Characterization of the *N*-Retinyl-3-silyl-*n*-propylamine Moiety in Solution by NMR Spectroscopy and Covalently Bound to Celite by Hydrogen Ion Binding

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Binding of hydrogen ions by *N*-retinyl-Celite indicates an approximate p*K* of 5.6 for the secondary amino group which is consistent with the previously observed pH dependence of *β*-lactoglobulin binding by this affinity matrix. *N*-Retinyl-3-(triethoxysilyl)-*n*-propylamine was synthesized as a soluble model compound for the affinity ligand. ^{13}C NMR and ^{1}H NMR spectra of this compound and its precursors, (3-aminopropyl)triethoxysilane, *all-trans*-retinal, and the Schiff base, confirmed formation of the Schiff base and its selective reduction with $NaCNBH_3$. Furthermore, ^{1}H NMR spectra confirmed that protonation of the secondary amine in *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine occurred upon treatment at pH 5.1.

Keywords: *N-Retinyl-Celite; N-retinyl triethoxysilyl n-propylamine; ^{13}C NMR spectra; ^{1}H NMR spectra; hydrogen ion binding*

INTRODUCTION

Affinity matrices have been widely used for purification of enzymes and proteins for research purposes (Sundaram and Eckstein, 1978; Jakoby and Wilchek, 1974; Chaiken et al., 1983). However, development of this technology is of potential commercial importance to the food and pharmaceutical industries. If the affinity binding constant is large enough under appropriate conditions, bioselective adsorption can be accomplished during a loading period, followed by a change of conditions to cause desorption. In some cases, interaction with a bioselective hydrophobic ligand can be accentuated by an additional electrostatic interaction resulting in high-affinity binding, a process termed "amphiphilic chromatography" (Scouten, 1981).

We have previously reported characteristics of the bioselective adsorption of *β*-lactoglobulin to all-*trans*-retinal moieties covalently immobilized on porous glass (Jang and Swaisgood, 1990) and on Celite (Wang and Swaisgood, 1993). Studies with *N*-retinyl-Celite indicated a strong pH dependence of the affinity such that adsorption could be accomplished at pH 5.1 and desorption at pH 7.0 (Wang and Swaisgood, 1993). The purpose of the present study was to confirm the immobilization chemistry by NMR spectral analysis and to test for protonation of the secondary amine by NMR spectral and hydrogen ion binding analyses.

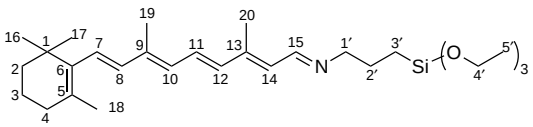
MATERIALS AND METHODS

Immobilization of *all-trans*-Retinal on Celite Beads. Celite beads (R649, 50/100 mesh from Celite Corp., Lompoc, CA) were dried at 110 °C for 2 h. Beads were derivatized with organosilane according to methods developed by Janolino and Swaisgood (1982) for controlled-pore glass and as modified by Wang and Swaisgood (1993) for Celite. The concentration of immobilized amino groups was assayed with *o*-phthalaldehyde (OPA) (Sigma Chemical Co., St. Louis, MO) as described by Janolino and Swaisgood (1992).

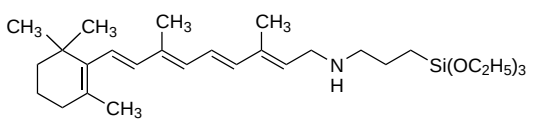
all-trans-Retinal was covalently attached to aminopropyl-Celite as described by Wang and Swaisgood (1993) with some modifications to accommodate larger quantities. Beads (70 g) were incubated with retinal (1 g in 1.5 L of methanol) for 20 h at room temperature (23 °C), after which 2.2 g of $NaCNBH_3$ was added to selectively reduce the Schiff bases formed. Following reduction for 7 h at room temperature, the beads were extensively washed successively with methanol and acetone, air-dried, and stored in the dark. All procedures were performed under low-intensity light to minimize possible isomerization of the *trans*-retinal moiety.

Hydrogen Ion Binding to (*N*-Retinyl-3-silyl-*n*-propylamino)-Celite. Dried (110 °C, 2 h) *N*-retinyl-Celite (1 g) was slurried with 20 mL of 3.4 M NaCl adjusted to various pHs for 2 h at room temperature (23 °C). After the beads were removed, 15 mL of the solution was titrated back to the original pH with 0.1 M HCl using 20−50 *μ*L increments. The number of moles of HCl required reflects the amount of protons bound by the matrix.

