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Effects of Surfactants on the Interaction of Vitamin B_{12a} with Cysteine and *N*-Alkanoylcysteines in Water and in Benzene. Influence of Aqueous Micelles and Solvent Restrictions

Faruk Nome and Janos H. Fendler*

Contribution from the Department of Chemistry, Texas A&M University, College Station, Texas 77843.

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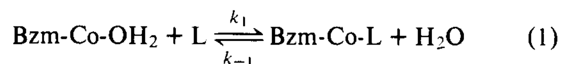
Abstract: Aqueous micellar hexadecyltrimethylammonium bromide, CTABr, and sodium dodecyl sulfate, SDS, show only a modest rate retardation of the anation of vitamin B_{12a} by L-cysteine. Rate constants for the interaction of vitamin B_{12a} with *N*-butanoyl-L-cysteine, NBC, *N*-octanoyl-D,L-cysteine, NOC, and *N*-decanoyl-D,L-cysteine, NDC, are markedly affected, however, by these micelles. CTABr has the most pronounced effect on the reactivities of NOC and NDC; anation rate constants are smaller by factors of 58 and 185 than those in water. SDS decreases the rate constants for the attack of NBC and NOC on vitamin B_{12a} by factors of 6 and 2, but it does not influence the reactivity of NDC. The determined partition coefficients for the reactant between the micellar pseudo-phase and bulk water account well for the observed micellar effects. Vitamin B_{12a} and L-cysteine do not bind at all to micellar CTABr. Binding constants for the interaction of NBC, NOC, and NDC with CTABr are: 100, 2600, and 5500 M^{-1}, respectively. Micellar SDS binds vitamin B_{12a} ($K = 125\ M^{-1}$), but it does not appreciably bind any of the thiols investigated. Effects of CTABr on the interaction of NOC with vitamin B_{12a} has been satisfactorily treated in terms of equations which describe micellar effects on bimolecular reactions. Rate constants for the anation of vitamin B_{12a} by NOC and by NDC in 0.55 M dodecylammonium propionate surfactant solubilized water pools in benzene are factors of 1100- and 1400-fold faster than those of vitamin B_{12b}. In water the rate constant for anation of vitamin B_{12a} by L-cysteine is only eightfold faster than that of vitamin B_{12b}. Reversed micellar effects on anation are essentially due to concentrating the thiols in the micelle solubilized water pools. Decomposition of the vitamin B_{12} thiol complexes are, however, 3000-fold faster in the reversed micelle solubilized water pool in benzene than that in bulk water.

The bioinorganic chemistry of vitamin B_{12a} and related molecules are well documented.[1-4] Kinetic parameters for anation, governed by k_1, of vitamin B_{12a}, aquocobalamin (Bzm-Co-OH_2), and those for the aquation of the formed vitamin B_{12} complexes, Bzm-Co-L, governed by k_{-1}, have been determined for the ligands (L) N_3^-, OCN^-, SCN^-, SO_3^{2-}, NCO^-, I^-, Br^-, imidazole, and glycine:[5-11]



$$\text{Bzm-Co-OH}_2 + \text{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Bzm-Co-L} + \text{H}_2\text{O} \qquad (1)$$

Interest in vitamin B_{12} mediated enzymatic processes has prompted investigations of the interactions of thiols with aquo and alkylcobalamins.[12-17] We have recently determined rate and equilibrium constants for the interaction of L-cysteine with vitamin B_{12a} in water as a function of pH.[18] The obtained data allow us to examine the effects of surfactant aggregates or micelles on this reaction.

Surfactant aggregates in water[19] and in nonpolar solvents[20] have been utilized to mimic the microenvironments of biomacromolecular ensembles. Indeed, the effective polarity of the environment of vitamin B_{12} in dodecylammonium propionate solubilized water pools in benzene has been found to vary between those resembling water and benzene.[11] Changes in microscopic polarities depended on the concentration of the cosolubilized water, i.e., on the size of the water pool. The larger the water pool, the more closely its polarity approximated that of bulk water. Rate constants for reaction 1, using glycine, imidazole, and sodium azide as ligands, were found to be substantially and selectively affected in surfactant solubilized water pools in benzene.[11] As expected, the most pronounced rate effects were observed in the smallest water pool. Conversely, these rate constants were only slightly altered by aqueous micellar hexadecyltrimethylammonium bromide and sodium dodecyl sulfate. The rate effect in surfactant solubilized water pools in benzene is the consequence of partitioning both reactants in the restricted water pool, whose effective polarity differs from that of bulk water and wherein favorable and often concerted proton transfer and dipole–dipole interactions facilitate the reaction. Effects of aqueous micelles essentially originate in electrostatic interactions.

