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STUDY OF THE RETENTION MECHANISMS FOR BASIC COMPOUNDS ON SILICA UNDER "PSEUDO-REVERSED-PHASE" CONDITIONS

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SUMMARY

The performance characteristics of a set of nitrogenous bases were studied on a number of silicas using aqueous organic mobile phases. The retention characteristics were complex functions of the organic solvent and buffer concentrations as well as pH. The retention mechanisms were shown to be a combination of ion-exchange and interaction with siloxane and silanol groups over the entire range of concentration of organic solvent. The differences in retention on silica were due largely to the differences in ion-exchange strength of the silanol groups and the surface concentration of the siloxane bridges. The same solutes were studied on a range of C_8-bonded phases of varying surface coverage, where the same interactions were observed as seen on silica with the addition of reversed-phase interactions. The unexpected relation between capacity factor and bonded-phase coverage was explained by interaction of the several retention mechanisms involved. The retention of small proteins on pure silica was also related to its ion-exchange strength. The performance of the silicas was related to that of the bonded-phase packings prepared from them, the retention of the proteins again being related to the ion-exchange characteristics.

INTRODUCTION

The analysis of bases by high-performance liquid chromatography (HPLC), especially under reversed-phase conditions, is characterised by long and variable retention times, poor efficiency of separation and excessive peak tailing. Many strategies have been used in attempts to control these effects, the most popular being ion-pair chromatography and the so-called ion-suppression chromatography. Whilst these are successful in many cases, a few problems remain. Some of these can be eliminated by the use of alternative modifiers, such as triethylammonium salts[1], which are thought to act as competitors for the "active sites" that cause the chromatographic problems. In all cases the problem is usually assigned to some form of interaction with unbonded silanol groups which remain on the silica surface. It has been demonstrated that at least a part of this interaction is ionic in nature, the silica surface of a C_{18} packing acting as a weak cation-exchanger for aromatic bases over a very wide range of organic solvent concentrations[2,3]. In order to study the phe-

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nomena involved in such interactions between bases and silanol groups it is clearly necessary to begin with a study of the interactions in which pure silica is used in order to remove any variability in results due to small differences in bonded-phase coverage resulting from variability in the synthesis or from hydrolysis of the bonded-phase under the test conditions.

The first report on chromatography of bases on pure silica packings under conditions related to reversed-phase systems was by Jane[4], who performed the analysis of basic drugs of abuse in methanol-rich aqueous ammonium nitrate at high pH. Other researchers[5–7] have described similar systems. A more thorough study of the use of these aqueous eluents with silica as well as with octadecylsilyl-bonded phases for the chromatography of bases at methanol compositions above 50% has been reported by Sugden *et al.*[8]. This group concluded that ion-exchange mechanisms were less important than other interactions between protonated species and silanol groups and ion-pair interactions. Since this time, Flanagan and Jane[9] have reported on the chromatography of basic amines and quaternary ammonium compounds on silica, mostly at high organic solvent concentration. Although ion-exchange mechanisms were postulated, a few observations were made which could not be reconciled with them. Very recently, work which extends these studies to a wider range of solvent compositions and to alumina was reported[10].

To date, little work has been reported which relates the chromatographic to the physical properties of silicas. It is known that silicas prepared by different routes do possess different properties[11]. Data on retention of diethylaniline in unbuffered systems[11] and of basic peptides[12] have been presented, although these investigations were carried out on bonded phases and no chromatographic data for the pure silicas were obtained.

The work reported in this paper attempts to address a number of questions. The mechanisms of retention of basic compounds on silica were shown to be more complex than merely ion exchange. The influence of increasing surface coverage of bonded phase on the separations were found to be unexpected and increasingly complex. Differences between different silicas were shown to be due largely to different ion-exchange capacities and/or strengths and were reflected in the performance of bonded-phase packings which were prepared from the silicas for the separation of proteins.

THEORETICAL

The surface of silica is not simple[13]. Although only two types of group exist on the surface (the silanols and the siloxane bridges), there are many and complex interrelationships. Silanol groups are known to be either isolated or hydrogen bonded. The majority are weakly acidic, although a small (currently undefined) proportion is strongly acidic and is ionised even at low pH[11]. In addition to single silanol groups, there is an appreciable proportion of geminal silanols. It is believed that these are among the first to react with bonded-phase reagents[11], although how they are distributed between the different types of silanols is not known. The siloxane bridges are frequently assumed to be inactive or to contribute to the retention by some "hydrophobic" chromatographic mechanism[14]. Little is known about these groups other than that they are formed by dehydration of silica and that they can be rehydrated

to generate silanol groups under suitable conditions. Since silica is characterised as a "living polymer"[13] it is not unreasonable to suppose that the silanols and siloxane groups exist in some kind of slow equilibrium. Against this background, it is not surprising that the chromatography performed on silica-based materials is not always reproducible or explicable, especially when the compounds being separated interact with the silica surface as well as with a chemically bonded phase.

Since the major interaction which is likely to occur with the silanol groups is ion exchange, it is useful to consider aspects of the ion-exchange equilibrium. For cation exchange (which is involved here) the ion-exchange process can be represented as [15]

$$SiO^-M^+ + NH^+ \rightleftharpoons SiO^-NH^+ + M^+$$

The ion-exchange equilibrium constant K_{ix} is given by

$$K_{ix} = [SiO^-NH^+][M^+]/[SiO^-M^+][NH^+]$$

If the solute is ionogenic, the pH of the system controls the concentration of the cation through its ionisation constant K_a. If one assumes that only the charged form of the solute is present in the stationary phase, then the distribution coefficient, D_{ix}, for the process can be derived. This turns out to be

$$D_{ix} = \frac{[SiO^-NH^+]}{[NH^+] + [N]} = K_{ix} \cdot \frac{[SiO^-M^+]}{[M^+]} \cdot \frac{1}{1 + \dfrac{K_a}{[H^+]}}$$

Thus, the distribution coefficient is, to a first approximation, related to the ion-exchange equilibrium constant and to the number of accessible ionised silanol groups (which also depends upon the pH and the pK_a of the silica surface). It is also related to the inverse of the concentration of counter-ions in solution. Thus, a plot of capacity factor (k'), which is directly related to the distribution coefficient, against the inverse of counter-ion concentration should be a straight-line graph, passing through the origin (assuming that no other retention mechanism exists), with a slope proportional to the ion-exchange equilibrium constant and the number of ionised silanol groups on the silica surface. The presence of other retention mechanisms not related to ionic processes will result in an intercept on the k' axis which corresponds to an "infinite" competing-ion concentration.

Interacting retention mechanism

The majority of HPLC experiments are performed under conditions where it is hoped that essentially only one retention mechanism predominates. In this case, it is relatively simple to understand how retention is affected by changes in mobile phase composition, parameters relating to the packing material and the nature of the solute. In addition, it is frequently possible to predict k' and selectivity from a knowledge of the structure of the solutes[16]. The situation becomes more complex when several mechanisms of retention come into play. It is quite unreasonable to assume that a solute molecule is retained by only one interaction at any one time—indeed, chiral separations, which require a three-point contact with the surface, would be

impossible under such a circumstance[17]. Thus, a polar molecule could be retained by a reversed-phase and a silanol interaction at the same time, acting on different parts of the molecule. If it is assumed that each individual interaction acts only on a specific part of the molecule, independently of the presence (or absence) of other interactions, then the total energy of interaction would be the sum of the individual energies of interaction.

