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Binding characteristics of various neurochemicals to glassy carbon

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ABSTRACT

The determination of acetylcholine and choline using liquid chromatography with electrochemical detection using the approach of Potter *et al.* [*J. Neurochem.*, 41 (1983) 188] normally requires isolation of the desired species before an analysis of tissue samples can be undertaken due to coeluting interferences afforded by other neutrochemicals. We have recently shown that this problem can be overcome by the use of a glassy carbon precolumn to effectively trap the interfering species [Ikarashi *et al.*, *J. Chromatogr.*, 575 (1992) 29]. We now report on the nature and mechanism of this adsorption onto glassy carbon for norepinephrine, dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid. For both the acidic and basic compounds which comprise this group, distinct Langmuir adsorption processes appear to be involved for both the neutral and ionic forms of the individual compounds. Using various data fitting approaches, we have attempted to derive appropriate adsorption constants for the two forms of each compound. Theoretical predictions employing these derived constants provided results which match reasonably well with the observed adsorption data in most cases.

INTRODUCTION

We recently reported [1] that interfering species seen in the determination of acetylcholine and choline using liquid chromatography with electrochemical detection (LC–ED) [2–5] could be eliminated by utilization of a precolumn packed with glassy carbon particles. The precolumn selectively removed catecholamines, indoleamines and related metabolites while not adsorbing any of the quaternary amine species being analyzed. Further, the precolumn substantially decreased the solvent front peak associated with tissue homogenates. Thus, the precolumn allows direct injection of tissue homogenates, without the need for extensive isolation/purification, in the LC–ED determination of acetylcholine and choline.

Glassy carbon is a fairly well-characterized hard, char material which results from treatment of polymeric precursors at temperatures exceeding 1000°C. This material is well-known to exhibit considerable chemical inertness, a mirror-

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like finish, reasonable electrical conductivity, a hardness in the isotropic state, a relative impermeability to gases and liquids, an ability to withstand high temperatures in non-oxidizing atmospheres, and an ability to withstand considerable thermal shock. These characteristics of glassy carbon are generally attributed to its structure, which is fundamentally a variety of carbon ribbons forming a network of microfibrils [6,7]. Yet, little information exists concerning the mechanism of adsorption of species like the neurochemicals described onto this glassy carbon material [8].

In the current report, we examine the mechanism of adsorption onto glassy carbon particles for norepinephrine (NE), dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA). The first three of these neurochemical species are notably basic compounds, while the latter three are acidic compounds. These fundamental acid–base properties are intimately related, as will be seen, to the adsorption characteristics of each compound.

MATERIALS AND METHODS

Reagents

The chemical reagents employed in this investigation were all obtained from commercial suppliers at the highest available purity and used without further purification. The only exception to this involves N,N-dimethyl-N-ethyl-3-amino-1-propanol, more commonly known as ethylhomocholine (EHC), which was prepared according to a previously described procedure [2,3]. The particular neurochemicals of concern were all purchased from Sigma (St. Louis, MO, USA). These included NE hydrochloride, DA hydrobromide, 5-HT creatinine sulfate monohydrate, HVA, DOPAC, 5-HIAA, acetylcholine (ACh) chloride and choline (Ch) chloride.

Glassy carbon particles

Glassy carbon particles used in these experiments were obtained from the Analytical Laboratories of IRICA Instruments (Kyoto, Japan). They are listed as IRICA type CP-2250. Physical investigations showed that the particles were of a glass-like, vitreous, hard carbon material [1]. They had an electrical resistivity of $4.5 \cdot 10^{-3}$ Ω/cm, a specific gravity of 1.52, a thermal expansion coefficient of $2.2 \cdot 10^{-6}$, and impurities totalling less than 0.008%. The particles were irregular, with typical dimensions on the order of 70 μm [1].

LC–ED system for determination of catecholamines, indoleamines and related metabolites

The liquid chromatographic system employed for the determination of NE, DA, 5-HT and the three related acid metabolites primarily employed components from Bioanalytical Systems (BAS, West Lafayette, IN, USA). These included a PM-60 pump, a CC-4 injector, a BioPhase ODS IV (3μm, 110 × 4.6 mm) analytical column, a dual glassy carbon electrode, and an LC-4B amperometric potentiostat. Temperature in the column was maintained by an LC-22 temperature controller to be 35 ± 1°C. The potential of the working electrode was set at +0.70 V *vs.* the Ag/AgCl reference electrode. The mobile phase was a 0.050 *M* citrate buffer of pH 3.2, containing 0.80 m*M* sodium 1-octanesulfonate and 0.50 m*M* disodium EDTA. The flow-rate was typically 0.80 ml/min. Data collection and processing were accomplished with the aid of an SIC Chromatocorder from Yokogawa (Tokyo, Japan). A typical chromatogram observed for the six components of interest is shown in Fig. 1A. Retention times observed, in order of elution, were: NE, 3.1 min; DOPAC, 4.3 min; DA, 6.2 min; 5-HIAA, 7.2 min; HVA, 10.2 min; and 5-HT, 15.3 min. Detection limits were 0.3–1.0 pmol for the species of concern when operating at the usual 20 nA full scale setting.