Preparation of *N*-Retinyl-3-(triethoxysilyl)-*n*-propylamine. This model compound and intermediates in the preparative reactions were synthesized to examine the reaction chemistry by NMR spectroscopy using a reductive amination procedure similar to that described by Lee and Lee (1980). A solution was prepared containing 120 *μ*L (0.5 mmol) of (3-aminopropyl)triethoxysilane and 120 mg (0.42 mmol) of *all-trans*-retinal dissolved in 2 mL of tetrahydrofuran. After reaction for 6 h at room temperature, solvent was removed from the dark red solution by rotary evaporation. The Schiff base product (**I**) was dissolved in 0.5 mL of $CDCl_3$. Reduction



I



II

of the Schiff base (**I**) was performed by addition of a 12-fold molar excess of $NaCNBH_3$ and reaction at room temperature for 24 h. Excess $NaCNBH_3$ was removed by extraction with

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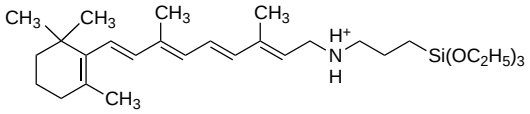
Table 1. Hydrogen Ion Binding to *N*-Retinyl-Celite[a]

Initial pH	$[MN^+H_2R]^b + [MN^+H_3]$ (mM)	$[MN^+H_2R]^c$ (mM)	$[MNHR]^d$ (mM)	$\log \frac{[MN^+H_2R]}{[MNHR]}$	pK^e
3.94	1.03 ± 0.02	0.72	0		
4.56	0.92 ± 0.02	0.61	0.11	0.74	5.30
5.61	0.69 ± 0.03	0.38	0.34	0.05	5.66
5.61	0.69 ± 0.03	0.38	0.34	0.05	5.66
6.12	0.55 ± 0.03	0.24	0.48	−0.30	5.82
6.78	0.37 ± 0.02	0.06	0.66	−1.04	5.74
6.78	0.35 ± 0.01	0.04	0.68	−1.23	5.55
6.96	0.31 ± 0.01	0	0.72		

[a] Dried *N*-retinyl-Celite (1.0 g) was slurried with 20 mL of 3.4 M NaCl adjusted to the pH indicated. [b] Total hydrogen ion concentration bound as measured by the concentration of OH^- released and titrated with HCl. M and R represents the Celite matrix and *N*-retinyl moiety, respectively. Standard deviations were calculated from triplicate measurements. [c] These values were calculated by assuming that $[MNH_2]_{total}$ is 0.31 mM, i.e. assuming at pH 6.96 all primary amino groups will bind protons and that none of the secondary amino groups will do so. [d] These values were calculated using a value of 0.72 mM for $[MNHR]_{total}$, i.e. by assuming that all primary and secondary amino groups bind protons at pH 3.94. [e] These values were estimated using the relationship $pK = pH + \log([MN^+H_2R]/[MNHR])$.

water and the *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine (**II**) was recovered in the organic phase.

The protonated form of the secondary amine was obtained by equilibrating 0.5 mL of the secondary amine solution in $CDCl_3$ with 5 mL of 0.4 M sodium phosphate, pH 5.1, by vigorous shaking. After phase separation, the aqueous phase was discarded and the equilibration was repeated eight times. The protonated form (**III**) was dried for NMR measurements.



III

NMR Spectroscopy. NMR spectra for compounds **I**, **II**, **III**, *all-trans*-retinal, and (3-aminopropyl)triethoxysilane were recorded on a GN 300 spectrometer (General Electric Co., Fremont, CA) operating at a magnetic field of 7.05 Tesla, ^1H frequency of 300.52 MHz, and ^{13}C frequency of 75.13 MHz. Chloroform ($CDCl_3$) was used to dissolve the compounds and to obtain a field frequency lock. Samples were placed in 5 mm diameter tubes, and ^{13}C NMR spectra were obtained with broad band proton decoupling without NOE, with a pulse width of 30°, a 4.3 μs pulse, and a spectral width of 66 kHz. One-dimensional ^{1}HMR spectra were obtained in the quadrature-phase detection mode with 8 K data points, 30° pulse width, 2.3 μs pulse, a recycle time of 1.2 s, and a spectra width of 3600 Hz.