Since the distribution coefficients are related to the free energy of the system by the relation

$$\Delta G = - RT \ln K$$

the overall distribution coefficient arising from the interacting retention mechanism would be equal to the product of the individual coefficients. For a retention equilibrium with distribution coefficient K_i, there is a phase ratio (φ_i) associated with it which depends upon the packing material and the mobile phase. In the case of a single retention mechanism this phase ratio is simply the ratio of the stationary and mobile phase volumes appropriate to that mechanism. If several mechanisms exist, the individual phase ratio will be a function of the number of sites on the packing material which interact with the solute within that equilibrium. If two or more retention mechanisms interact, there is an associated phase ratio which reflects the number of groups on the surface in a suitable configuration for such interaction. This ratio defines the extent of the interaction. Since the volume of the mobile phase in the column is not a function of the retention mechanism, the critical parameter in the phase ratio is the stationary phase volume for each interacting mechanism. This is simply seen as a parameter which relates to the frequency of occurrence of the interacting groups on the surface of the packing in a configuration close enough to allow concurrent interactions with one solute molecule.

In the case of uniform random distributions of the interactive sites on a packing material, the maximum distribution coefficient due to the interacting mechanisms should be observed when the concentrations of the sites are equal. If the sites are not uniformly distributed on the surface, *e.g.*, as has been postulated for reversed-phase chromatography where "rifle stacks" of bonded-phase molecules are thought to occur in the predominantly aqueous environment[18], then the interacting mechanisms would be more likely to occur in localised areas. In this example this would be around the edge of the "stacks" where both the bonded phase and the silica surface are easily accessible. For these interacting retention mechanisms the associated stationary phase volume must be a function of the product of the individual phase volumes.

These stationary phase volumes are difficult to assign, even in the case of a single retention mechanism, especially where there is some uncertainty concerning the actual mechanism of a retention process. Several proposals have been made in an attempt to solve this problem[19]; of these perhaps the most useful is to view the phase ratio as the ratio of molecules participating in the retention in the stationary phase to the total number of molecules in the mobile phase. This avoids the difficulty of having to cope with combinations of surface areas and volumes and, consequently, the derivation of distribution coefficients which have the dimension of reciprocal length.

The foregoing arguments imply that, if more than one retention mechanism

is involved, the observed capacity factor is determined not only by the sum of the products of the individual distribution coefficients and their phase ratios but also by the addition of the products of interacting distribution coefficients (K_i) and phase ratios (φ_i).

$$k' = K_1\varphi_1 + K_2\varphi_2 + \cdots + K_i\varphi_i + K_1K_2\varphi_1\varphi_2 + \cdots + K_iK_j\varphi_i\varphi_j + \\ + \cdots + K_1K_2K_3 \cdots K_i\varphi_1\varphi_2\varphi_3 \cdots \varphi_i$$

One way of studying these processes is to modify the individual phase ratios by changing the surface coverage of a bonded-phase packing and observing the retention changes in an otherwise fixed system. A number of papers describing such experiments have appeared[20–22], although the intention of their authors was not necessarily to study this question. One universal observation has been that the plots of k' against surface coverage or percent carbon of the bonded-phase for the ostensibly reversed-phase systems studied were not linear, as would be predicted from a single reversed-phase mechanism, although this aspect has not been addressed. The data can be rationalised by the presence of interacting mechanisms. In this case, the phase ratios for the various processes would be related to the surface coverage of the bonded phase: reversed-phase processes relating to the bonded phase would obviously show direct proportionality, whilst mechanisms involving the silica surface would be related to a function of the original silica surface area less that covered by the bonded-phase. Hence, k' in such a case would not be related linearly to the surface coverage, as would be expected for a "pure" reversed-phase mechanism, but would be a quadratic or higher power function of the surface coverage (depending on the number of interacting mechanisms), resulting in non-linear plots of k' against bonded-phase coverage.

EXPERIMENTAL

Instrumentation

A DuPont (Wilmington, DE, U.S.A.) 8800 "Sentinel" fully automated HPLC system with as DuPont spectrophotometric detector and a Hewlett-Packard (Avondale, PA, U.S.A.) 1090A HPLC system fitted with a filter photometric detector were used throughout this work. A Nelson Analytical (Cupertino, CA, U.S.A.) data system based upon a Hewlett-Packard 200 series computer, modified in house to give theoretical plate height and skew data, was used.

Columns

Stainless-steel columns (15 cm × 4.6 mm I.D.) were packed by proprietary procedures using a slurry method.

Mobile phases

Mobile phases were mixed dynamically from phosphate buffers (made from appropriate mixtures of mono- and dibasic sodium phosphate and orthophosphoric acid to achieve the desired pH at the required sodium ion concentration) and methanol for the amine studies. Protein separations were performed using a gradient from 0.01% aqueous trifluoroacetic acid to 60% aq. acetonitrile containing 0.01% trifluoroacetic acid in 40 min.

Flow-rates were 2 ml/min for the amine studies and 1 ml/min for the proteins. All separations were performed at ambient temperature.

Materials

HPLC-grade solvents were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Protein standards were obtained from Sigma. Other chemical reagents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Packing materials

Silicas used in this study were experimental DuPont Process Grade silica, approximately 10 μm particle diameter and either 260 m^2/g surface area with 90 Å pore diameter (Type S) or 170 m^2/g surface area with 120 Å pore diameter (Type L).

Bonded-phase packings were synthesised by a minor modification of the method of Kinkel and Unger[23] using octyldimethylchlorosilane. Those materials which were capped after bonding were similarly treated with trimethylchlorosilane. Materials of restricted surface coverage were prepared by limiting the quantity of octyldimethylchlorosilane in the reaction mixture to the molar proportion, calculated from the desired coverage and the surface area of the silica. These packings were not capped.

RESULTS AND DISCUSSION

The solutes chosen for this study were intended to exemplify the different types of basic compound which may be expected to interact with silica surfaces. These were thiamine (a quaternary ammonium compound) morphine (a basic tertiary amine) and caffeine (which is not basic and is generally assumed to be "well-behaved" in reversed-phase systems).

Initial experiments were performed using a Type S silica. The effect of changing the methanol concentration in 0.05 *M* phosphate at pH 4.6 on the capacity factors of the three solutes is shown in Fig. 1. The U-shaped graphs for thiamine and morphine are typical of separations on silica. Curves of similar shape have been seen in the separation of crown ethers[14] as well as basic amino compounds[8–10]. The shape has generally been ascribed to hydrophobic interactions at low organic solvent concentrations and to changes in protonation[9] or to changes in counter-ion solvation[10] at high organic solvent concentration.