The purpose of the present work was to examine the effects of both solvent restrictions and aqueous micelles on the attack

Table I. Interaction of Vitamin B_{12a} with Thiols at 25.0 °C[a]

10^4[NBC], M, H_2O[b]	6.66	13.33	16.66	20.00	26.66	33.33			
$10^2 k_\Psi$, s^{-1}	2.20	3.27	3.93	4.80	6.41	7.37			
10^4[NOC], M, H_2O[b]	5.00	6.66	8.33	10.00	11.66	13.33	15.00	16.66	
$10^2 k_\Psi$, s^{-1}	2.06	2.56	3.15	4.07	4.68	5.58	5.78	6.93	
10^4[NDC], M, H_2O[b]	5.00	6.66	8.33	10.00	11.66	13.33	15.00	16.66	25.00
$10^2 k_\Psi$, s^{-1}	2.38	3.08	3.61	4.38	5.23	5.77	6.41	7.22	10.82
10^4[Cys], M, SDS[b,c]	6.66	13.33	16.67	20.00	23.33	26.66	30.00	33.33	
$10^3 k_\Psi$, s^{-1}	5.15	10.10	13.20	15.40	17.70	20.10	23.20	25.40	
10^4[NBC], M, SDS[b,c]	10.00	16.66	20.00	23.33	26.66	33.33			
$10^3 k_\Psi$, s^{-1}	3.93	6.25	7.80	8.40	9.60	11.40			
10^4[NOC], M, SDS[b,c]	5.00	6.66	8.33	10.00	11.66	13.33	15.00	16.66	
$10^2 k_\Psi$, s^{-1}	1.17	1.56	1.99	2.43	2.98	3.12	3.60	4.12	
10^4[NDC], M, SDS[b,c]	5.00	6.66	8.33	10.00	11.66	13.33	15.00	16.66	
$10^2 k_\Psi$, s^{-1}	2.25	2.65	3.98	4.20	5.09	5.25	6.24	7.53	
10^4[Cyst], M, CTABr[b,d]	6.66	13.33	16.67	20.00	26.67	30.00	33.33		
$10^3 k_\Psi$, s^{-1}	7.60	14.20	16.30	22.00	26.30	29.20	33.80		
10^3[NBC], M, CTABr[b,d]	1.00	1.33	1.66	2.00	2.66	3.33			
$10^2 k_\Psi$, s^{-1}	1.65	2.06	2.56	2.72	3.85	4.62			
10^3[NOC], M, CTABr[b,d]	1.00	1.66	2.33	3.00	3.83	5.00			
$10^4 k_\Psi$, s^{-1}	7.20	12.50	16.00	20.20	26.80	36.00			
10^3[NDC], M, CTABr[b,d]	1.00	1.66	2.33	3.00	3.66	4.33	5.00	5.00	
$10^4 k_\Psi$, s^{-1}	3.21	4.41	6.19	7.22	9.06	10.65	11.78	12.40	

[a] Abbreviations: NBC = *N*-butanoyl-L-cysteine; NOC = *N*-octanoyl-L-cysteine; NDC = *N*-decanoyl-L-cysteine; CTABr = hexadecyltrimethylammonium bromide; SDS = sodium dodecyl sulfate; Cys = L-cysteine. [b] Stoichiometric $[B_{12a}] = 5 \times 10^{-5}$, 0.10 M CH_3COONa buffer, pH 5.5. [c] [SDS] = 0.10 M. [d] [CTABr] = 0.10 M.

of L-cysteine and *N*-alkanoylcysteines on vitamin B_{12a}. We report substantial micellar effects in both aqueous and reversed micellar systems.

Experimental Section

The best available grade of vitamin B_{12a}, aquocobalamin (Merck), was used as received. Its purity was established by spectrophotometry and by obtaining rate constants for ligand exchange reactions in water identical with those reported previously (vide infra).[10] Preparation and purification of dodecylammonium propionate (DAP), sodium di-(2-ethylhexyl)sulfosuccinate (Aerosol-OT), hexadecyltrimethylammonium bromide (CTABr), and sodium dodecyl sulfate (SDS) have been described.[19]

Aqueous solutions were prepared in doubly distilled water. The pH was determined by a Radiometer PHM-26 instrument. Reagent grade benzene was distilled from sodium and stored over Linde 5A molecular sieves. Stock solutions of vitamin B_{12a} [usually $2–8 \times 10^{-3}$ M] were made up in water and stored in the refrigerator. Individual solutions for spectral and kinetic determinations were prepared by injecting appropriate volumes of the aqueous vitamin B_{12a} stock solutions to benzene solutions of the surfactants. Final concentrations of vitamin B_{12a} ranged between 1×10^{-5} and 5×10^{-5} M. Water concentrations were carefully controlled and monitored by gas-liquid partition chromatography using a Poropak Q column.[21]

L-Cysteine and D-cysteine were purchased from Sigma Chemical Co. and used without further purification.