LC–ED system for determination of ACh, EHC and Ch

The liquid chromatographic system employed for the determination of these three quaternary amines employed at LC100P pump and an LC100S injector from Yokogawa, an LC-4A amperometric detector with a dual platinum

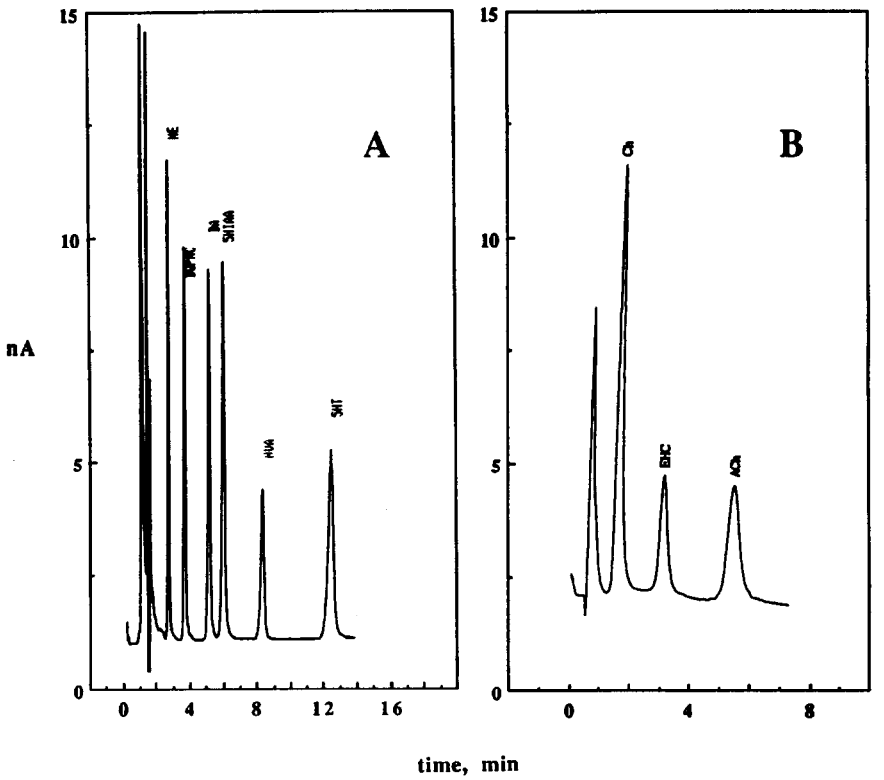

Fig. 1. Typical chromatograms obtained from mixture of (A) indoles/catechols and (b) quaternary amines. Injection of 4.0 μl of sample containing 2.00 nmol/ml of each substance identified.

working electrode from BAS, an Acetylcholine Separation analytical column (3 μm, 60 × 4 mm, polymeric styrene based packing material) from BAS, and an immobilized post-column enzyme reactor (5 × 4 mm) containing acetylcholinesterase and choline oxidase from BAS. Data collection and processing employed an LC100W/F work station from Yokogawa. The mobile phase for this system was a 0.050 *M* phosphate buffer, pH 8.4, containing 1.0 m*M* disodium EDTA and 0.40 m*M* sodium 1-octanesulfonate. The flow-rate was typically 0.80 ml/min, and the working electrode potential was set to 0.50 V *vs.* Ag/AgCl. Temperature of the column and post-column were maintained at 35 ± 1°C by a BAS Model LC-22 temperature controller. A typical chromatogram obtained for the compounds of concern is presented in Fig. 1B. Pertinent retention times, in order of elution, were: Ch, 2.2 min; EHC, 4.5 min; and ACh, 8.4 min. Detection limits were 2–5 pmol at the usual setting of 20 nA full scale.

Adsorption of neurochemicals onto glassy carbon

Three separate sets of experiments were performed to determine the binding characteristics of the neurochemicals of concern. In all three, a mixture of indoles/catechols and a mixture of quaternary amines were separately exposed to glassy carbon particles. For the indoles/catechols, each sample incorporated a pre-determined amount of glassy carbon and a 1.00-ml aliquot of 0.100 *M* phosphate buffer containing 2.00 nmol each of NE, DA, 5-HT, HVA, DOPAC and 5-HIAA. For the quaternary amines, each sample incorporated the glassy carbon and a 1.00-ml aliquot of 0.100 *M* phosphate buffer containing 2.00 nmol each of ACh, Ch and EHC. After the

described agitation, the glassy carbon particles with the adsorbed species were separated by filtration through a 0.45-μm Millipore filter, and a 4.00-μl aliquot of the filtrate was injected into the appropriate LC–ED system for quantitation. The results for each substance were compared to the LC–ED results obtained from equivalent mixtures which had not been exposed to any glassy carbon particles. This procedure yielded a percent adsorbed for each species, calculated as:

$$\% \text{ Adsorbed} = \frac{(PH_{\text{no GC}} - PH_{GC})}{PH_{\text{no GC}}} \times 100$$

where $PH_{\text{no GC}}$ = LC–ED peak height observed for the species of concern when no glassy carbon particles were included

PH_{GC} = LC–ED peak height observed for the species of concern when glassy carbon particles were included.

The fraction adsorbed, f, is simply equal to the percent adsorbed divided by 100. All results were calculated and reported as the mean ± S.D. for at least 3 separate determinations.

The first set of experiments was designed to examine the importance of the time of exposure on the amount of adsorption. In both groups associated with these experiments, a 1.00-ml pipet of the compounds, in a pH 8.40 phosphate buffer, was added to 100 mg of the glassy carbon particles. In the first group, the samples were shaken on a vortex mixer for a few (≤5) seconds prior to filtration. In the second group, the samples were shaken on a mechanical shaker for 10 min prior to filtration.

The second set of experiments was designed to determine the effect of the amount of glassy carbon on the adsorption observed. In this case, a 1.00-ml pipet of the compounds, again contained in a pH 8.40 phosphate buffer, was added to 0, 10, 20, 50, 100 or 200 mg of the glassy carbon particles. After shaking on a vortex mixer for a few (≤5) seconds, the samples were filtered, and the filtrate was subjected to LC–ED analysis.

