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BONDED-PHASE SELECTION IN THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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SUMMARY

The importance of several parameters in the reversed-phase high-performance liquid chromatography of proteins was investigated. Branching of the bonded phase, as in neohexyl *versus* *n*-hexyl, was found to be beneficial in reducing interaction of residual surface silanols with small basic analytes, but detracted from the chromatography of proteins. A deactivation procedure which reduced deleterious interaction of small, basic analytes with the packing also improved the chromatography of proteins. Varying the bonded-phase chain length from octyl to hexyl and then to butyl had relatively little effect on peak shape and recovery. Shortening the bonded-phase chain length, however, did decrease protein retention, particularly for proteins with molecular weights of 35,000 and 77,000 daltons. Increasing silica pore size from 12 nm to 30 nm appeared to improve the chromatography of proteins.

INTRODUCTION

At the first meeting in this series[1], it became apparent that reversed-phase high performance liquid chromatography (RP-HPLC) was rapidly becoming accepted as an excellent technique for conducting peptide separations and could also be applied to larger molecules. While the vast majority of HPLC applications utilizes the octadecyl reversed-phase bonded on small (3, 5 or 10 μm) particle, 10-nm pore size silica, it has become clear that other column packings may be quite suitable for protein separations[2,3].

With bonded-phase packings based on totally porous silica, most of the surface area is within the pores. Such pores must be of sufficient size to permit entry of the analytes of interest. Regnier's group[2,3] have convincingly made the case for the use of wide-pore silicas, *i.e.* 30 nm, in protein separations. Most of the data relating bonded-phase characteristics such as linear chain length, branching and presence of aromatic groups, derive from studies with small molecules[4–11]. There are, however, several reports[2,12,13] suggesting that relatively short-chain bonded phases, on wide-pore silica may provide advantages in protein chromatography.

Other factors important in RP-HPLC separations are the degree of surface coverage and the effect of residual surface silanol groups[14,15]. The present investigation considered the effect of bonded-phase chain length, chain branching, silica

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pore size and a deactivation procedure which reduces undesirable interaction of basic compounds with the packings on the RP-HPLC of proteins.

EXPERIMENTAL

Materials

Bonded-phase packings used in this investigation were prepared on 5-μm spherical silicas with either 12- or 30-nm pores (Supelcosil LC-Si and Supelcosil LC-3Si, respectively) and were supplied by Supelco (Bellefonte, PA, U.S.A.). In all cases, the packings were endcapped with a trimethylsilyl reagent and, where designated by the notation DB, the packings were deactivated to reduce undesirable interaction with small basic analytes. The nature of this deactivation procedure has not been publicly disclosed but can be readily deduced from a close reading of Iler's text on silica[16]. All packings were filled into 5 cm × 4.6 mm I.D. columns by Supelco. The *n*-hexyl phase was prepared from *n*-hexylmethyldichlorosilane; all other bonded phases were prepared from the corresponding alkyldimethyl monochlorosilanes, supplied by Petrarch Systems (Levittown, PA, U.S.A.). Caffeine, procainamide (PA) and N-acetyl procainamide (NAPA), were obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.). Proteins were obtained from Sigma (St. Louis, MO, U.S.A.).

Instruments

The studies with the basic analytes were conducted with a Waters Model 590 pump, a Rheodyne Model 7125 injection valve and a Spectroflow 757 variable-wavelength detector (Kratos, Ramsey, NY, U.S.A.). For the work with the proteins, a DuPont 870 pump module and Series 8800 gradient controller were used with a Rheodyne Model 7125 injection valve and a Spectra-Physics Model 770 variable-wavelength detector.

Chromatography

For the tests with caffeine, PA and NAPA isocratic elution was used, the mobile phase A containing 10 m*M* potassium dihydrogen phosphate (KH_2PO_4) in an aqueous solution of 400 μl triethylamine per liter and 5 to 20% methanol as needed to produce comparable k' values on the various packings. Other parameters were: flow-rate, 2 ml/min; temperature, ambient; detection, UV at 254 nm, 0.2 a.u.f.s.; chart-speed, 1 in./min; sample injected, 20 μl, at a concentration of 0.25 mg/ml each analyte.