N-Octanoyl-D,L-cysteine and *N*-decanoyl-D,L-cysteine were synthesized as already described.[22] *N*-Butanoyl-L-cysteine was prepared from *S*-benzyl-L-cysteine methyl ester HCl according to the following procedure. *S*-Benzyl-L-cysteine methyl ester HCl (0.02 mol) was dissolved in 100 ml of $CHCl_3$ containing 0.05 mol of triethylamine followed by the dropwise addition of 0.04 mol of butanoyl chloride. The reaction mixture was refluxed for 3 h. Ethanol (0.04 mol) was then added and the solution was further refluxed for 30 min. All the fractions below 70 °C were distilled and finally rotatory evaporated to dryness. The reaction products were extracted with CCl_4 in a Soxhlet apparatus, rotatory evaporated, and the liquid residue poured on an alumina column (one-third cationotropic, two-thirds anionotropic). $CHCl_3$ was used to elute the product from the column. The first fraction was collected and rotatory evaporated to dryness. The product was identified with IR and NMR as *N*-butanoyl-*S*-benzyl-L-cysteine methyl ester. This ester was mixed with 20 ml of 10^{-3} M NaOH and was allowed to react at room temperature for 12 h. The solution was acidified to pH 2 with HCl and the precipitate was filtered, washed with ice-cold water, and dried in vacuo over P_2O_5. IR and NMR showed that *S*-benzyl and *N*-butanoyl functionalities are retained, while methyl ester had been removed. *N*-Butanoyl-*S*-benzyl-L-cysteine (1.0 g) was dissolved in 50 ml of liquid ammonia and reduced with sodium. The ammonia was allowed to evaporate and the residue dissolved in dry ethanol and carefully neutralized with 6 N aqueous HCl to pH 5 or 6. The solution was filtered, and *N*-butanoyl-L-cysteine(NH_4) salt was crystallized from ethanol. The reaction product was identified by IR, NMR, elemental analysis for C, H, and N, and titration of the *SH* with 2-chloromercury-4-nitrophenol (Eastman). All other chemicals used were the best available reagent grade.

Spectrophotometric determinations were carried out using a Cary 118-C spectrophotometer. The complete spectral range was recorded, generally, on the 0–1.0 *A* scale at a speed of 10 nm/in. and 0.2 nm/s. Kinetic data were obtained on either the Cary 118-C or the Beckman Kintrac VII spectrophotometer. The faster runs were followed by a Durrum Model 110 stop-flow spectrophotometer. Temperatures for the kinetic runs were maintained at 25.0 ± 0.1 °C by water circulation. Individual pseudo-first-order rate constants, k_Ψ values, were obtained from linear plots of $\log(A_\infty - A_t)$ vs. time. All of these plots were linear for at least 90% of the reaction.

Partition coefficients, *K* values, were determined by gel filtration on Sephadex G-25.[23] Typically, 1.95 × 32 cm column with a total volume, V_t, of 96 cm^3 was employed. Using Blue Dextran 2000, the void volume, V_0, of the packed column was determined to be 40.6 cm^3. The imbibed volume, V_i, was calculated to be 65 cm^3 using tritiated water.[24] Prior to each run the column was equilibrated with distilled water or with the appropriate detergent solution. Runs were initiated by the addition of approximately 0.5 ml of 10^{-3} M thiol or vitamin B_{12a} to the column. Elution with the appropriate surfactant concentrations followed at a rate of 0.5 cm^3/min. Fractions of 1.0 cm^3 were collected by means of an automatic fraction collector and the absorbance, at the appropriate wavelength, of each fraction was determined manually in the thermostated cell compartment of a Cary 118-C spectrophotometer. The elution volume, V_e, was calculated from the maximum absorbance of the emerging vitamin B_{12a} at 350 nm. The thiols were reacted with Ellman's reagents (5,5′-dithiobis(2-nitrobenzoic acid))[25] and absorbances of the product were determined at 412 nm. In the determination of the imbibed volume, V_i, the radioactivity of each sample was determined by a Beckman LS-100 liquid scintillation system.

Results

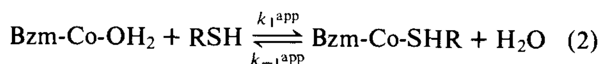
Addition of excess L-cysteine, D-cysteine, *N*-butanoyl-L-cysteine (NBC), *N*-octanoyl-D,L-cysteine (NOC), and *N*-decanoyl-D,L-cysteine (NDC) to an aqueous buffered solution of vitamin B_{12a} resulted in marked alteration of the absorption

Table II. Interaction of Vitamin B_{12a} with *N*-Octanoyl-D,L-cysteine in Aqueous CTABr[a] at 25.0 °C

	10^2k_Ψ, s^{-1}					
10^4[CTABr], M	5.0×10^{-4} M NOC	1.0×10^{-3} M NOC	1.5×10^{-3} M NOC	2.0×10^{-3} M NOC	2.5×10^{-3} M NOC	k_1^{app}, M^{-1} s^{-1}
0.0[b]						41.6
5.00	2.37	4.44	6.93	9.36	11.6	46.2
7.00	2.31	4.52	7.00	9.41	11.4	46.1
20.00	1.25	2.46	3.63	5.00	6.07	24.6
30.00	0.88	1.63	2.27	3.85	4.62	17.4
50.0	0.54	1.01	1.57	1.92	2.84	10.5
70.00	0.29	0.60	0.91	1.23	1.46	5.98
100.00	0.15	0.29	0.43	0.58	0.72	2.89
250.00	0.070	0.14	0.21	0.28	0.36	1.42
1000.00[b]						0.71