The third set of experiments was designed to examine the effect of pH on the adsorption process. In this case, a 1.00-ml pipet of the compounds was added to 100 mg of the glassy carbon particles, the mixture was shaken on a vortex mixer for a few (≤5) seconds, the samples were filtered, and the resultant filtrate was subjected to LC–ED analysis. The pH values employed for these experiments were 1.00, 3.00, 5.00, 7.00 and 9.00.

Calculations

Cursory examination of the data from the second and third sets of experiments indicated a Langmuir adsorption type behavior for the six indoles/catechols, while no adsorption was observed for any of the quaternary amines under any of the conditions investigated. The attempted fitting of the empirical data to simple and multiple versions of Langmuir adsorption involved two basic approaches. In the first, the non-linear least squares (NLLSQ) method of Christian and Tucker [9], based on the original strategy of Marquardt [10], was employed. In the second, a successive approximation was employed, in which sum of the squares of the deviations of the calculated values from the experimental values, Σd_i^2, was directly minimized. In both approaches, we sometimes considered only one and sometimes considered two separate adsorption processes to be concurrently active. Only the approaches which provided the final reported results are given immediately below. Alternative attempts are briefly covered in the text.

Method 1. This calculation used the non-linear least squares approach and assumed that, at the pH value of 8.40, only one adsorption process was significant for the compound under the conditions utilized. The one adsorption process involved the neutral form of the compound for the basic compounds and the ionic form of the compound for the acidic compounds. For the bases, the equation of concern, derived below, can be expressed as:

$$f = \frac{m}{C_{\text{init}}} \left(\frac{K_{\text{ads,neut}} C^*_{\text{neut}} C_{\text{neut}}}{1 + K_{\text{ads,neut}} C_{\text{neut}}} \right) \quad (1)$$

where f is the fraction of the compound adsorbed, m is the number of milligrams of carbon

used per ml of solution, $K_{ads,neut}$ (ml/nmol or μM^{-1}) is the adsorption constant for the neutral species, C^*_{neut} is the number of available adsorption sites (nmol/mg carbon), C_{neut} is the concentration of the unbound neutral species in solution (nmol/ml), and C_{init} is the initial total concentration (ionic + neutral forms) of the compound (nmol/ml). For the acids, a completely analogous equation was employed in which the terms given above for the neutral form of the compound were replaced by those representative of the ionic form of the compound.

Method 2. Having obtained values of $K_{ads,ion}$ and C^*_{ion} for the acidic species at pH 8.40 using method 1, we were able to simultaneously consider the more complex adsorption situation involving both the ionic and neutral species at lower pH values. Using the empirical data for the fraction adsorbed at pH values of 3.00 and 5.00 along with the previously derived values of $K_{ads,ion}$ and C^*_{ion} provided two equations containing two unknowns ($K_{ads,neut}$ and C^*_{neut}), which were readily solved for the latter constants. A similar approach was not successful in an attempted determination of the constants for the ionic form of the basic compounds.

Method 3. Calculation of the theoretical values for the percent adsorbed was accomplished for the acids using the derived values for $K_{ads,neut}$, C^*_{neut}, K_{ion} and C^*_{ion} and using a successive approximations technique. In this case, we started by assuming that the concentration of unbound compound, representing the sum of the neutral and ionic forms of the compound, was initially equal to the value present in the absence of any adsorbent, *i.e.*, C_{init}. In the first step, we calculated the fraction adsorbed considering only the adsorption of the ionic form and the appropriate form of the quadratic expression for f (see below); this yielded a modified value for the total concentration of unbound compound. In the second step, the modified value of the unbound compound was used with a second form of the quadratic expression considering adsorption of the neutral form of the compound only, yielding a further modified value for the unbound concentration of the compound. If the further modified value was within 0.001% of the initial value for the unbound concentration, the iteration was considered complete; if not, the initial value was set equal to the further modified value, and the process was repeated. Since we did not determine the values of K_{ion} and C^*_{ion} for the basic compounds, this process involved only a simple and single solution of the appropriate quadratic expression.

RESULTS AND DISCUSSION

Initial experiments focused on the time required to achieve equilibration in the adsorption of various neurochemicals onto glassy carbon particles. The amount of glassy carbon employed was a constant 100 mg. This and the following set of experiments notably employed a pH 8.40 phosphate buffer as the solvent for the neurochemicals, since this solution is identical to the eluting solvent employed in the LC–ED determination of ACh and Ch using the glassy carbon precolumn [1]. The time allowed for contact between the glassy carbon particles and the solutions was either a few ($\leq$5) seconds or 10 min, respectively.

The results for the indoles/catechols, as shown in Fig. 2, indicate a convenient subdivision of these species into acids (HVA, DOPAC and 5-HIAA) and bases (NE, DA and 5-HT). The bases were adsorbed in amounts exceeding 90%, while the acids showed adsorption varying between 25 and 80%. However, for each compound investigated, there was no significant difference between the results obtained for the percent adsorbed when comparing the brief shaking with a vortex mixer ($\leq$5 s) to the extended shaking period of 10 min (two-tailed t-test, $P > 0.05$). Thus, the shorter time was used for all subsequent investigations. The comparable experiments for the quaternary amines (ACh, Ch and EHC) showed no significant adsorption onto the glassy carbon particles using either the short or long shaking times.