Mobile phase composition for the proteins was linearly programmed from 100% A to 20% B in 20 min, A being a 9:1 mixture of a 0.1% aqueous trifluoroacetic acid solution and 2-propanol, and B a 1:9 mixture of the same components. Other parameters were: flow-rate, 2.0 ml/min; temperature, 45°C; detection, UV at 220 nm, 0.4 a.u.f.s.; chart-speed, 1 cm/min; sample injected, 20 μl at a concentration of 0.33 mg/ml for each protein.

RESULTS

Small, basic analytes

The retention of caffeine is reduced and peak symmetry for this compound is improved by endcapping of an octyl packing. Deactivation of endcapped RP-HPLC

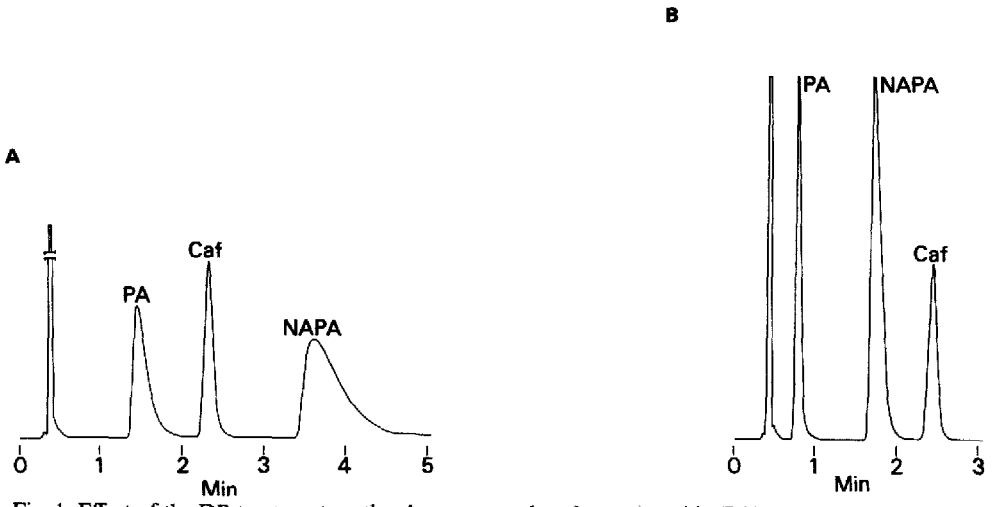

Fig. 1. Effect of the DB treatment on the chromatography of procainamide (PA), N-acetyl procainamide (NAPA) and caffeine (Caf) on (A) Supelcosil LC-8 column, (B) Supelcosil LC-8DB column (see experimental section for conditions).