RESULTS AND DISCUSSION

Binding of Hydrogen Ions to *N*-Retinyl-Celite. Addition of dry *N*-retinyl-Celite to aqueous solutions between pH 3 and 7 resulted in binding of hydrogen ions to the primary and secondary amino groups with a resulting increase in pH of the suspending solution. No pH change was detectable when underivatized Celite was added to these solutions. Results listed in Table 1 indicate that a basic group bound protons in the pH range examined. The concentration of secondary amino groups $[MNHR]_{total}$ was chemically determined by OPA analysis of the immobilized primary amino groups before and after attachment of the retinyl moiety. This difference indicated that $[MNHR]_{total}$ was 0.6 mM in a 20 mL slurry containing 1.0 g of beads. Allowing for experimental error (typically 5%, Janolino and Swaisgood (1992)), comparison of this value with that of 0.72 mM estimated from hydrogen ion binding suggests that the assumptions made to calculate the values in Table 1 are not unreasonable. Furthermore, OPA analysis of primary amino groups on the aminopropyl-Celite prior to addition of retinal yielded a concentration of 6.3 μmol/mL beads. Using a specific volume of 3 mL/g beads, a concentration of 0.95 mM is obtained for the 20 mL slurry which agrees with the value (1.03 mM) for total primary and secondary amino group concentration listed in Table 1.

Table 2. Natural Abundance ^{13}C NMR Spectral Data Obtained in $CDCl_3$

	chemical shift, ppm			
assignments[a]	retinal	silane	Schiff base	*N*-retinyl-3-(triethoxysilyl)-*n*-propylamine
C(1)	34.22		34.15	34.12
C(2)	39.55		39.52	34.49
C(3)	19.13		19.13	19.13
C(4)	33.09		32.99	32.76
C(5)	130.51		129.67	130.15
C(6)	137.59		137.39	136.59
C(7)	129.35		127.73	126.34
C(8)	137.04		135.97	135.59
C(9)	141.33		137.91	137.39
C(10)	132.61		129.93	129.54
C(11)	128.93		127.73	128.70
C(12)	134.45		135.97	135.61
C(13)	155.00		143.95	137.75
C(14)	129.73		129.35	129.37
C(15)	191.22		159.53	65.76
C(16,17)	28.84		28.85	28.82
C(18)	21.71		21.65	21.62
C(19)	12.96		12.73	12.60
C(20)	13.09		12.93	12.86
C(1′)		44.66	64.72	58.65
C(2')		26.79	33.00	32.93
C(3')		7.08	7.98	7.82
C(4')		57.87	58.29	58.29
C(5')		17.84	18.22	18.19

[a] These assignments were made according to earlier assignments for *all-trans*-*N*-retinylidene-*n*-butylamine made by Birge et al. (1987). $CDCl_3$ were used as internal reference standards for chemical shift correction; ^{13}C (77 ppm) and ^1H (7.26 ppm).

Estimation of the pK for proton binding to the secondary amino group in *N*-retinyl-Celite yielded an approximate value of 5.6. This conclusion is consistent with the observed rapid increase in binding affinity of β-lactoglobulin to *N*-retinyl-Celite as the pH is lowered from 6 to 5.1 (Wang and Swaisgood, 1993). Effects of pH and ionic strength on the binding affinity of β-lactoglobulin with *N*-retinyl-Celite led to a proposed electrostatic contribution to the affinity.

^{13}C NMR Spectral Analysis. Formation of the Schiff base between (3-aminopropyl)triethoxysilane and *all-trans*-retinal was confirmed by ^{13}C NMR spectral analysis. The structure of the Schiff base is shown, compound **I**, and the spectral assignments are listed in Table 2. The recorded spectra were very similar to that previously obtained with *all-trans*-*N*-retinylidene-*n*-butylamine in $CDCl_3$ (Birge et al., 1987), and the assignments were used as a basis for assignments in this study. The ^{13}C NMR spectrum of the Schiff base is shown in Figure 1. Comparison of the spectra for (3-aminopropyl)triethoxysilane, *all-trans*-retinal, and the Schiff base indicates that significant chemical shifts occur for carbon atoms near the Schiff base. Thus, upon Schiff base formation, C(15), which is part of the Schiff base, was shifted approximately 30 ppm upfield relative to its spectrum in retinal. The other odd-numbered polyene carbons are also shifted upfield relative to the