A second set of experiments was intended to investigate the effect of varying amounts of glassy carbon particles on the adsorption phenomena. While investigating Langmuir type adsorption phenomena as a function of adsorbent is a bit unusual (one would normally examine adsorption as a function of the solution concen-

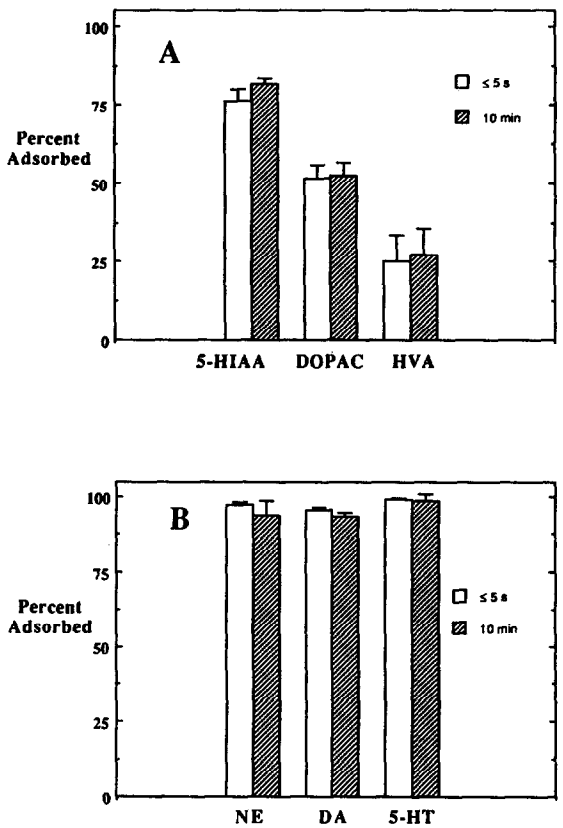

Fig. 2. Effect of adsorption time on percent adsorbed onto glassy carbon for various neurochemicals. Compounds are grouped as acids (A) and bases (B).

tration of the adsorbed species), we felt this approach was more relevant to the intended use of these materials; users of the mentioned precolumns [1] have some control over the amount of carbon employed but virtually no control over the concentration of the adsorbing species. Thus, in this second set of experiments, 1.00 ml of the test solution of indoles/catechols or quaternary amines, in a pH 8.40 phosphate buffer, was briefly shaken with 0 to 200 mg of the particles prior to filtration and analysis of the filtrate. The results, as presented in Table I, again indicate that there was no significant adsorption for the quaternary amines, even at the highest amounts of glassy carbon employed. With the indoles/catechols, the three bases (NE, DA and 5-HT) show a rapid rise in the percent adsorbed with increasing amounts of glassy carbon at small amounts of glassy carbon used, followed by an asymptotic approach to 100% adsorbed at the higher values of glassy carbon used. The three acids (DOPAC, HVA and 5-HIAA) exhibit a more moderate rise in percent adsorbed with increasing amounts of glassy carbon at the small amounts of carbon used; the results at the higher amounts of carbon, however, indicate an eventual asymptotic approach to 100% adsorbed. Thus, for both the acids and bases at pH 8.40, we observe a Langmuir type of adsorption curve for each of the indole/catechol species involved.

In the third set of experiments, we briefly examined the effect of the pH on the adsorption process. As seen in Table II, no significant adsorption was again observed for any of the three quaternary amines at any pH value between 1.0 and 9.0. For the indoles/catechols, however, there was a marked pH dependence observed. For the bases, the largest amounts of adsorption were observed at pH 9.0, with substantially greater amounts being adsorbed at this value than the low pH values. The adsorption of the bases decreased with decreasing pH, although the decrease observed was not linear and not reminiscent of a classic pH titration curve. In the case of the acids, the percent adsorbed appears to be fairly constant for each compound in the pH range of 1.0 to 3.0; the percent adsorbed then modestly declines in the pH range of 3.0 to 7.0 and becomes relatively constant, although slightly lower, in the pH range of 7.0 to 9.0. These results for the acids are reminiscent of classic pH titration curves.

In examining the results obtained in Tables I and II, we can initially and unequivocally state that adsorption of ACh, Ch and EHC was not significant for any of the pH values or amounts of carbon investigated. This means that the glassy carbon precolumn previously described [1] will in no way impede the determination of these three targeted compounds.

However, understanding the results of Tables I and II with respect to the behavior of the indoles/catechols turned out to be somewhat more difficult. Preliminary observation of the results for, particularly, Table II indicates that there is clearly some effect(s) associated with the individual forms, neutral or ionic, of the compound of concern. For the bases, the deproto-

TABLE I

PERCENT OF COMPOUND ACTUALLY ADSORBED (AND THEORETICALLY CALCULATED) AS A FUNCTION OF THE AMOUNT OF GLASSY CARBON EMPLOYED

Each experimental value represents the mean ± S.D. derived from three determinations. See text for calculation of theoretical values.