Because of the way in which the mobile phase was mixed and the assumed ion-exchange mechanism, at least part of the change was believed to be due to a dilution of the buffer by the addition of methanol. This was addressed by performing the same experiment with concentrations of between 0.2 and 0.01 *M* phosphate and plotting k' values against the inverse of the calculated concentration of sodium ion in the mobile phase at various methanol concentrations. All of these plots (see Fig. 2) are linear, indicating that the mechanism is indeed ionic, and show a positive intercept on the k' axis. Ion-exchange theory[15] predicts that for a pure ion-exchange mechanism the plot of k' against inverse competing-ion concentration should give straight lines passing through the origin of the plot, the slope being proportional to the ion-exchange equilibrium constant and the ion-exchange capacity of the packing. An interesting point to note is the change of the slope of the plots with changes in

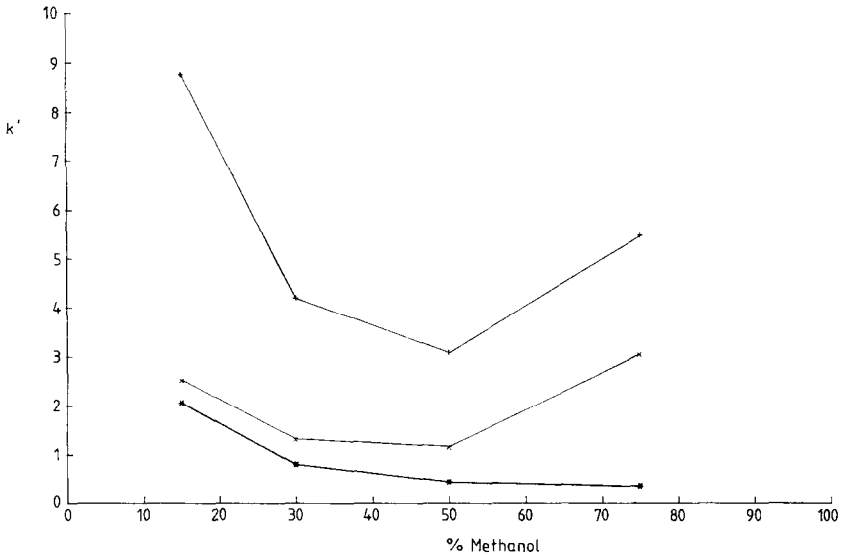

Fig. 1. Plot of k' against methanol concentration. Type S silica, mobile phase made from mixtures of methanol with 0.05 M sodium phosphate buffer (pH 4.6). + = Thiamine; * = caffeine; × = morphine.

methanol concentration. The initial increase in methanol concentration from 15 to 50% results in a decrease in slope. At higher methanol concentrations the slope of the plot increases. An experiment to measure the pH of the solutions at the methanol concentrations employed showed that the pH (uncorrected for changes in electrode performance with change in methanol concentration) increased with added methanol, reaching a value of 6.4 at 75%. This means that the silica surface is increasingly ionised at high methanol concentration, and this is probably the cause of the in-

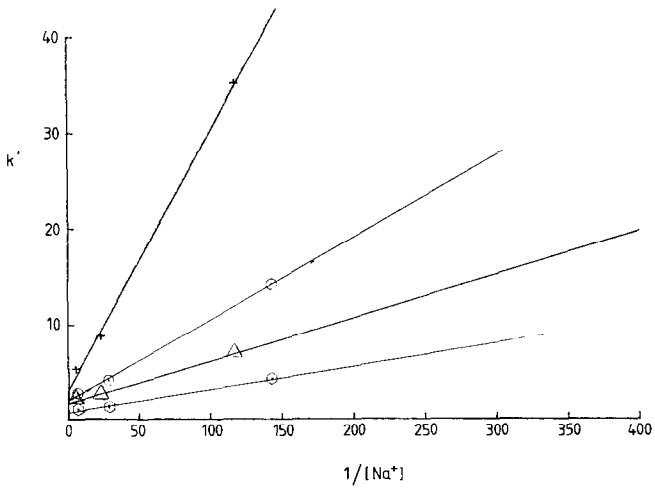

Fig. 2. Plot of k' against inverse buffer concentration. Type S silica, mobile phase 15 and 30% methanol in phosphate buffer (pH 4.6). + = Thiamine, 15% methanol; △ = thiamine, 30% methanol; ○ = morphine, 15% methanol; ⬡ = morphine, 30% methanol.

creased retention of thiamine and morphine. Since the pH of the solution slowly increases with methanol concentration, at low methanol concentrations the pH of the solution is not appreciably affected by the addition of methanol and it is reasonable to suppose the number of ionised silanol groups on the silica surface does not appreciably increase. Thus, the decrease in slope with low methanol concentration appears to be a result of change in the ion-exchange equilibrium constant. The reduction in retention with added organic solvent concentration under ion-exchange conditions has been noted in the past[24].

The positive intercept of the plot of k' *versus* inverse competing-ion concentration can be taken as evidence for additional retention mechanism(s) which exist at "infinite" buffer concentration, *i.e.* not influenced by the concentration of the ions in solution and therefore not ionic in nature. A plot of k', measured at infinite buffer concentration, against methanol concentration (see Fig. 3) has the same form as the other plots. Since in this case the ion-exchange effects are eliminated, this plot must arise from the other mechanisms which exist. Possible changes in solute ionisation which could influence the retention of morphine may be discounted due to the similarity in behaviour of morphine and thiamine which as a quaternary salt is fully ionised under these conditions. Additionally, the pH of the mobile phase is at all times well below the pK of morphine and so changes in the level of ionisation are expected to be minimal. At low methanol concentrations, the plot follows the expected form of a reversed-phase separation, whilst at high concentrations the k' values increase. This increase is probably due to non-ionic interactions of the solutes with silanol groups, in some form of normal-phase or adsorption mode. Evidence for such a mechanism was seen in the chromatography of crown ethers[14]. In contrast, at first sight, caffeine shows no evidence of ionic interactions or adsorption and

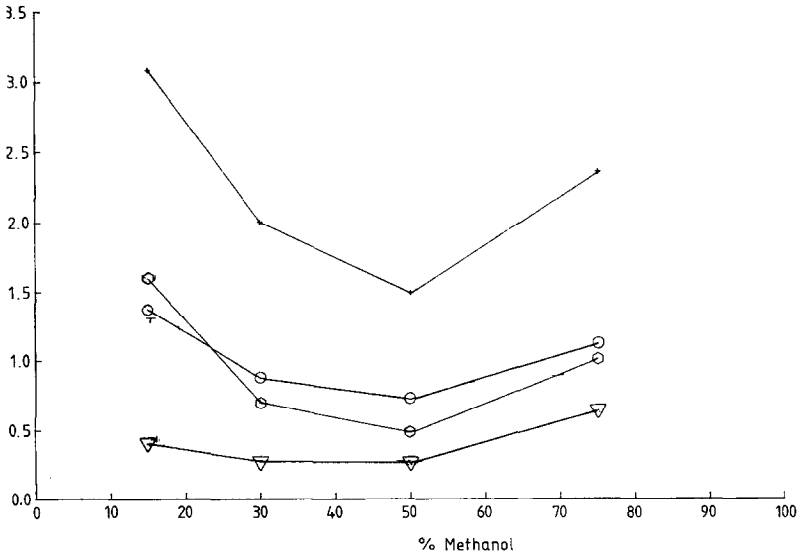

Fig. 3. Plot of k' against methanol concentration at "infinite" buffer concentration (pH 4.6). + = Thiamine, Type S silica; ○ = morphine, Type S silica; ○ = thiamine, rehydroxylated Type L silica; ▽ = morphine, rehydroxylated Type L silica.

appears to behave as if the only retention mechanism involved were reversed-phase partition. Its behaviour is entirely consistent with such a mechanism in that the retention increases with increasing buffer concentration, corresponding to expected salting-out effects.