[a] In the presence of 0.10 M sodium acetate at pH 5.5. [b] See Table I.

spectra. The spectra of the products formed had absorption maxima at 370, 532, and 552 nm. These same maxima occurred in both aqueous and nonaqueous micellar solutions and were identical with those observed for vitamin B_{12} thiol complexes.[17,18] Development of absorbances at 370 and 552 nm at the expense of absorbances at 495 and 525 nm was dependent upon the thiol concentration in *all* systems. Increasing concentrations of thiol resulted in increased absorbance changes up to a point, above which the absorbances remained constant. These observations indicate, of course, the equilibrium formation of vitamin B_{12} thiol complexes:



$$\text{Bzm-Co-OH}_2 + \text{RSH} \underset{k_{-1}^{app}}{\overset{k_1^{app}}{\rightleftharpoons}} \text{Bzm-Co-SHR} + \text{H}_2\text{O} \quad (2)$$

where Bzm-Co-OH_2 and Bzm-Co-SHR represent vitamin B_{12a} and its thiol complex, respectively; k_1^{app} and k_{-1}^{app} are the apparent second-order and first-order rate constants for the formation and decomposition of the vitamin B_{12} thiol complex. Under favorable conditions ([vitamin B_{12a}] $\ll$ [RSH]), the pseudo-first-order rate constant for the attainment of equilibrium 2, k_Ψ, could be determined from absorbance changes at 350 nm as a function of time. Values for k_Ψ in water and in aqueous micellar CTABr and SDS are given in Table I. Table II presents kinetic data for the interaction of *N*-octanoyl-D,L-cysteine with vitamin B_{12a} in water as a function of CTABr concentration. Rate constants for the equilibrium attainment of the vitamin B_{12} thiol formation, k_Ψ, are related to k_1^{app} and k_{-1}^{app} by the equation:

$$k_\Psi = k_1^{app}[\text{RSH}] + k_{-1}^{app} \quad (3)$$

Plots of k_Ψ vs. thiol concentration gave good straight lines whose slopes and intercepts allowed the calculation of anation, k_1^{app}, and aquation, k_{-1}^{app}, rate constants. Equilibrium constants for the vitamin B_{12}–thiol complex formation, K^{app}, were obtained from k_1^{app}/k_{-1}^{app}. Since the anation rate of vitamin B_{12a} by L-cysteine is independent on the hydrogen ion concentration in the pH 3–6.5 region,[18] k_1^{app} and k_{-1}^{app} in micellar solutions were determined at pH 5.5. Under these conditions, k_Ψ values remained the same upon degassing. Apparent rate and equilibrium constants for the interaction of L-cysteine, *N*-butanoyl-L-cysteine, *N*-octanoyl-D,L-cysteine, and *N*-decanoyl-D,L-cysteine in water and in aqueous micellar CTABr and SDS are given in Table III.

Binding constants between the substrates and micellar surfactants were determined as partition coefficients by gel filtration on Sephadex G-25 (see Experimental Section). It is well known that solutes with molecular weight greater than 4000–5000 are excluded from Sephadex G-25.[26] The aggregated surfactant moves on Sephadex G-25, therefore, just like any macromolecule. If the column is equilibrated with detergent solutions at concentrations well above the critical micelle concentration, the movement of any additional solutes with molecular weight smaller than 4000–5000 will depend on the partition coefficient of this solute between the bulk aqueous and micellar pseudo-phases.[27] Equation 4 relates quantitatively the observed elution volume with the partitioning coefficient, K:

Table III. Rate and Equilibrium Constants for the Interaction of Vitamin B_{12a} with Thiols

Medium	Thiol	k_1^{app}, M^{-1} s^{-1}	k_{-1}^{app}, s^{-1}	K^{app}, M^{-1}
H_2O	Cys[a]	11.50	5.22×10^{-5}	2.2×10^5
	NBC	20.30	7.19×10^{-3}	2.8×10^3
	NOC	41.60	1.52×10^{-3}	2.7×10^4
	NDC	42.20	1.98×10^{-3}	2.1×10^4
0.10 M SDS	Cys	7.60	1.78×10^{-4}	4.3×10^4
	NBC	3.21	9.60×10^{-4}	3.3×10^3
	NOC	24.80	7.00×10^{-4}	3.5×10^4
	NDC	42.50	4.80×10^{-4}	8.9×10^4
0.10 M CTABr	Cys	9.56	1.33×10^{-3}	7.2×10^3
	NBC	12.90	3.44×10^{-3}	3.7×10^3
	NOC	0.71	4.60×10^{-5}	1.5×10^4
	NDC	0.23	7.90×10^{-5}	2.8×10^3

[a] Taken from ref 18.