Compound	Percent adsorbed (calculated)					
	Amount of carbon (mg)					
	0	10	20	50	100	200
NE	0.0 ± 3.3(0)	25.8 ± 0.6(17.3)	32.8 ± 1.9(34.2)	83.6 ± 0.7(76.6)	94.5 ± 0.5(95.1)	98.4 ± 0.5(98.3)
DA	0.0 ± 0.7(0)	51.6 ± 2.5(24.9)	51.9 ± 0.8(48.8)	93.7 ± 1.0(91.8)	96.9 ± 1.1(97.9)	99.2 ± 0.2(99.2)
5-HT	0.0 ± 2.7(0)	78.5 ± 0.8(65.2)	90.8 ± 1.3(93.9)	98.5 ± 0.7(98.8)	99.5 ± 0.4(99.5)	100.0 ± 0.0(99.8)
DOPAC	0.0 ± 1.6(0)	13.1 ± 1.3(7.0)	15.1 ± 0.9(13.8)	30.5 ± 1.4(32.5)	54.2 ± 1.5(56.8)	80.2 ± 0.6(80.2)
HVA	0.0 ± 2.1(0)	18.4 ± 2.7(5.1)	21.5 ± 3.2(9.8)	29.3 ± 2.9(21.3)	36.7 ± 2.2(35.1)	49.8 ± 2.3(52.0)
5-HIAA	0.0 ± 1.3(0)	18.8 ± 1.7(17.4)	23.8 ± 1.5(31.7)	60.4 ± 1.2(58.8)	78.2 ± 1.7(77.2)	88.4 ± 0.7(88.4)
ACh	0.0 ± 1.5	−0.9 ± 5.0	−3.4 ± 2.2	−0.3 ± 1.5	1.8 ± 3.5	1.2 ± 1.6
Ch	0.0 ± 1.7	−0.1 ± 1.2	−0.6 ± 1.4	1.2 ± 1.7	2.5 ± 2.2	−2.0 ± 1.2
EHC	0.0 ± 4.1	−1.0 ± 0.9	−3.3 ± 2.9	−0.7 ± 3.0	−2.0 ± 3.1	−4.0 ± 3.5

nated form appears to have a much stronger attraction for the glassy carbon than the protonated form, while for the acids, the protonated form appears to have a much stronger attraction for the glassy carbon than the deprotonated form. One could alternatively say that, in both cases, the neutral form has a stronger attraction for the glassy carbon than does the ionic form. Thus, we clearly must consider the deprotonation constants for these compounds. These

TABLE II

PERCENT OF COMPOUND ACTUALLY ADSORBED (AND THEORETICALLY CALCULATED) AS A FUNCTION OF pH EMPLOYED

Each experimental value represents the mean ± S.D. derived from three determinations. Theoretical values only calculated for the acids (see text).

Compound	Percent adsorbed (calculated)					
	pH					
	1.0	3.0	5.0	7.0	8.4	9.0
NE	6.7 ± 2.1	25.7 ± 0.9	35.9 ± 1.7	58.1 ± 1.7	94.5 ± 0.5	97.3 ± 0.6
DA	32.8 ± 1.4	56.0 ± 1.4	69.6 ± 2.0	76.1 ± 2.0	96.9 ± 1.1	98.8 ± 0.5
5-HT	95.0 ± 0.9	97.2 ± 0.5	98.3 ± 0.4	98.6 ± 0.3	99.5 ± 0.4	99.5 ± 0.2
DOPAC	90.0 ± 8.9(85.1)	84.8 ± 7.8(84.8)	69.0 ± 1.9(69.0)	49.9 ± 0.9(57.0)	54.2 ± 1.5(56.8)	44.3 ± 1.9(56.8)
HVA	82.2 ± 0.5(79.7)	79.1 ± 1.5(79.1)	55.1 ± 3.5(55.1)	28.0 ± 3.3(35.3)	36.7 ± 2.2(35.1)	26.8 ± 4.5(35.1)
5-HIAA	96.8 ± 0.4(96.8)	96.7 ± 1.0(96.7)	92.2 ± 1.8(92.2)	74.6 ± 1.7(78.3)	78.2 ± 1.7(77.2)	76.4 ± 1.9(77.2)
ACh	−4.9 ± 4.1	1.0 ± 0.9	−4.6 ± 7.5	0.6 ± 1.0	1.8 ± 3.5	3.6 ± 4.9
Ch	−2.4 ± 2.9	1.0 ± 1.6	3.3 ± 3.1	−2.8 ± 5.0	2.5 ± 2.2	−4.9 ± 4.4
EHC	−6.4 ± 6.7	1.5 ± 3.4	−5.3 ± 4.8	−8.7 ± 7.4	−2.0 ± 3.1	−5.9 ± 3.5

TABLE III

ACID DISSOCIATION CONSTANTS

For the first three compounds (NE, DA and 5-HT), the pK_a refers to deprotonation of the ammonium ion, while for the last three compounds (DOPAC, HVA, 5-HIAA), the pK_a refers to deprotonation of the neutral acid.

Compound	pK_a
NE	8.61
DA	8.88
5-HT	9.85
DOPAC	4.14
HVA	4.31
5-HIAA	4.14

constants are presented in logarithmic form in Table III. These values were all obtained from the reference work by Smith and Martell [11–13], with the exception of that for 5-HIAA. Since no pK_a value could be found for 5-HIAA, we assumed a value of 4.14 to be reasonable after examining a number of different carboxylic acids in which the acid functionality was located one methylene group away from an aromatic structure; we estimate that this pK_a value for 5-HIAA is accurate within ±0.5, which is more than sufficient for the current discussion. For the remainder of the species, the reported pK_a values were determined at ionic strengths between 0.1 and 0.37 and at temperatures of 20–30°C, both of which are entirely appropriate to the current investigation.