In order to investigate reversed-phase separations on silica further, an experiment was carried out on the same silica column measuring the retention of toluene and uracil as a function of methanol concentration. Toluene was expected to show a reversed-phase type of behaviour as has been observed for butylbenzene[14], whilst uracil was originally chosen as a dead-volume marker. Fig. 4 shows the plot of capacity factors of uracil and toluene against methanol concentration observed in this experiment. Toluene clearly shows a close approximation to the expected relationship between log k' and methanol concentration. The data for caffeine (not shown) plotted as log k' *vs.* percent methanol does not show such a clear linearity. A curvature relative to the plot obtained for toluene suggests strongly that some adsorption at high methanol concentration does take place. Uracil clearly does not function as an unretained marker in this system (unlike its behaviour on fully bonded and capped reversed-phase packings) and shows a U-shaped plot similar to the other bases. Since, in this case, the mobile phase was unbuffered, the increase in retention at high methanol concentrations is probably due to adsorption.

The plots of k' *vs.* methanol concentration at other pH values show increased retention as the pH increases, corresponding to the expected increase in the number of ionised silanol groups on the silica surface. Over the pH range studied (2.1 to 7.0) this increase was exponential for thiamine, an observation similar to that of Lingeman *et al.*[10]. The plots of k' against the inverse of buffer concentration were linear at these other pH values, although the slopes and intercepts differ. The values of slopes and intercepts are reported in Table I. The slopes of the plots are clearly increased between pH 2.1 and 4.6, reflecting increased ionisation of the silica, even though this range is well below the pK of silica and reflects the inhomogeneity of the

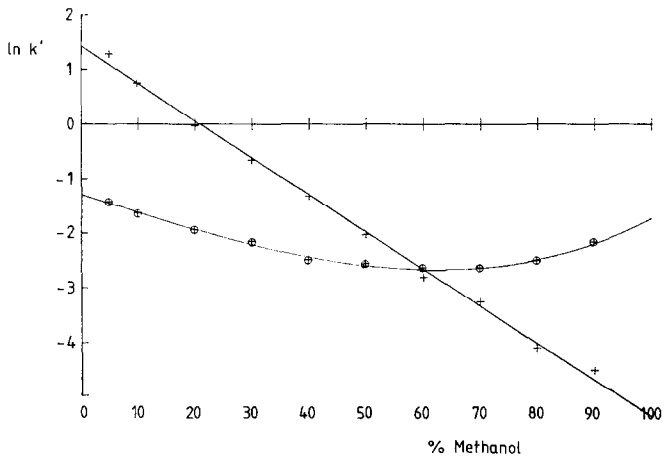

Fig. 4. Plot of ln k' of toluene and uracil against methanol concentration. Type S silica. Mobile phase, methanol–water mixtures. + = Toluene; ○ = uracil.

TABLE I

SLOPES AND INTERCEPTS OF k' *vs.* 1/[BUFFER] PLOTS: TYPE S SILICA

pH	*Methanol concentration (%)*	*Thiamine*		*Caffeine*		*Morphine*	
		Slope	*Intercept*	*Slope*	*Intercept*	*Slope*	*Intercept*
2.1	15.0	0.00975	1.28	−0.0002	1.19	0.00474	1.12
4.6	15.0	0.271	3.09			0.0453	1.59
	30.0	0.0845	2.00			0.0247	0.70
	50.0	0.0448	1.49			0.0197	0.48
	75.0					0.0263	1.01
7.0	15.0	2.03	8.12	0.0153	0.73	1.152	6.43

surface. The intercepts, too, are larger, although by a much smaller factor. As expected, the slopes increase markedly between pH 4.6 and 7, due to the enhanced ionisation. The data for caffeine show an interesting feature in that at pH 7 there is a change in mechanism. Some ion-exchange character is introduced since the slope of the plot changes to a positive (although extremely small) value.

In addition to the silica used for the above data, two Type L silicas, known to have differing degrees of surface rehydroxylation, were examined. The first ("packing a") was a material which had been heat-treated at excessive temperature before rehydroxylation by a standard acid treatment and the second ("packing b") was a silica prepared by the procedure of Köhler and Kirkland[25]. A plot of k' against 1/buffer concentration for thiamine and morphine is shown in Fig. 5. Data for the slopes and intercepts of these packings are shown in Table II. It is of interest to observe that

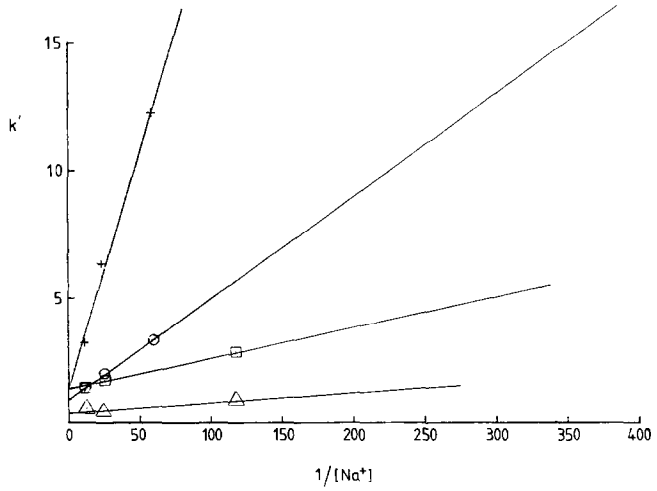

Fig. 5. Plot of k' against 1/buffer concentration. Type L silicas. + = Thiamine, packing a; ○ = morphine, packing a; □ = thiamine, packing b; △ = morphine, packing b.

TABLE II

SLOPES AND INTERCEPTS OF k' *vs.* 1/[BUFFER] PLOTS: TYPE L SILICAS

Methanol concentration		*Thiamine*		*Morphine*	
Silica	*(%)*	*Slope*	*Intercept*	*Slope*	*Intercept*
a	15	0.186	1.390	0.040	0.931
	30	0.064	1.156	0.020	0.447
	50	0.035	0.805	0.016	0.262
	75	0.036	0.922	0.021	0.434
b	15	0.014	1.368	0.004	0.397
	30	0.007	0.869	0.003	0.272
	50	0.005	0.716	0.003	0.255
	75	0.007	1.121	0.004	0.641

the two silicas have essentially the same intercept on the k' axis and markedly different slopes for thiamine. Morphine, however, displays a smaller intercept as well as a similarly reduced slope. Thus, the conclusion can be drawn that the major difference between these silicas is simply the ion-exchange character of the materials. This agrees well with the hypothesis that the surface treatment modifies the silanols in such a way as to increase the number of hydrogen-bonded (less acidic) silanols at the expense of the more acidic, free groups. At the same time, it appears that the other mechanisms of retention for thiamine are essentially the same for the two silicas. Caffeine, however, is retained to a much smaller extent on the rehydroxylated silica, as is morphine at "infinite" buffer concentration. Since at this pH the retention is not influenced by ion-exchange, it appears that the sites of interaction of caffeine with the surface are decreased in number or strength by the treatment. This implies that caffeine is retained either by interaction with the strongly ionised surface silanols or by siloxane bridges which are converted to silanols by the rehydroxylation process. Interaction with the silanol groups would explain the slightly reduced retention of caffeine at pH values above 4.8 (see Table I) in that the majority of silanols begin to ionise above this value. The form of such an interaction is not so easy to deduce.