$$\frac{V_i}{V_e - V_0} = \frac{\bar{v}(K-1)}{k'K_d}[\text{M}] + \frac{1}{k'K_d} \quad (4)$$

where V_i, V_e, and V_0 are the imbibed (stationary), elution, and void volumes, respectively, $\bar{v}$ is the partial specific volume of the detergent molecule in the micelle and it is assumed to be 0.995 for CTABr[28] and 0.91 for SDS,[29] K_d is the "molecular sieving" constant and is equal to the ratio of the solute concentration in imbibed liquid to the concentration in the nonmicellar portion of the external liquid, k' is the proportionality constant between the solute absorbed per unit volume of gel matrix and equilibrium concentration of the monomer solute in liquid, and [M] is the concentration of the micelles ([M] = C_D − cmc) where C_D is the stoichiometric surfactant concentration and cmc is the critical micelle concentration. In the absence of any micellar and adsorption effects eq 4 reduces to the usual gel filtration equation:

$$V_i/(V_e - V_0) = 1/K_D \quad (5)$$

Table IV gives the values of $V_i/(V_e - V_0)$ at different concentrations of surfactants and K values calculated from eq 4. The data are plotted in Figures 1 and 2. As expected, at low surfactant concentrations eq 5 is obeyed and values of $V_i/(V_e$

Table IV. Gel Filtration Parameters

		$V_i/(V_e - V_0)$								
	H_2O	5.0×10^{-4} M CTABr	1.0×10^{-3} M CTABr	2.0×10^{-3} M CTABr	2.5×10^{-3} M CTABr	3.0×10^{-3} M CTABr	5.0×10^{-3} M CTABr	10^{-2} M CTABr	2.0×10^{-2} M CTABr	K, M^{-1}
B_{12a}	2.13							1.70	1.71	0
Cys	1.53							1.26	1.25	0
NBC	1.37	1.40					1.85	2.62	3.84	100
NOC	1.31	1.32	1.34	2.10	3.20	6.70	10.2	10.2	10.2	2600
NDC	1.30	1.32	1.32	3.51	7.50	10.20	10.20	10.20	10.20	5500

		$V_i/(V_e - V_0)$					
	H_2O	5.0×10^{-4} M SDS	5.0×10^{-3} M SDS	2.0×10^{-2} M SDS	6.0×10^{-2} M SDS	10×10^{-2} M SDS	K, M^{-1}
B_{12a}	2.13	2.14	3.96	6.91	16.6		125
Cys	1.53	1.52		1.46		1.40	0
NBC	1.37	1.37		1.49	1.56	1.65	3
NOC	1.31	1.31		1.49	1.65	2.10	8
NDC	1.30	1.31		1.65	2.13	2.78	13

Table V. Interactions of Vitamin B_{12b} with Thiols in 0.10 M DAP Entrapped Water Pools in Benzene[a]

10^4[NOC], M	2.66	4.00	4.66	5.66	6.00	6.60	
$10^3 k_\psi$, s^{-1}	4.44	5.96	6.67	7.64	7.90	8.32	
k_1^{app}, M^{-1} s^{-1}	9.75						
$10^3 k_{-1}^{app}$, s^{-1}	2.00						
K^{app}, M^{-1}	4.8×10^3						
10^4[NDC], M	2.00	2.66	3.00	3.66	4.33	4.66	6.00
$10^3 k_\psi$, s^{-1}	2.88	3.28	3.49	3.86	4.48	4.70	5.54
k_1^{app}, M^- s^{-1}	6.91						
$10^3 k_{-1}^{app}$, s^{-1}	1.45						
K^{app}, M^{-1}	4.8×10^3						

[a] At 25.0 °C, containing 0.55 M H_2O; [Vitamin B_{12b}] = 7.0×10^{-6} M.

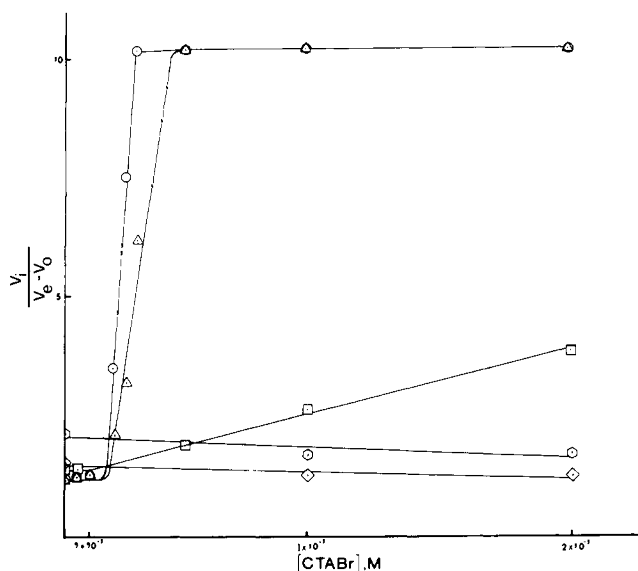


Figure 1. Plots of $V_i/(V_e - V_0)$ vs. (CTABr) concentration for vitamin B_{12a} (○); L-cysteine (◇); NBC (⊡); NOC (△); and NDC (⊙).