Initial attempts to fit the indoles/catechols data to a Langmuirian conceptual framework considered both the neutral and ionic forms of the compound of concern. The adsorption process for the neutral species, neut or N, is given as:

$$N + A \rightleftharpoons NA \tag{2}$$

where A represents an unoccupied adsorption site on the glassy carbon particle and NA represents an adsorption site occupied by the neutral species. The associated adsorption constant is given as

$$K_{ads,neut} = \frac{C_{NA}}{C_{neut} C_A} \tag{3}$$

and has the units of $(nmol/ml)^{-1}$ or μM^{-1}. The total number of available adsorption sites for the neutral species, given in units of nmols per milligram of glassy carbon adsorbent, is correspondingly labeled as C^*_{neut}. Thus,

$$mC^*_{neut} = C_{NA} + C_A \tag{4}$$

where m is the number of milligrams of carbon per ml of solution used in the experiment. The fraction adsorbed is simply

$$f = \frac{C_{NA}}{C_{init}} \tag{5}$$

where $C_{init} = 2\ \mu M$ is the initial concentration, prior to adsorption, employed for all the compounds of concern. Rearrangement of eqns. 3–5 provides the originally presented eqn. 1, which may be directly fitted to the observed data (see below). For the determination of the C_{neut} term in eqn. 1, we define the fraction of the total amount of unbound compound which is in the neutral form as

$$g_{neut,base} = \frac{C_{neut}}{C_{neut} + C_{ion}} = \frac{1}{1 + 10^{pK_a - pH}} \tag{6}$$

for the basic compounds investigated. Then,

$$C_{neut} = g_{neut,base} C_{unbound} \tag{7}$$

where $C_{unbound}$ is the measured, total concentration of unbound species ($=C_{neut} + C_{ion}$).

By analogy to that given above, adsorption of the ionic form of the compound, represented as I or ion, to a second adsorption site, B, is

$$I + B \rightleftharpoons IB \tag{8}$$

$$K_{ads,ion} = \frac{C_{IB}}{C_{ion} C_B} \tag{9}$$

$$mC^*_{ion} = C_{IB} + C_B \tag{10}$$

with the corresponding fractional concentrations for a basic compound being

$$g_{ion,base} = \frac{C_{ion}}{C_{neut} + C_{ion}} = \frac{1}{1 + 10^{pH - pK_a}} \tag{11}$$

$$C_{ion} = g_{ion,base} C_{unbound} \tag{12}$$

Consideration of simultaneous adsorption of both the neutral and ionic forms of the compound requires modification of the fraction adsorbed in eqn. 5 to

$$f = \frac{C_{NA} + C_{IB}}{C_{init}} \quad (13)$$

Then, utilization of eqns. 3, 4, 9 and 10 leads to

$$f = \frac{m}{C_{init}} \left(\frac{K_{ads,neut} C^*_{neut} C_{neut}}{1 + K_{ads,neut} C_{neut}} + \frac{K_{ads,ion} C^*_{ion} C_{ion}}{1 + K_{ads,ion} C_{ion}} \right) \quad (14)$$

which is a comprehensive expression for the fraction adsorbed for basic compounds incorporating both neutral and ionic adsorption sites. Eqn. 1 is simply a shortened version of this in which the right-hand expression inside the parentheses of eqn. 14, corresponding to adsorption of the ionic species, is eliminated.

Consideration of the acidic compounds likewise leads to an expression for f which is identical to eqn. 14. However, in this case, the corresponding fractional concentrations of unbound species are

$$g_{neut,acid} = \frac{C_{neut}}{C_{neut} + C_{ion}} = \frac{1}{1 + 10^{pH - pK_a}} \quad (15)$$

and

$$g_{ion,acid} = \frac{C_{ion}}{C_{neut} + C_{ion}} = \frac{1}{1 + 10^{pK_a - pH}} \quad (16)$$

which are employed as above with the $C_{unbound}$ to yield the values of C_{neut} and C_{ion} to be used in eqn. 14. We will return to these comprehensive results shortly.

For both the acids and the bases, one may, in an alternative approach, note that the values of C_{neut} and C_{ion} can be expressed in terms of the fraction adsorbed. For the basic compounds, these values would be given as

$$C_{neut} = g_{neut,base} C_{unbound} = g_{neut,base}(1 - f) C_{init} \quad (17)$$

and

$$C_{ion} = g_{ion,base} C_{unbound} = g_{ion,base}(1 - f) C_{init} \quad (18)$$

Utilization of these equations, along with eqns. 3, 4, 9, 10 and 13, leads to a cubic equation in f when one considers simultaneous adsorption of both the neutral and ionic forms of the compound at two separate sites. The only other parameters appearing in this cubic expression are the two unknown K_{ads} values, the two unknown C^* values, the known pK_a value, and the pH. A comparable cubic equation also results for the acidic compounds. Since these cubic equations are intractable (see below) and since our primary focus was on the adsorption processes occurring at the pH of 8.40 [1], we also proceeded along this same general path but considered adsorption of only the prominent species at this important pH. For both the basic and acidic compounds, this results in a quadratic equation for f, the solution for which is the usual

$$f = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (19)$$

where, for the basic compounds, focussing on the neutral form,

$$a = K_{ads,neut} C_{init} g_{neut,base} \quad (20)$$

$$b = -(C_{init} + mC^*_{neut})(g_{neut,base} K_{ads,neut}) - 1 \quad (21)$$

$$c = g_{neut,base} K_{ads,neut} mC^*_{neut} \quad (22)$$

and, for the acidic compounds, focussing on the ionic form,

$$a = K_{ads,ion} C_{init} g_{ion,acid} \quad (23)$$

$$b = -(C_{init} + mC^*_{ion})(g_{ion,acid} K_{ads,ion}) - 1 \quad (24)$$

$$c = g_{ion,acid} K_{ads,ion} mC^*_{ion} \quad (25)$$

Armed with theoretical frameworks to attack the data in a number of appropriate ways, we began by trying to fit all the data of Tables I and II simultaneously. Using NLLSQ or successive approximations along with the two adsorption sites outlined above yielded non-convergence for all of the six compounds attempted. The corresponding cubic equation mentioned was solved, in both of these attempts, through the use of the Newton–Gauss method. Trying either NLLSQ or successive approximations with eqn. 14 also failed to yield convergence for any of the compounds. Similar attempts were undertaken by assuming two different adsorption sites for the

neutral species and none for the ionic species; these attempts were also unsuccessful.