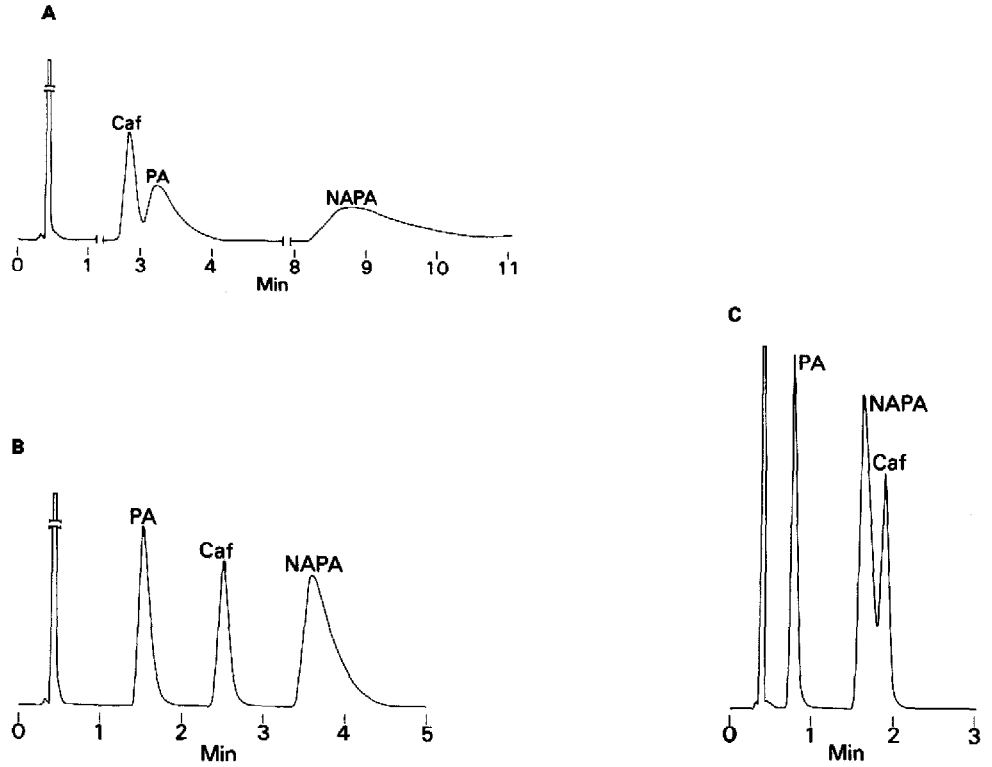

Fig. 2. Effect of bonded-phase carbon chain branching and the DB treatment on the chromatography of procainamide (PA), N-acetyl procainamide (NAPA) and caffeine (Caf) on (A) *n*-hexyl bonded phase, (B) neohexyl bonded phase, (C) neohexyl bonded phase on a DB packing, (see experimental section for conditions).

packings to reduce retention of basic compounds has little effect on the chromatography of caffeine but has been found to greatly decrease the retention of basic compounds, such as PA and NAPA. A comparison of the chromatograms obtained with the commercially available (Fig. 1a) Supelcosil LC-8 and Supelcosil LC-8DB (Fig. 1b) columns illustrates such data. With a mobile phase of methanol–10 m*M* KH_2PO_4 (15:85) plus 400 μl triethylamine/l, caffeine is eluted from these two columns in 2.31 and 2.40 min respectively. On the Supelcosil LC-8 column PA and NAPA have retention times relative to caffeine of 0.55 and 1.66 respectively (Fig. 1a). Undesirable interaction with the packing is clearly evident from the marked tailing of the peaks for the basic drugs. Deactivation of the packing (Fig. 1b) reduces the retention times of PA and NAPA relative to caffeine to 0.18 and 0.64, respectively. While the height of the caffeine peak remains constant, the PA and NAPA peaks are 3–4 times higher than on the previous chromatogram due the the decrease in retention and tailing.

Branched-chain bonded phase

Only a portion of surface silanols of silica reacts with the silyl reagent used to produce a bonded phase, but analyte contact with the residual silanols is to some extent prevented by a steric screening provided by the bonded phase. The possibility that this steric effect could be increased by increasing the cross sectional area of the alkyl chain by chain branching was investigated. A branched-chain octyl phase (2,2,4-trimethylpentyl) has been described[10] but the necessary silyl bonding reagent is not commercially available. A comparison was therefore undertaken of packings prepared with commercially available *n*-hexyl and neohexyl (3,3-dimethylbutyl) bonding reagents. With the *n*-hexyl phase the retention times relative to caffeine of PA and NAPA are 1.17 and 3.46 respectively (Fig. 2a). For the neohexyl phase, the corresponding values for relative retention times are 0.65 and 1.92 (Fig. 2b). Peak heights increase approximately 2.5-fold, but, while there is a very marked improvement in peak symmetry, tailing is still evident. Deactivation of the neohexyl packing (Fig. 2c) resulted in reduction of the relative retention times of PA and NAPA to 0.20 and 0.47, respectively, and a marked improvement in peak symmetry.

Proteins

The proteins used in this study had molecular weights of 6000 (insulin), 13,700 (ribonuclease A), 14,000 (lysozyme), 35,000 (β-lactoglobulin) and 77,000 daltons (transferrin). These proteins were chromatographed on three commercially available columns, Supelcosil LC-8, Supelcosil LC-8DB and Supelcosil LC-308. The first two columns contained packings prepared from silica with 12-nm pores while the third column contained a packing prepared from silica with 30-nm pores. All packings prepared (by the supplier) from 30-nm pore silica had received the DB treatment.

Recoveries of the two largest proteins, transferrin and β-lactoglobulin, were relatively low (Table I) on the Supelcosil LC-8 column (Fig. 3a) with the mobile phase used in this investigation. Better peak shapes and recoveries were obtained with columns filled with packings having the octyl bonded phase on DB-treated packings with either 10-nm (Fig. 3b) or 30-nm pores (Fig. 3c). Shortening the chain length of the linear phases from octyl to *n*-hexyl (Fig. 3d) or *n*-butyl (Fig. 3f) did not appear to adversely effect peak shape or recovery. Peak shapes and recovery of trans-

TABLE I

RECOVERY OF PROTEINS FROM VARIOUS HPLC COLUMNS

Recoveries are stated relative to integrator counts on peaks for the Supelcosil LC-8DB and Supelcosil LC-308 columns. Triplicate samples were analyzed on each column to determine recoveries and reproducibility.