A hydrogen-bonding interaction would be expected to decrease with increasing water concentration. The alternative is that the number of siloxane bridges which are postulated to sorb by hydrophobic interaction may be reduced by the rehydroxylation. This reduction would therefore lead to lower retention of caffeine, although this mechanism does not explain the reduced k' at high pH, since it should remain invariant. In order to elucidate this further, the "pseudo-reversed-phase" retention of toluene on silica was studied further. In this experiment toluene was chromatographed with a range of methanol concentrations at two different pH values (2.1 and 7.0). The capacity factors under the two sets of conditions were identical, proving that the retention is invariant with changes in ionisation of the surface. A second experiment was carried out in which retention of toluene was compared on two silicas, one prepared by the standard technique and the other by a rehydroxylation procedure analogous to that of Köhler and Kirkland. In this case, the retention of

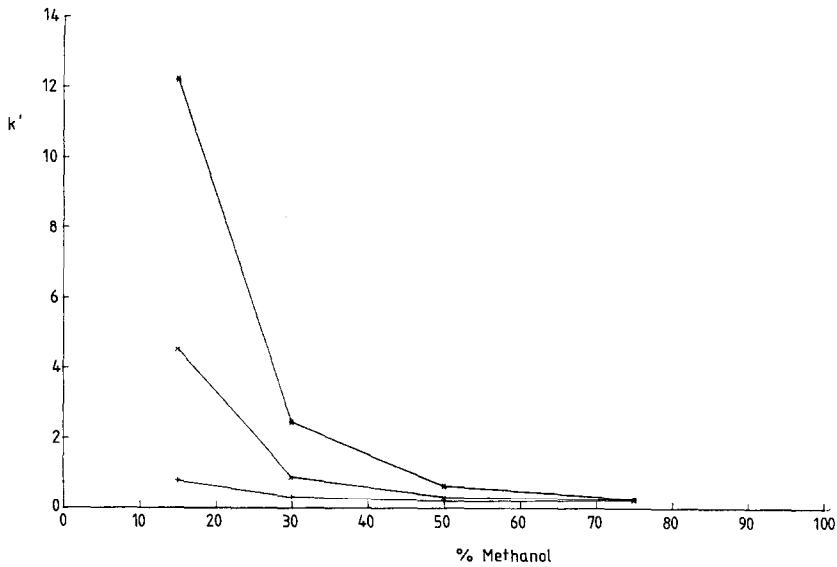

Fig. 6. Plot of k' against methanol concentration. Fully covered C_8-bonded phase. All other conditions as Fig. 1. + = Thiamine; * = caffeine; × = morphine.

toluene was very markedly reduced on the rehydroxylated silica, indicating that retention occurred on the surface siloxane bridges, the number of which were reduced by the process. This implies that the retention of caffeine and, in part, that of morphine also occurs by interaction with the siloxane groups.

Preparation of a C_8 bonded-phase with high surface coverage (3.37 μmoles/m^2) from the Type S silica changes its performance in the chromatography of the test solutes dramatically. This is illustrated in Fig. 6. The retention of caffeine at 15% methanol in 0.05 *M* phosphate buffer was increased relative to silica while thiamine and morphine were retained to a much smaller extent. This may be attributed to reversed-phase interactions of caffeine and reduction of the ion-exchange retention of basic compounds by virtual elimination of ionic silanols through bonding or steric masking with the bulky bonded-phase molecules. Morphine is retained more than thiamine by virtue of reversed-phase interactions, although in this case the relative participation of siloxane and C_8 interactions is not clear. A more interesting result is observed when the transition between silica and a bonded phase with maximum coverage is investigated. A range of C_8 phases was prepared from the Type S silica with a wide range of surface coverage. Their properties are reported in Table III. The capacity factors and plate counts for thiamine, caffeine and morphine were determined for 15, 30, 50 and 75% methanol in 0.05 *M* phosphate buffer. Fig. 7 shows a plot of k' against surface coverage (α_{exp}) at 15% methanol. It is clear that these curves do not fit the plots of log k' against percent carbon which have been reported for packings at low values of surface coverage[20–22]. Most of these data were collected from C_{18}-bonded phases which had surface coverage values of less than 1.6 μmoles/m^2. Since, according to the idealised mechanism of reversed-phase chromatography, the capacity factor should be related directly to the bonded-phase coverage

TABLE III

PROPERTIES OF C_8-BONDED-PHASE PACKINGS

Designation	*Carbon (%)*	α_{exp} *(μmoles/m²)*
1	0.00	0.00
2	2.37	0.78
3	3.49	1.17
4	4.78	1.63
5	5.47	1.89
6	6.90	2.43
7	7.61	2.71
8	8.25	2.97
9	9.19	3.36

through the phase ratio (assuming the reversed-phase distribution coefficient remains constant throughout the range of coverage), it is hard to see why the plots should turn out to be logarithmic, especially over a wide range of coverage. The shape of the curves was unexpected, and clearly mechanisms other than reversed-phase partition are operating on the bonded-phase packings. It is noteworthy that at low surface coverages (< 1.6 μmoles/m²) the plots follow the same general form as those reported earlier. It is at the higher values of coverage where the assumed log function no longer applies. Fig. 8 shows data on the efficiency, as measured by the number of theoretical plates for the three test solutes. Since all of these columns showed between 5000 and 6000 theoretical plates for toluene, chromatography of the solutes is clearly

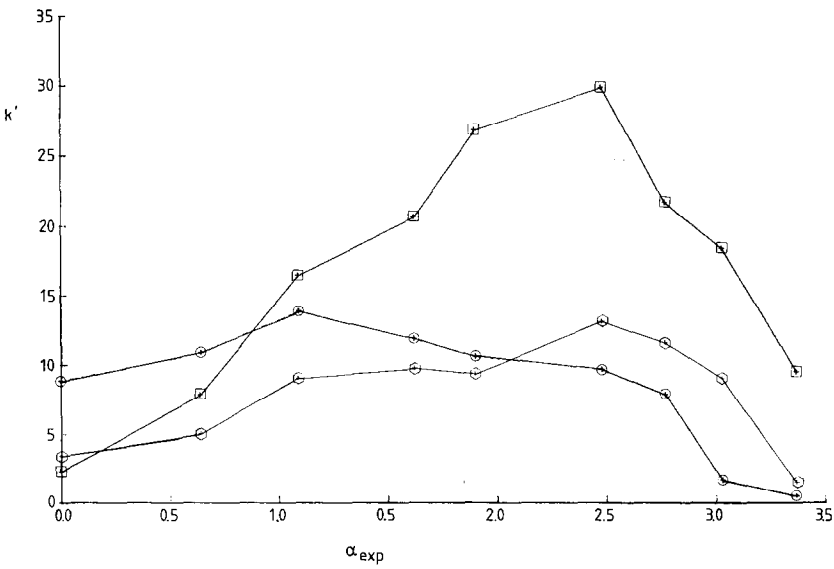

Fig. 7. Plot of k' against surface coverage (α_{exp}). Type S silica; mobile phase, 15% methanol in 0.05 *M* phosphate buffer (pH 4.6). ○ = Thiamine; □ = caffeine; ⬡ = morphine.