Having obtained unacceptable results using the comprehensive considerations of two adsorption processes simultaneously, we narrowed our efforts to the most important pH 8.40 data presented in Table I considering only the adsorption of a single form of the compound in question, the species of interest being the neutral compound for the bases and the anion for the acids. Starting with the appropriate form of the quadratic eqn. 19, the NLLSQ approach failed to converge for any of the compounds. On the other hand, the successive approximations approach did converge for all the compounds of concern. Comparing the theoretical data using the obtained constants to the actual data of Table I graphically seemed to show reasonable results for most of the compounds. However, there is no estimation of the precision with which the derived adsorption constants are known when using this approach. At this point, we moved to the single adsorption process represented by eqn. 1 for the neutral form of the bases and the corresponding equation for the anionic form of the acids. Again, restricting ourselves to the data of Table I, we were successful in obtaining convergence for all of the compounds as well as estimates of the precision associated with the constants. Further calculations, using data for the selected pH values of 3.0 and 5.0 for the acids, yielded two equations with two unknowns (method 2) allowing the determination of both $K_{ads,neut}$ and C^*_{neut} for the three acidic compounds. Unfortunately, efforts to obtain the $K_{ads,ion}$ and C^*_{ion} values for the basic compounds did not yield satisfactory results.

The values of the derived constants are presented in Table IV. Graphical comparisons of theoretical curves obtained using these constants are shown in Fig. 3 along with the experimental points. As can be seen, the theoretical curves fit the empirical data reasonably well for all the compounds of concern, except in the case of HVA. For HVA, the fit is quite poor, and, as noted in the footnote to Table IV, we seriously doubt the reliability of the numerical constants derived for this compound. The reasonably good fit for the other five compounds shown in Fig. 3 are particularly pertinent when one considers that the primary use of the glassy carbon in the determination of ACh and Ch employs a completely comparable phosphate buffer at the identical pH of 8.40. Thus, the theoretical approach described should be useful in predicting

TABLE IV

ADSORPTION CONSTANTS AND NUMBER OF ADSORPTION SITES FOR ADSORPTION OF NEUROCHEMICALS ONTO GLASSY CARBON

Values derived from NLLSQ are presented as the converged result ± the standard error estimate. Values for the neutral form of the acids determined using method 2. N.D. = Values not determined.

Compound	Neutral form		Ionic form	
	$K_{ads,neut}$ (μM^{-1})	C^*_{neut} (nmol/mg)	$K_{ads,ion}$ (μM^{-1})	C^*_{ion} (nmol/mg)
NE	29.0 ± 6.0	0.036 ± 0.005	N.D.	N.D.
DA	59.0 ± 10.0	0.052 ± 0.007	N.D.	N.D.
5-HT	466.0 ± 138.0	0.14 ± 0.03	N.D.	N.D.
DOPAC	0.093	0.63	2.13 ± 0.53	0.0175 ± 0.0024
HVA	0.74[a]	0.069[a]	[a]	[a]
5-HIAA	59.0	0.024	0.66 ± 0.16	0.066 ± 0.014

[a] The values obtained for HVA are highly questionable and considered generally unreliable, since the standard error estimates resulting from the NLLSQ fitting are 5–6 orders of magnitude greater than the values. The values obtained were: $K_{ads,ion} = 1.6 \cdot 10^{-5} \pm 0.79$ and $C^*_{ion} = 334 \pm 1.6 \cdot 10^7$.

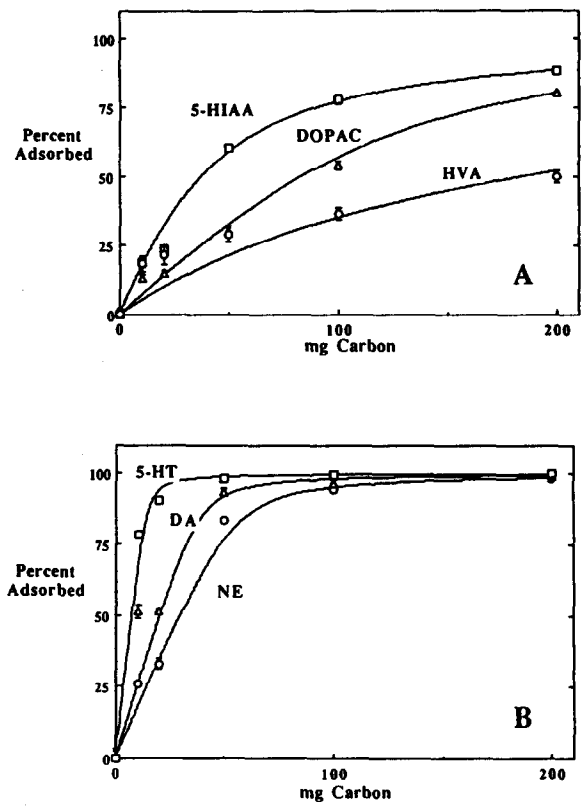

Fig. 3. Effect of amount of glassy carbon on percent adsorbed for various neurochemicals. The theoretical lines shown were derived using the constants listed in Table IV. For the bases (B), quadratic eqn. 19 was used for the theoretical curve, while for the acids (A) method 3 described in the text was employed. Empirical values are shown as mean ± S.D. for NE (○), DA (△), 5-HT (□), HVA (○), DOPAC (△) and 5-HIAA (□).

the adsorption behaviour of the six indoles/catechols and the useful lifetime of glassy carbon precolumns in such applications.