Protein	*Column type**						
	LC-8	*LC-8DB*	*LC-308*	*LC-306*	*LC-306b*	*LC-304*	*LC-304b*
Ribonuclease A	0.87	1.0	1.0	0.96	0.91	1.15	0.95
Insulin	0.87	1.0	1.0	0.99	0.96	1.03	1.00
Lysozyme	0.94	1.0	1.0	0.93	1.03	0.97	0.99
Transferrin	—**	1.0	1.0	0.72	0.92	0.93	0.34
β-Lactoglobulin	0.31	1.0	1.0	0.93	0.95	0.98	—***

* The first three columns listed are commercially available: Supelcosil LC-8, Supelcosil LC-8DB, and Supelcosil LC-308. The designations LC-306, LC-306b, LC-304 and LC-304b refer, respectively, to *n*-hexyl, neohexyl, *n*-butyl and *tert.*-butyl phases bonded on Supelcosil 3Si, a 30-nm pore silica. All packings on this 30-nm pore silica have undergone a DB treatment to reduce undesirable interaction of basic analytes with the packing. All columns are 5 cm × 4.6 mm I.D.

** Recovery low and not readily quantifiable.

*** Recovery high but erratic.

ferrin, however, were adversely influenced by chain branching in the *tert.*-butyl phase (Fig. 3g) but not in the neohexyl phase (Fig. 3e). There was a slight decrease in retention of the proteins, particularly transferrin and β-lactoglobulin with decreasing bonded-phase chain length (Table II).

DISCUSSION

Column comparisons

It is rather difficult to relate new data to previously published data on RP-HPLC of proteins. Indeed it frequently is difficult to compare various items of published data one against the other. The initial surge in interest in RP-HPLC of proteins corresponded in time to a period during which the nature of the column packings was undergoing a major transition. Older packings were usually prepared with di- and trifunctional bonding reagents, and endcapping was not considered an obligatory step in packing preparation. Such packings range from materials with low surface coverage to materials of unknown coverage with high loading of partially polymeric phase. Newer packings are for the most part prepared from monofunctional silanes, have high surface coverage and are very frequently endcapped. Investigations using different columns of the same nominal phase compositions from different manufacturers or from the same manufacturers at different times[17] may yield drastically different data. This has been shown to be the case with small molecules[18], and is also evident in the work of O'Hare *et al.*[12] and Cooke *et al.*[13] with proteins.

Branched-chain bonded phases

Branched-chain phases have been prepared in several laboratories[5,9,19] but have not been extensively investigated for use in the chromatography of basic ana-