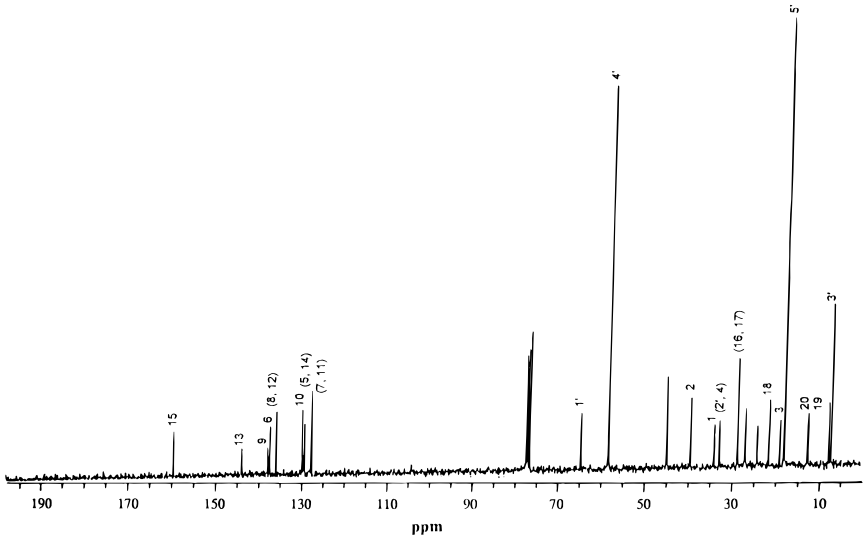


Figure 1. ^{13}C NMR spectrum obtained for the Schiff base, *N*-retinylidene-3-(triethoxysilyl)-*n*-propylamine, in $CDCl_3$. The lines not identified are derived from unreacted organosilane or from the solvent.

retinal spectrum because of the substitution of oxygen by the less electronegative nitrogen atom, with the resulting release of electron density into the polyene chain.

On the other hand, formation of the C═N bond caused a downfield shift of the spectum of all carbon atoms in the silyl structure relative to the spectrum for (3-aminopropyl)triethoxysilane (Table 2). This results from the substitution of hydrogens by the more electronegative carbon atom.

Results of ^{13}C NMR sprectral analysis of *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine are also listed in Table 2. The data confirm that selective reduction of the Schiff base occurred with the conditions used. Thus, addition of two less electronegative hydrogens resulted in a large upfield shift (94 ppm) for C(15) upon reduction of the double bond and a smaller upfield shift (6 ppm) for C(1′) in the propyl chain. No shift in the spectum was observed for carbon atoms in the polyene chain.

Protonation of *N*-Retinyl-3-(triethoxysilyl)-*n*-propylamine as Detected by ^1H NMR Spectral Analysis. Further proof that the secondary amino group in *N*-retinyl-Celite is protonated at pH 5.1 was obtained by analysis of ^1H NMR spectra for *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine obtained with the protonated (treated at pH 5.1) and unprotonated form (Figure 2). Introduction of a positive charge on the nitrogen atom changed the ^1H-N chemical shift to 7.28 ppm which was the largest observed in the ^1H NMR spectrum (Table 3). Small downfield shifts of other ^1H resonances [H−C(15) and 2H−C(1′)] also appeared to be caused by protonation of the secondary amine. These spectra were similar to those of *all-trans*-retinylpyrrolidiniminium perchlorate (Birge et al., 1987), protonated methylamine or ethanolamine Schiff base derivatives of retinal (Livnah and Sheves, 1993), and protonated *N*-butyl(*all-trans*-retinylidene)amine (Pattaroni and Lauterwein, 1981). Spectral assignments reported for these and other compounds (Jendrasiak et al., 1990) were used as a basis for assignment of resonances in this study.

It was also noted that the ethoxy groups appeared not to be hydrolyzed during any of the chemical synthesis. However, these groups were hydrolyzed by treatment at pH 5.1 (Figure 2b). Thus, the ^1H NMR resonances assigned to these groups did not change, nor did the ^{13}C