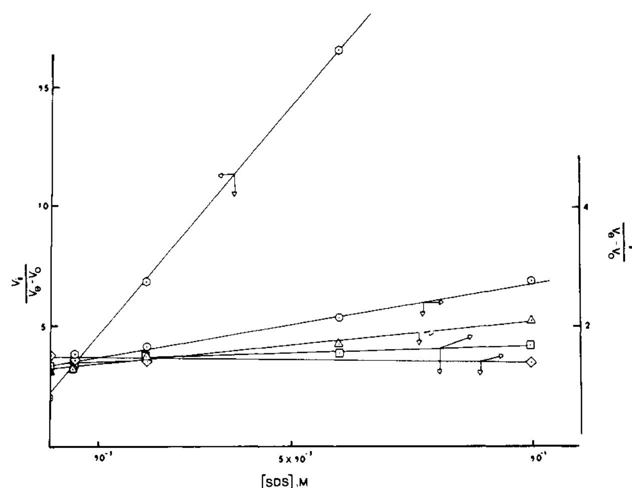


Figure 2. Plots of $V_i/(V_e - V_0)$ vs. SDS concentration for vitamin B_{12a} (○); L-cysteine (⊡); NBC (⊡); NOC (△); and NDC (⊙).

$- V_0)$ remain independent of the concentration of the detergent. Above the cmc, however, the data are governed by eq 4 and values of $V_i/(V_e - V_0)$ increase linearly with increasing micelle concentrations.

Intersections of the lines in Figure 1 define critical micelle concentration of CTABr in the presence of solutes and Sephadex G-25. The critical micelle concentration is up to three times greater than that determined in water.[29] Such discrepancy may arise from effects of the solutes or of the gel or indeed of both on the critical micelle concentration of CTABr. Even at a relatively low concentration of CTABr, constant values of $V_i/(V_e - V_0)$ are reached for NOC and NDC (Figure 1) as expected for substrates which bind strongly to the micelle (Table IV). Conversely, micellar SDS binds rather poorly to NBC, NOC, and NDC (Figure 2). Consequently, it was not possible to assess critical micelle concentrations from these plots.

Vitamin B_{12a} has previously been observed to be solubilized in benzene by dodecylammonium propionate aggregates.[11]

Table VI. Interaction of Vitamin B_{12a} with Thiols in 0.10 M DAP Entrapped Water Pools in Benzene[a]

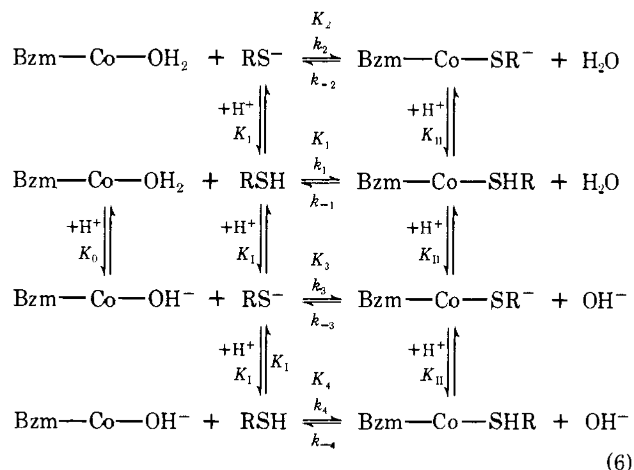
10^4[L-cysteine], M	2.50	5.00	7.50	10.0	12.5	
k_Ψ, s^{-1}	1.60	2.95	4.20	5.70	6.85	
k_1^{app}, $M^{-1}\,s^{-1}$	5.4×10^3					
k_{-1}^{app}, s^{-1}	0.25					
K^{app}, M^{-1}	2.16×10^4					
10^4[D-cysteine], M	2.50	5.00	7.50	10.0	12.5	
k_Ψ, s^{-1}	1.82	3.46	4.85	6.10	7.56	
k_1^{app}, M^{-1}	5.7×10^3					
k_{-1}^{app}, s^{-1}	0.30					
K^{app}, M^{-1}	0.9×10^4					
10^4[NOC], M	5.0	10.0	15.0	24.2	30.0	35.2
k_Ψ, s^{-1}	12.6	15.4	20.4	29.3	36.5	44.7
k_1^{app}, $M^{-1}\,s^{-1}$		1.07×10^4				
k_{-1}^{app}, s^{-1}		5.0				
K^{app}, M^{-1}		2.14×10^3				
10^4[NDC], M	5.0	10.0	15.0	24.2	30.0	35.2
k_Ψ, s^{-1}	12.4	15.1	21.0	28.7	35.0	43.1
k_1^{app}, $M^{-}\,s^{-1}$		9.94×10^3				
k_{-1}^{app}, s^{-1}		6.0				
K^{app}, M^{-1}		1.66×10^3				

[a] At 25.0 °C, containing 0.55 M H_2O and 0.082 M $HClO_4$; [Vitamin B_{12a}] = 7.0×10^{-6} M.