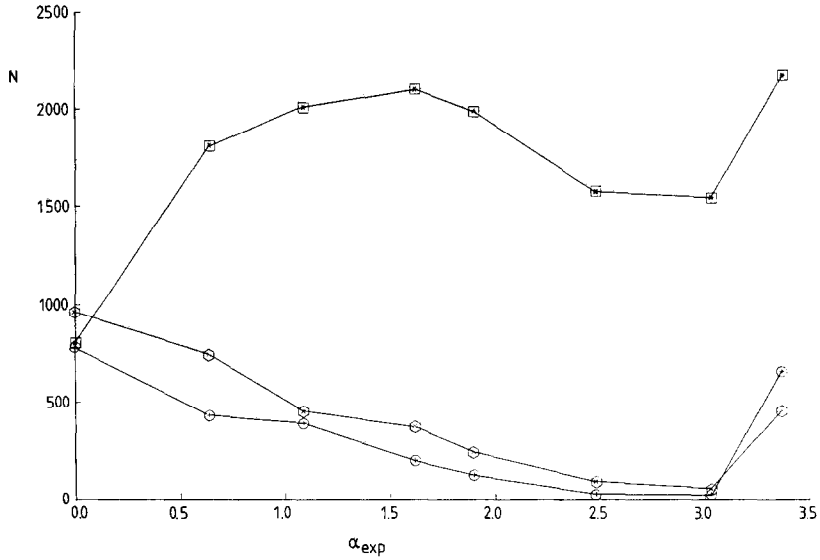

Fig. 8. Plot of plate number (N) against surface coverage. All other conditions as in Fig. 7.

not optimal, the maximum number of plates being only a little over 2000 for caffeine and the minimum number being below 100 for thiamine. It is also significant that the minima in the efficiency plots coincide with the maxima in the retention plots. This suggests that the maxima in the plots of k' are due to interaction of retention mechanisms which severely reduce the desorption rates and thereby increase the plate heights. From the silica data it is clear that cation exchange is the major retention mechanism for thiamine. This view is supported by plots of k' against inverse sodium concentration for a C_8-bonded-phase silica with 1.63 μmoles/m^2 loading (Fig. 9). The plots for thiamine and morphine are consistent with the proposed ion-exchange

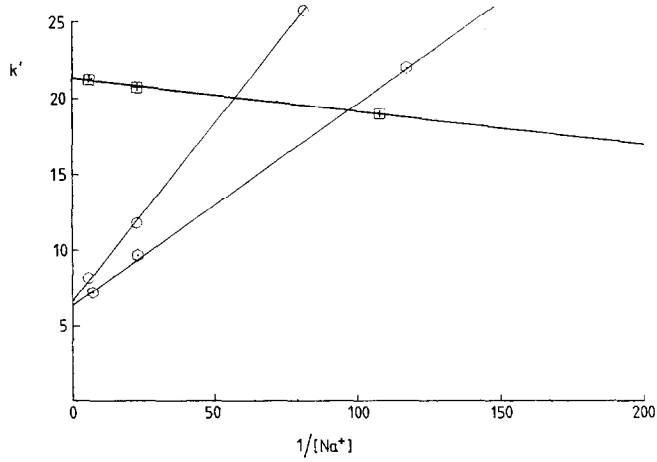

Fig. 9. Plot of k' against 1/buffer concentration on a partially bonded C_8 packing. Mobile phase, 15% methanol in phosphate buffer (pH 4.6). ○ = Thiamine; □ = caffeine; ⬡ = morphine.

mechanism, whilst that for caffeine shows a slope of the opposite sign (indicating salting out), as has been seen for separations on silica. The retention at "infinite" sodium concentration shows that there are additional mechanisms for all three test substances, one of which appears to be a hydrophobic type, occurring at low methanol concentrations and the other being possibly an adsorption (or silanophilic) type which is measurable at higher methanol concentrations. The plots of log k' against methanol concentration (Fig. 10) show clearly the marked curvatures which suggest this. Thiamine does not appear to have a particularly great affinity for the hydrocarbon chain of the bonded-phase packing, according to the very low retention on a fully bonded material. Thus, any addition to the interacting retention mechanisms arising from the traditional reversed-phase interaction is at best small. This lack of reversed-phase character for thiamine is also shown at other mobile phase pH values. At higher methanol concentrations any contribution by reversed-phase or hydrophobic interactions would be expected to be minimal. At 75% methanol the plot of k' against α_{exp} for thiamine is simplified and follows more closely a straight line, as shown in Fig. 11, suggesting that hydrophobic processes which are largely eliminated at high methanol concentrations are small but important for thiamine. Although these do not immediately appear to be related to the bonded phase, they cannot result from interaction with the silica surface, since changing the number of siloxane bridges does not particularly influence the retention of thiamine on silica as seen from the intercepts of the k' *vs.* 1/[buffer] plots. Morphine shows a little higher retention on the bonded-phase packing and thus it may be concluded that reversed-phase interaction with the bonded phase is more important for this compound. This is further borne out by its performance at higher pH. When chromatographed on a C_8-bonded phase in a mobile phase prepared from pH 7 buffer (Fig. 12), the retention

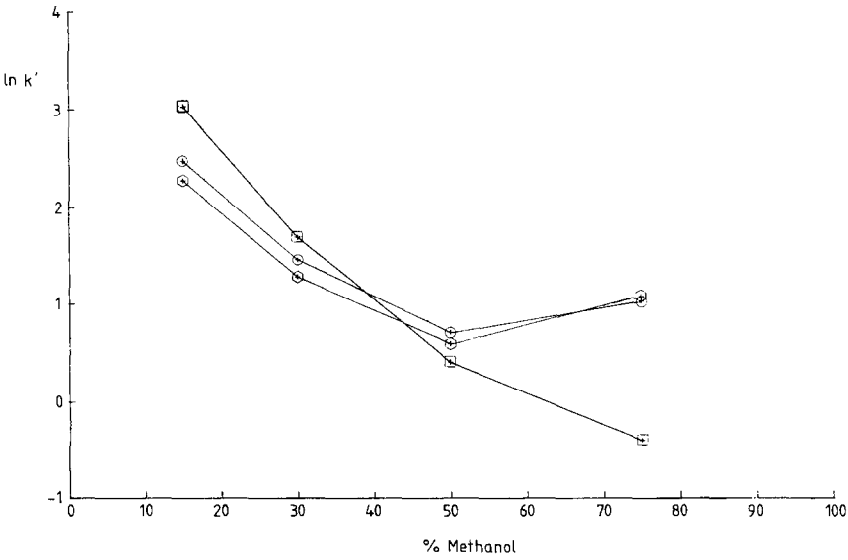

Fig. 10. Plot of log k' against methanol concentration for a partially bonded C_8 packing. Other conditions as in Fig. 1. ○ = Thiamine; □ = caffeine; ⬡ = morphine.