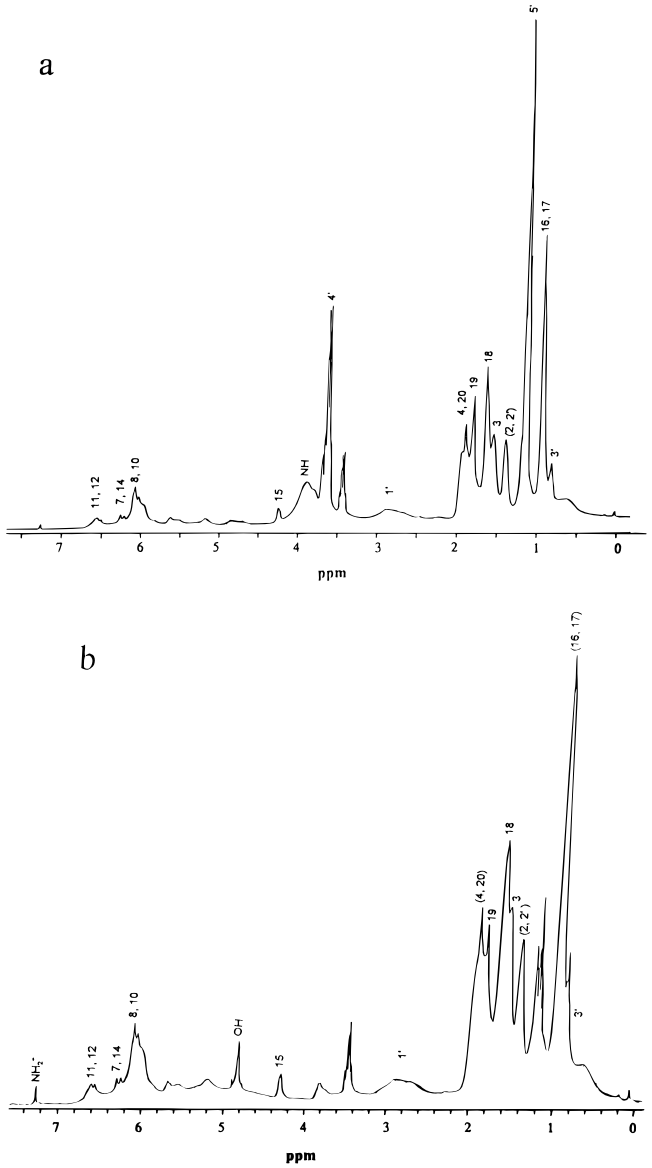


Figure 2. ^1H NMR spectra obtained for *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine (a) and its protonated form produced by treatment at pH 5.1 (b). Resonances due to H−C(4′) and H−C(5′) in the ethoxy group disappear after acidification, indicating hydrolysis.

Table 3. ^{1}H NMR Chemical Shifts of the Major Lines That Undergo Change upon Protonation of *N*-Retinyl-3-(triethoxysilyl)-*n*-propylamine

		N-retinyl-3-(triethoxysilyl)-*n*-propylamine	
assignment[a]	Schiff base	pH 7	pH 5.1
H−N		3.70	7.28 7.27
H−C(15)[b]	8.27	4.73	4.80
2H−C(1′)[c]	2.67	2.66	2.72

[a] All other lines around 6.0, 1.6, and 1.1 ppm corresponding to hydrogens in the polyene chain, the ring, and the methyl groups, respectively, did not change significantly upon protonation. These assignments were based upon previous assignments for *all-trans*-retinylpyrrolidiniminium perchlorate (Birge et al. 1987), for *all-trans-N*-retinlyidenemethylamine and *all-trans-N*-retinylideneethanolamine (Livnah and Sheves, 1993), for amiodarone (Jendrasiak et al., 1990), and for protonated *N*-butyl(*all-trans*-retinylidene-)amine (Pattaroni and Lauterwein, 1981). $CDCl_3$ were used as internal reference standards for chemical shift correction; ^{13}C (77 ppm) and ^{1}H (7.26 ppm). [b] The chemical shift for this hydrogen in *all-trans*-retinal was 10.08 ppm. [c] The chemical shift for this hydrogen in (3-aminopropyl)triethoxysilane was 2.54 ppm.

NMR spectrum unless the compound was exposed to acid pH. This observation suggests an immobilization procedure alternative to that previously reported (Wang and Swaisgood, 1993). The (3-aminopropyl)triethoxysilane could be reacted with *all-trans*-retinal in solution, the resulting Schiff base could be selectively reduced, and the retinyl-3-(triethoxysilyl)-*n*-propylamine could be used as the silanization reagent for derivatization of Celite. This approach may avoid leaving unreacted surface primary amino groups that could cause nonspecific adsorption by general ion-exchange behavior, thus improving the selectivity of β-lactoglobulin adsorption on *N*-retinyl-Celite.

Conclusion. Hydrogen ion binding by *N*-retinyl-Celite suggested an approximate p*K* for the secondary amino group of 5.6, which is consistent with a previously observed (Wang and Swaisgood, 1993) rapid increase in the affinity for binding of β-lactoglobulin as the pH is decreased from 6.0 to 5.1 and the proposed participation of this protonated ligand in binding enhancement. Both ^{13}C and ^{1}H NMR studies of the reaction chemistry for synthesis of *N*-retinyl-3-(triethoxysilyl)-*n*-propylamine, as a solution model for synthesis of *N*-retinyl-Celite, indicated that selective reduction of the Schiff base occurred. Furthermore, ^{1}H NMR spectra confirmed that the protonated form of the secondary amine was formed by treatment at pH 5.1

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