Here the corrinoid is localized in the surfactant entrapped water pool. Indeed, its microscopic polarity depends markedly on the size of this water pool.[11] In 0.55 M water solubilized by 0.10 M DAP in benzene, vitamin B_{12a}, in the absence of any additives, immediately dissociates to hydroxocobalamin, vitamin B_{12b}. In this environment, the interaction of NOC and NDC with vitamin B_{12b} has been kinetically examined (Table V). Unfortunately, lack of solubilites precluded examination of the interaction of vitamin B_{12b} with L-cysteine in this micellar system. Addition of $HClO_4$ does not only shift the equilibrium in favor of vitamin B_{12a},[30] but allows the solubilization of L-cysteine in 0.55 M H_2O pools entrapped by 0.10 M DAP in benzene. Table VI contains the kinetic data for the interaction of D-cysteine, L-cysteine, NOC, and NDC with vitamin B_{12a} in reversed micellar DAP. No difference was observed, within experimental error, between the reactivities of D- and L-cysteine.

Discussion

Interactions in Water. Interactions of thiols with vitamin B_{12} are given by eq 6,[18] where K_0, K_I, and K_{II} are the dissociation constants for vitamin B_{12a}, the thiol, and the vitamin B_{12} thiol complex, respectively;



(6)

k_1, k_2, k_3, and k_4 are the pH independent anation and k_{-1}, k_{-2}, k_{-3}, and k_{-4} are the pH independent aquation rate constants. Equation 7 describes the observed rate constant for anation, k_1^{app}, at any given pH values:[18]

$$k_1^{app} = \frac{k_1}{1 + (K_0/[H^+])}\left(\frac{[RSH]}{[RSH] + [RS^-]}\right) + \frac{k_2}{1 + (K_0/[H^+])}\left(\frac{[RS^-]}{[RSH] + [RS^-]}\right) + \frac{k_3}{1 + ([H^+]/K_0)} \times \left(\frac{[RS^-]}{[RSH] + [RS^-]}\right) + \frac{k_4}{1 + ([H^+]/K_0)}\left(\frac{[RSH]}{[RSH] + [RS^-]}\right) \quad (7)$$

Values for pK_0 and pK_{II} have been determined to be 7.60[1] and 10.9.[18] Although the sites of protonation of L-cysteine are not known with certainty,[31,32] the seven microscopic dissociation constants for the pH dependence of the concentrations of the eight species have been calculated.[32,33] These dissociation constants allow the assessment of the contributions of the different reacting species at any pH to the observed rate. Under the present experimental conditions, pH 5.5, 99% of the observed anation rate is due to k_1.[18]

Rate constants for the interaction of thiols with vitamin B_{12a} increase with increasing chain length of the thiols (Table III). It should be pointed out that the concentration range of the long chain thiols used (Table I) are well below their cmc.[22] These thiols are, therefore, present as monomers. The observed similarities in the elution volumes of L-cysteine, NBC, NOC, and NDC in water (Table IV) substantiates this postulate. Rate enhancements are not, therefore, explicable in terms of regular micellar effects. Hydrophobic effects between the corrinoid ring and presumably the long chain thiols, to be present as premicellar aggregates, are likely to be responsible for this modest rate enhancement.

Interactions in Aqueous Micelles. Rate constants for the formation of vitamin B_{12}-L-cysteine complexes are slightly retarded both by micellar CTABr and SDS. The effect of the anionic surfactant is more pronounced, however (Table III). Similar micellar effects have been observed in the ligand exchange reactions between vitamin B_{12a} and sodium azide, imidazole, and glycine.[11] In order to assess hydrophobic interactions, reactions of long chain *N*-alkanoylcysteines with vitamin B_{12a} have been investigated in aqueous micellar CTABr and SDS (Table III). The rate constant for the anation of vitamin B_{12a} by NBC is smaller than that by L-cysteine in micellar SDS. Conversely, in micellar CTABr k_1^{app} is greater for NBC than for L-cysteine. Higher chain length *N*-alkanoylcysteines reverse the trend. This behavior is illustrated in Figure 3. The observed kinetic behavior can be rationalized