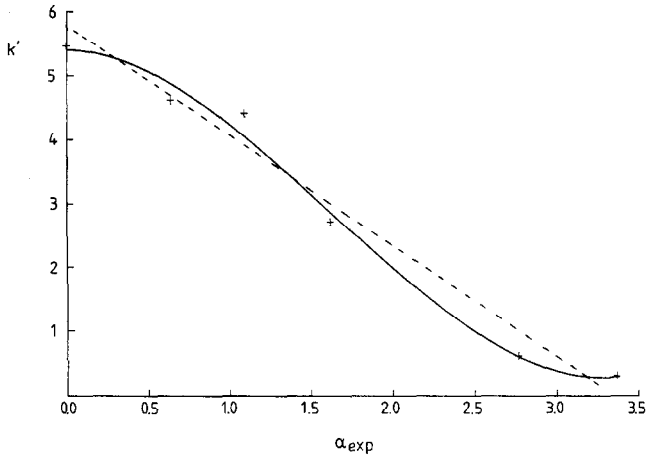

Fig. 11. Plot of k' of thiamine against surface coverage at 75% methanol. Other conditions as in Fig. 7.

of morphine increases dramatically to exceed that of caffeine. This presumably is due to changes in the extent of ionisation of morphine, despite the fact that the pH of the system is below the pK of morphine. Thiamine, in contrast, does not show this effect, indicating that ion-exchange processes are not important here. Thus, each of the test solutes displays a different variety of mechanisms. Thiamine is retained mainly by ion-exchange processes throughout the range of bonded-phase coverage from silica to C_8. In addition, there is evidence for a silanol interaction at high methanol

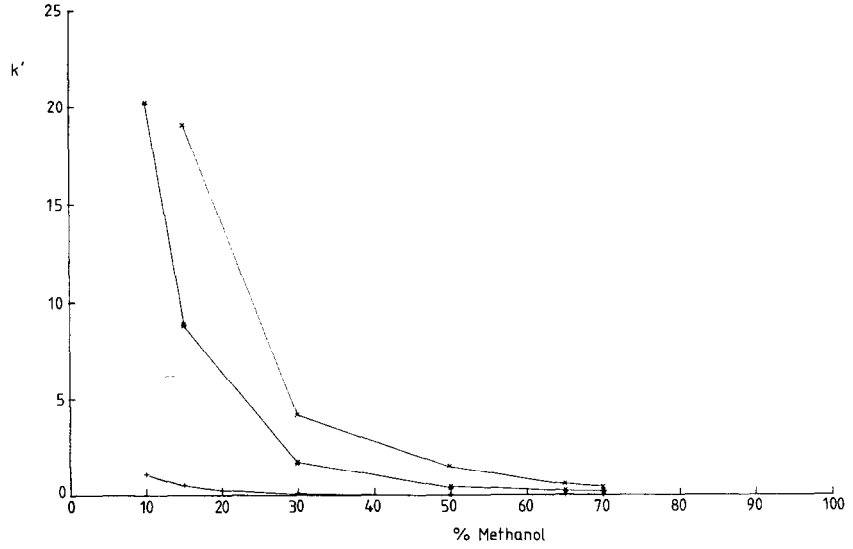

Fig. 12. Plot of k' against methanol concentration at pH 7. C_8-Bonded phase; other conditions as in Fig. 1. + = Thiamine; * = caffeine; × = morphine.

concentrations, minimal hydrophobic interaction with the silica and a little interaction with the bonded phase. For each of the two main processes it appears that the surface with which thiamine interacts is that of the silica and only that small part of the retention which is governed by the reversed-phase process is directly related to the concentration of bonded phase. Hence, the retention of this compound is largely a function of the freely accessible silanol groups on the surface. If the bonded-phase molecules are randomly distributed over the silica surface during the bonding process, then the decrease in accessible silanol concentration should be linearly related to the increase in bonded-phase coverage. This implies that the curvature of the k' *vs.* surface coverage plot is not due to non-linear changes of the concentration of the silanol groups and must therefore be caused by the proposed interaction of retention mechanisms. It is not possible to explain the shape of this and the plots for morphine and caffeine by summation of capacity factors or distribution coefficients. Only functions of higher order than unity can fit the curves. This means that several mechanisms must interact in this system. The case for morphine is very similar, except for the greater reversed-phase character in the retention mechanism. This is reflected in the movement of the maximum in the k' *vs.* surface coverage plot to higher coverages relative to thiamine. The plots for all solutes at higher methanol concentrations also show shifts in the maximum, in this case to lower values of coverage, which is expected from the decrease in reversed-phase retention. Caffeine behaves differently in that ion exchange is relatively unimportant in its retention relative to reversed-phase interactions. This is clear from its greater retention as surface coverage of the C_8 phase increases. The decrease in retention at high values of coverage is clear evidence for a coupling of retention mechanisms, since this should not occur if only reversed--phase interaction with the bonded phase were important. In order to ensure that these observations were not an artifact of the bonded phase, a number of simple compounds were tested under reversed-phase conditions using the set of columns with a range of surface coverage. The k' *vs.* coverage plots are shown in Fig. 13. Toluene shows essentially a straight-line plot under these conditions, suggesting that for a pure reversed-phase separation the k' value is, as is expected, linearly related to the bonded-phase coverage. Other, simple molecules show less than linear plots which again can be taken as evidence for additional retention mechanisms, especially as the curvature increases with the polarity of the molecule. Similar effects can be seen in the data of Hennion *et al.*[20], Miller *et al.*[21] and Kaliszan *et al.*[22] at the lower surface coverages they employed. It is interesting to note that the curves relating k' and surface coverage for most of these solutes have essentially the same shape and can be approximated by a polynomial which is third-order in surface coverage. Work in progress in our laboratories in this area will form the basis of a forthcoming paper.

Protein retention studies

Following the discovery that silicas prepared by different procedures and rehydroxylation processes show different ion-exchange characteristics it was thought that even on well-bonded reversed-phase silicas these ion-exchange properties may remain in evidence for separations. Accordingly, a range of proteins were chromatographed on C_8-bonded-phase columns, prepared from normally synthesised silica, the silica which displayed highly ionic character (reported above) and from silica prepared by procedures analogous to those of Köhler and Kirkland[25]. A 0.1% tri-

TABLE IV

RETENTION TIMES (t_R), PEAK WIDTHS (w_b) AND PEAK SKEW VALUES FOR PROTEIN STANDARDS ON C_8 COLUMNS PREPARED FROM SILICAS OF DIFFERING SLOPES

ND = Not determined.

Slope of k' vs. 1/[ ]	*Bovine insulin*			*Lysozyme*			*Ribonuclease A*			*Cytochrome c*		
	t_R *(min)*	w_b *(min)*	*Skew*	t_R *(min)*	w_b *(min)*	*Skew*	t_R *(min)*	w_b *(min)*	*Skew*	t_R *(min)*	w_b *(min)*	*Skew*
0.014	27.8	0.42	1.19	32.8	1.15	1.28	26.8	0.75	0.78	30.7	0.93	1.34
0.081	28.6	0.62	1.72	33.9	1.91	1.86	27.2	0.99	1.57	31.5	1.63	1.93
0.101	32.1	1.73	1.94	41.4	5.85	1.39	31.4	2.68	1.56	37.9	2.14	ND
0.186	35.8	2.03	1.85	*	–	–	*	–	–	*	–	–

* Peak not eluted under these chromatographic conditions.