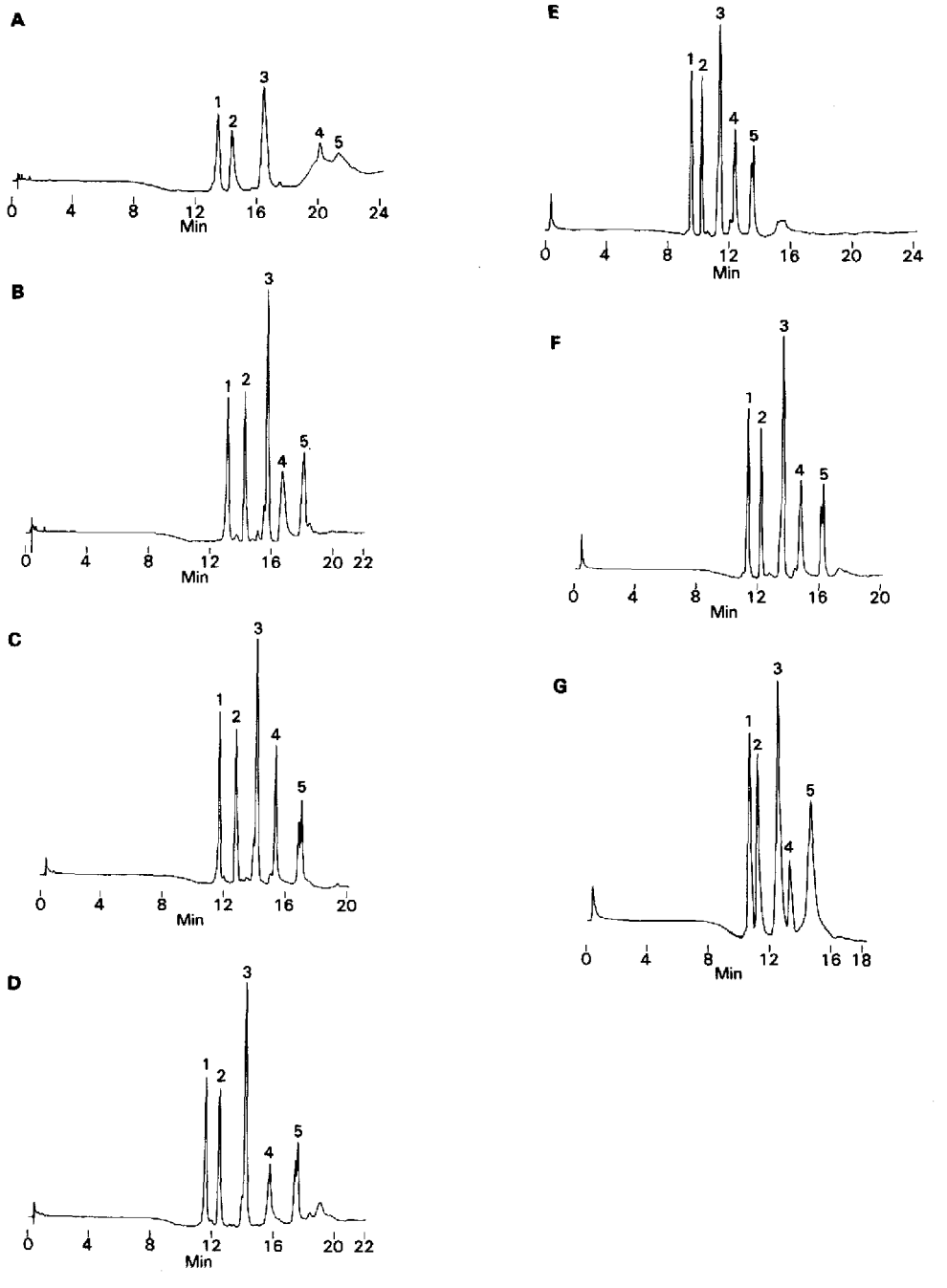

Fig. 3. Effect of chain length, chain branching, DB treatment and packing pore size of bonded phases on the chromatography of proteins, (A) Supelcosil LC-8 column, (B) Supelcosil LC-8DB column, (C) Supelcosil LC-308 column, (D) *n*-hexyl bonded phase on 30-nm pore silica, (E) neohexyl bonded phase on 30-nm pore silica, (F) *n*-butyl bonded phase on 30-nm pore silica, (G) *tert.*-butyl bonded phase on 30-nm pore silica. Peak sequence: 1 = ribonuclease A, 2 = insulin, 3 = lysozyme, 4 = transferrin, and 5 = β-lactoglobulin. Mobile-phase composition was linearly programmed from 100% A to 20% A and 80% in 20 min. A being a 9:1 mixture of a 0.1% aqueous trifluoroacetic acid solution and 2-propanol and B an 1:9 mixture of the same components.

TABLE II

INFLUENCE OF BONDED PHASE CHAIN LENGTH ON PROTEIN RETENTION

The first three columns listed are commercially available: Supelcosil LC-8, Supelcosil LC-8DB, and Supelcosil LC-308. The designations LC-306, LC-306b, LC-304 and LC-304b refer, respectively, to *n*-hexyl, neohexyl, *n*-butyl and *tert.*-butyl phases bonded on Supelcosil 3Si, a 30-nm pore silica. All packings on this 30-nm pore silica have undergone a DB treatment to reduce undesirable interaction of basic analytes with the packing. All columns are 5 cm × 4.6 mm I.D.

Protein	*Column type**						
	LC-8	*LC-8DB*	*LC-308*	*LC-306*	*LC-306b*	*LC-304*	*LC-304b*
Ribonuclease A	13.1	11.9	11.4	11.7	11.2	9.7	10.7
Insulin	14.0	12.9	12.4	12.6	11.9	10.4	11.2
Lysozyme	16.2	14.0	13.8	14.2	13.4	12.0	12.6
Transferrin	–**	15.7	15.0	15.6	14.8	13.5	13.6
β-Lactoglobulin	19.5	17.4	16.7	17.2	16.1	14.2	14.9

* Retention time in min. With the gradient program used, a one-min increase in retention time corresponds to an increase of 3.7% isopropanol.