As an example of the use of adsorption constants derived in connection with Fig. 3, one might make a crude approximation of the capacity of a precolumn packed with 1 g of such carbon materials in the determination of ACh and Ch in whole mouse brains. A typical whole mouse brain weighs *ca.* 0.5 g and contains approximately 1.5 nmol NE, 3.0 nmol DA, 2.5 nmol 5-HT, 1.5 nmol 5-HIAA, 0.5 nmol HVA and 0.5 nmol DOPAC. Simply considering the C^* values to represent the maximal capacity of the 1-g carbon column for each of these compounds yields individual capacities of 36 nmol NE, 52 nmol DA, 140 nmol 5-HT, 18 nmol DOPAC, >1000 nmol HVA and 66 nmol of 5-HIAA. We can further reasonably assume that each whole mouse brain is homogenized in 1.0 ml of solution, and the normal injection volume is 5 μl per sample. Then, the precolumn thus considered would have a capacity for *ca.* 3500 injections with the first predicted break-through occurring for DA. Of course, this is an overly optimistic estimation of the column lifetime, since it ignores adsorption equilibrium with the moving mobile phase.

For the remaining pH data, a theoretical curve (calculated with method 3) is shown along with the experimental data for each of the three acids in Fig. 4A, while a graphical presentation of the empirical data only for the bases is given for reference in Fig. 4B. As can be seen, the data for the acids is moderately well described by the four adsorption related constants form Table IV. In the case of the bases, however, the adsorption observed as a function of pH is even more complex. Simple consideration of two adsorption processes for the bases, independent of the values of the constants involved, would lead to an S-shaped theoretical curve similar to an acid/base titration of pH *vs.* ml. Indeed, the empirical data of Fig. 4B seems to indicate some of this character, with strong adsorption of the neutral species at pH values of 9.0, and, after the percent adsorbed drops off as the pH is lowered, the three bases each seem to indicate the beginnings of an expected leveling associated with the predominance of the cationic species at a pH value of approximately 5.0. But, as the pH falls below 5.0 toward 1.0, the adsorption for each of the three compounds falls substantially instead of remaining level in this range. This deviation at pH values below 5.0, seen with the bases only, is ascribed to probable surface structural features of the glassy carbon and/or simple competitive displacement. It is likely that the glassy carbon contains adsorption sites for the protonated forms of the bases which consist of oxidized forms of carbon [14–17]. Such oxygenated forms of carbon would include aldehyde, ketone, quinone, or carboxylic functionalities which could exhibit protonation at lower pH leading to less

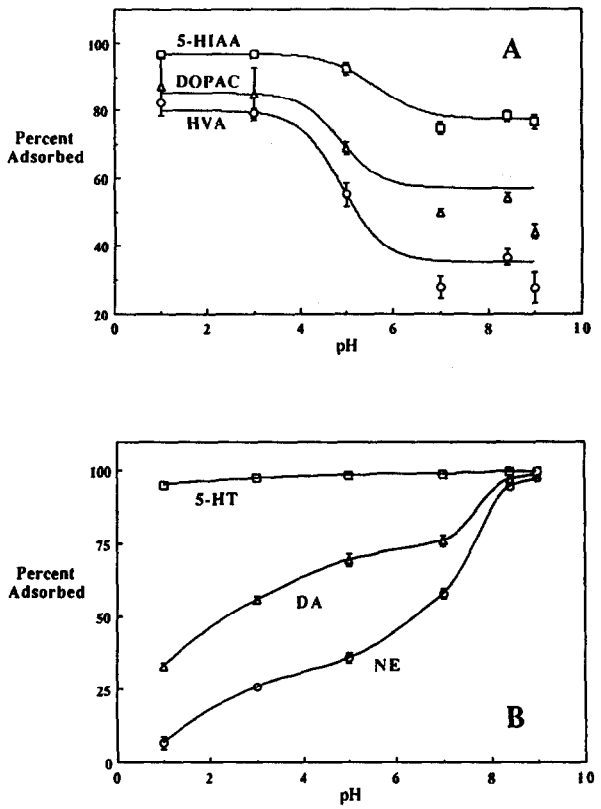

Fig. 4. Effect of pH on percent adsorbed for various neurochemicals. The theoretical lines shown for the acids (A) were derived using the constants listed in Table IV and calculation method 3 The empirical results are simply connected for the bases (B); no theoretical fit was obtained for the three basic compounds. Empirical values are shown as mean ± S.D. for NE (○), DA (△), 5-HT (□), HVA (○), DOPAC (△) and 5-HIAA (□).

adsorption of the protonated amines. But, perhaps a more feasible explanation simply concerns competition for the cationic binding sites by the increasing concentration of H^+ ions which exists at lower pH [16,17]. In any case, this low pH adsorption phenomena for the basic compounds was not pursued any further.

In short, however, the Langmuir adsorption framework incorporating consideration of separate adsorption of both the neutral and ionic forms of the indoles/catechols provides a reasonable theoretical approach by which the adsorption of these species onto glassy carbon may be understood. The low pH data for the bases indicate the existence of one or more adsorption phenomena which were not further investigated. But, the adsorption data for the acids at low and high pH and the data for the bases at high pH are at least adequately explained by the approach given. While there is almost certainly competition between the neurochemicals investigated for adsorption sites, this concept notably did not need to be invoked to fit the data. Finally, while most of the efforts in this paper were concerned with determination of the binding characteristics of the indoles/catechols, it should be emphasized that no adsorption of the quaternary amines onto the glassy carbon occurred under any of the conditions examined. Thus, the glassy carbon material is entirely appropriate to be used as a precolumn in the determination of acetylcholine and choline.

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