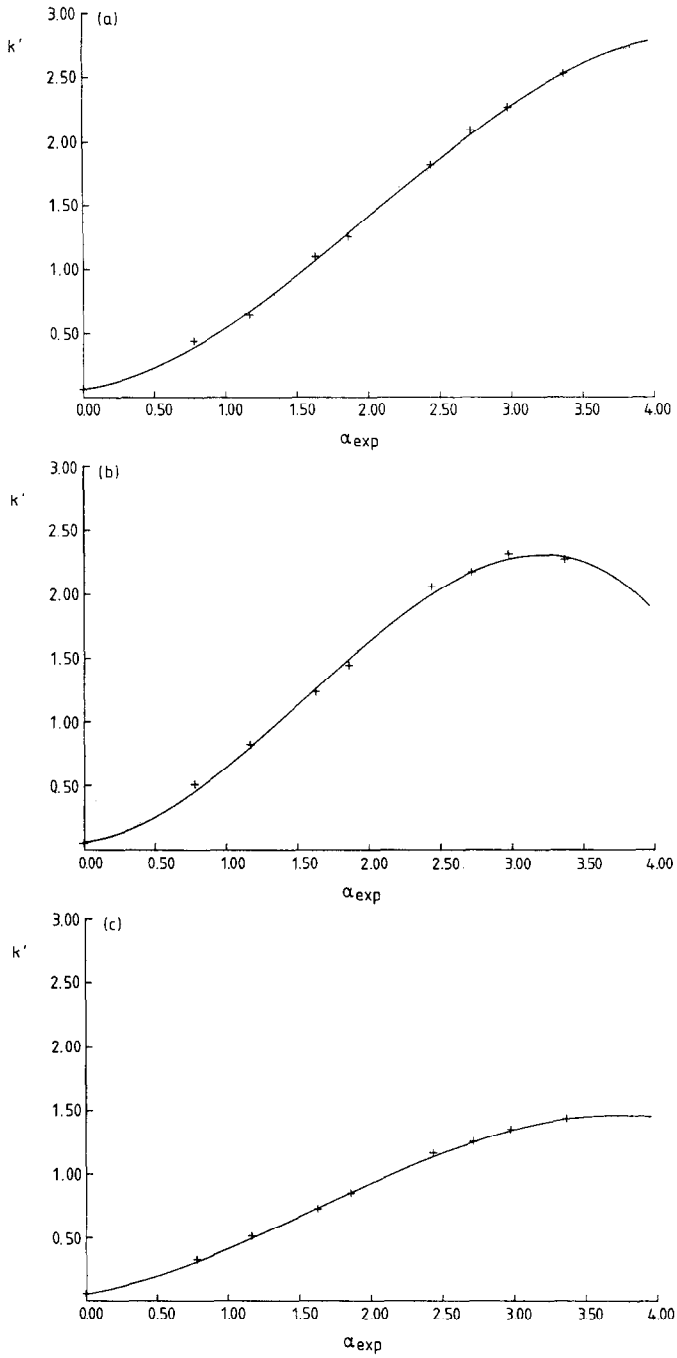

Fig. 13. Plots of k' against surface coverage for non-basic solutes. Mobile phase, 70% aq. methanol. (a) Toluene; (b) allyl phthalate; (c) anisole.

fluoroacetic acid-containing acetonitrile–water gradient was chosen in order to achieve elution under relatively low ionic strength conditions, which was expected to show the maximum effect of the underlying silica properties. Table IV shows the retention times, baseline peak widths (measured in minutes) and peak skew of four protein standards under a standard set of gradient conditions. Fig. 14 shows the retention times of insulin plotted against the value of the slope of the *k' versus* 1/buffer concentration plots, derived above for these and other silicas. The other parameters shown in Table IV follow similar trends. It is, therefore, clear that silicas with higher slope values give rise to bonded-phase packings with poor elution properties for the proteins. In these cases, and probably in the case of certain basic small molecules, the ionic character of the silica is important when mobile phases of low ionic strength are employed. In order to study the performance of the silicas for protein separations further, columns packed with silica were tested with insulin under the same conditions. A much wider range of retention was seen in comparison with the bonded-phase packings which suggested that the difference in the underlying silica retention was modifying the retention on the derived bonded-phase materials. The plot of retention time against slope of the *k' vs.* 1/buffer concentration plots is shown in Fig. 15. Again, a direct relation between retention time and slope was seen, those materials which had the smaller slopes also showing shorter elution times. These data are shown as a plot of retention time against slope in Fig. 15 and demonstrate the importance of the ion-exchange process on the retention. There is hence a clear influence of the ion-exchange character of the silica on the chromatography of peptides and proteins on bonded-phases. In order to obtain more reproducible packing materials, the ion-exchange effects should be minimised. This can be done by the use of high-ionic-strength buffers or by modification of the silica. It has been suggested that rehydroxylation techniques can enhance the recovery of certain peptides and proteins from bonded-phase silicas[11]. The data here show that there are links between the hydroxylation process and the ion-exchange character of the silica and between the ion-exchange character and the chromatographic behaviour of some

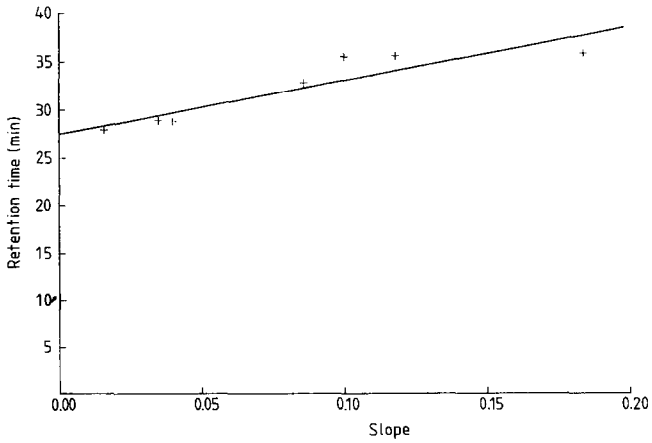

Fig. 14. Plot of insulin retention time on C_8-bonded phase packings against slope of the *k' vs.* 1/[buffer] curves for the silicas. For gradient conditions see text.

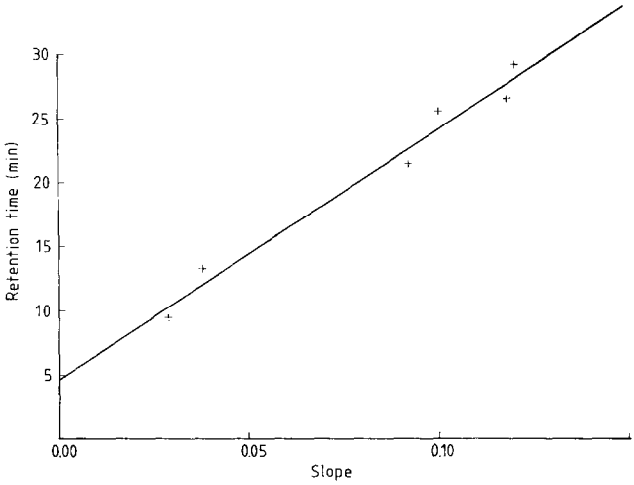

Fig. 15. Plot of insulin retention time on silicas against slope of the k' *vs.* 1/[buffer] curves. For gradient conditions see text.

proteins on the derived bonded-phase packing. Other, as yet unknown, parameters are certainly also involved in influencing the retention behaviour of proteins on silica-based packings, but it is evident that ion-exchange effects are an important modifier of the hydrophobic effects normally considered to determine their retention.

CONCLUSIONS

We have investigated the mechanisms of retention of basic compounds on silica and C_8 reversed-phase packings. These were shown to be mainly ion exchange for compounds ionised under the conditions of elution, and hydrophobic interaction for non-ionised materials. Separate hydrophobic interactions were seen with bonded-phase molecules and with the silica surface. Evidence was obtained which showed that these interactions were with the siloxane bridges and not the silanol groups. Study of the retention of the test solutes on a range of packings with differing bonded-phase coverages additionally gave strong evidence for the interaction between separation mechanisms. Silicas prepared by different routes were shown to have different ion-exchange properties and these were demonstrated to be important in the chromatography of proteins on bonded phases.

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