** Was not eluted.

lytes or proteins. *tert.*-Butyldimethylchlorosilane has also been investigated as a possible endcapping reagent[20]. In addition to working with the hexyl and neohexyl phases we have also prepared and tested DB-treated butyl and *tert.*-butyl phase packings with the procainamide probes. A large reduction in the retention of the basic drugs was noted following DB treatment of the butyl phase packing but this treatment had little if any effect on the *tert.*-butyl phase packing. For small basic molecules there may be advantages in the use of branched-chain phases. However, as noted below, the straight-chain phases are probably preferable for work with proteins.

DB treatment

A Supelcosil LC-18DB packing was included in a recent study by Sadek and Carr[21] designed to characterize the silanophilic interactions of a relatively large number of commercially available columns. These authors stated "Column 2 (Supelcosil LC-18DB) is a deactivated-base C_{18} support that has undergone extensive special treatment in the manufacturing procedure to minimize surface silanol groups. The superior performance of these columns is expected and supports the validity of this characterization procedure". DB packings have also been found to be useful in work with such other difficult-to-chromatograph basic drugs as levamisole[22], and clonidine[23].

The data of Barford *et al.*[24] would indicate that the percentage of organic modifier (isopropanol) needed in the mobile phase to elute a given protein (bovine serum albumin) decreases as the bonded-phase chain length is decreased from 18 to 8 and then to 1. Cooke *et al.*[13] eluted a series of proteins including bovine serum albumin from propyl and octyl bonded-phase columns with acetonitrile or 1-propanol gradients and found that the percentage of organic modifier needed was essentially independent of phase chain length. Pearson and Regnier[3] reported that a series of proteins, including bovine serum albumin, had comparable retention times on a series of columns when the phase carbon chain lengths were 2, 4, 8, 12, 18 and

22. In the present investigation, the retention of proteins, particular β-lactoglobulin and transferrin, decreased slightly as bonded-phase chain length was decreased. The present data were generated sequentially using the same batch of mobile phase and represent typical results of triplicate analyses. It is possible that this difference between our observations and these of Cooke[13] and Pearson and Regnier[3] is related to the DB treatment.

Recovery of proteins from 30-nm pore packing

O'Hare *et al.*[12] originally reported that the recovery of proteins from C_2 to C_6 bonded-phase packings with 30-nm pores was markedly less and in some cases virtually nil when compared to similar packings with 8- to 18-nm pores. Subsequently this same group[13] reported that recoveries were actually comparable if both sets of packings were maximally bonded and endcapped. Kato *et al.*[25] reported good recovery of proteins from butyl and phenyl bonded-phase packings prepared from a 10-μm silica with 25-nm pores.

Wilson *et al.*[26] reported that recovery of proteins larger than 15,000 daltons was greater on 30-nm pore packings than on 10-nm pore packings. Lewis *et al.*[27] found that packings with 33- and 50-nm pores and octyl, cyanopropyl or diphenyl phases were useful for larger (> 50,000 daltons) proteins.

In the present study recoveries of ribonuclease A, insulin and lysozyme were similar for all packings tested. When the DB treatment was included in packing preparation similar recoveries of β-lactoglobulin and transferrin were observed for all linear chain bonded-phase packings. The somewhat low value for transferrin on the *n*-hexyl phase is thought to be related to the use of a difunctional bonding reagent. Van der Venne *et al.*[18] reported that lower surface coverage was achieved with a difunctional octyl bonding reagent compound than with the corresponding monofunctional reagent. Branching of the phase chain resulted in poorer apparent chromatographic efficiency and a marked reduction in the recovery of transferrin. The present study utilized short, 5-cm, columns and small sample loadings, approximately 6–7 μg for each protein, and therefore did not address the question of packing capacity. This topic has been thoroughly described by Pearson *et al.*[2] and Pearson and Regnier[3]. The maximal loading capacity was reported to be higher for the C_4 and C_8 phase packings compared to the C_2, C_{12}, C_{18} and C_{22} phase packings.

CONCLUSIONS

Relatively short chain length linear but not branched-chain bonded phases appear to be of practical interest for protein chromatography. While very short (C_2 to C_4) chain length bonded phases may have special applications, the standard octyl phase appears to be well suited to work with proteins. Deactivation of the packing is desirable and 30-nm pore packings should be utilized for work with moderate size, 40,000–80,000 daltons, proteins.

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