive studies for the two and three component mixtures were consistent with the derived adsorption constants, the number of available sites, and the assumption of coincident adsorption sites when one also takes into consideration the fraction of the free (unbound) compound which exists in the appropriate form (protonated or deprotonated) for adsorption.

The amount and degree of adsorption of neurochemically related catechols and indoles onto glassy carbon, thus, is highly dependent on the nature of the starting materials and processing used in the formation of the final glassy carbon. Adsorption of these compounds appears to predominantly involve the graphite-like domains, as opposed to the diamond-like domains. Among the catechols and indoles, the adsorption is a competitive process, indicating accessibility of the same binding sites to more than one of the neurochemicals simultaneously. The 3000°C glassy carbon material obtained from either Mitsubishi Pencil Co. or Tokai Carbon Co. are suitable replacements as pre-column materials for the previously employed IRICA Type CP-2250 glassy carbon in the determination of ACh and Ch by direct injection of the homogenate supernate of tissue samples.

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