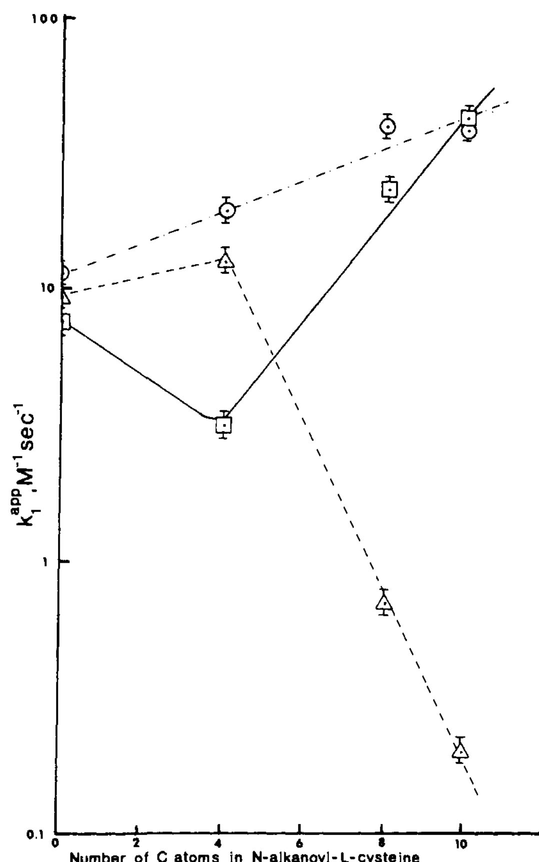


Figure 3. Plots of second-order rate constants for the anation of vitamin B_{12a} by thiols in H_2O (⊙), in 0.10 M SDS (⊡), and 0.10 M CTABr (▲) as a function of the alkanoyl chain length.

in terms of the determined binding constants between the substrates and the micelles (Table IV and Figures 1 and 2). It is seen that cationic micellar CTABr does not bind to vitamin B_{12a} or to L-cysteine. Lack of micellar effect on the ligand exchange reaction between vitamin B_{12} and L-cysteine in this surfactant is, therefore, not unexpected. Similarly, the meager binding of NBC to CTABr (Table IV) is entirely consistent with the observed modest rate decrease for the interaction of this thiol with vitamin B_{12a} in the presence of the cationic micellar surfactant. Increases in the binding constants between the longer thiols and vitamin B_{12a} are paralleled with the observed rate retardation (Tables III and IV). Indeed the attack of NDC on aquocobalamin is some 185-fold slower in micellar CTABr than that in water. Since vitamin B_{12a} does not partition into micellar CTABr, it is possible to calculate a value for the free energy of transfer of a methylene group from the bulk aqueous phase to the micelle from the relative reactivities of these thiols. The obtained value, 420 cal/CH_2, is in good agreement with that determined from other kinetic data.[36]

Unlike CTABr, micellar SDS does bind to vitamin B_{12a} (Table IV). This modest binding is explicable in terms of electrostatic attraction between the proposed net positive charge on vitamin B_{12a}[37] and the negatively charged micellar surface. It is envisaged that the benzimidazole moiety is in the environment of the first few CH_2 groups next to the surfactant headgroup and that the water in the sixth coordination position is in contact with and is hydrated by the water in the Gouy–Chapman electrical double layer. Figure 4 illustrates an oversimplified model of the proposed interaction between micellar SDS and vitamin B_{12a}. The observed rate inhibition for the reaction of methylcobalamin with metal halides by micellar SDS[38] is in accord with the proposed model. The fact that the value of K_0 (eq 6) is unaffected by micellar surfactants[11] also substantiates this postulate. The long chain thiols do not bind

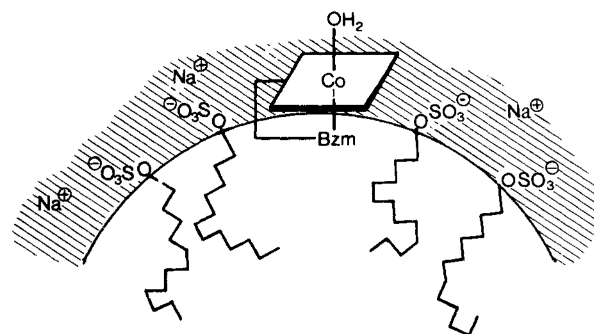


Figure 4. An oversimplified model indicating the proposed interaction of vitamin B_{12a} with micellar SDS.

appreciably to micellar SDS (Table IV). Binding of all substrates to these micelles can be satisfactorily rationalized in terms of electrostatic and hydrophobic interactions. The positively charged aquocobalamin is attracted by the negatively charged SDS micelle, but it is repelled by the positively charged CTABr micelle. The negatively charged long chain thiols are repelled by SDS, but they are attracted by CTABr. The more hydrophobic the thiols are, the greater the binding to micellar CTABr. Comicellization is not excluded.

Considerable advances have been made recently in the quantitative treatments of micellar "catalysis" and "inhibition" in aqueous solutions.[19,34,35] Using a pseudo-phase model Berezin and his co-workers[19] derived equations for uni- and bimolecular reactions in micellar solutions. These equations fitted a large number of experimental data. Romsted proposed a modified treatment for micellar effects on the reactions of neutral molecules and small hydrophilic ions.[34] In his treatment, micelles are considered as a separate but uniformly distributed phase in water. Significantly, the concentration of the counterion in the Stern layer is considered to remain constant. Although this latter treatment is more powerful, unfortunately it is not applicable to the present reaction, since it involves a bimolecular reaction between a large organic substrate and large hydrophobic ions. Our data could, however, be satisfactorily rationalized by Berezin's treatment.[19]

Equation 8 is the most general form to account for micellar effects on bimolecular reactions:[19]

$$k_1^{app} = \frac{k_m P_{Bzm\text{-}Co\text{-}OH_2} P_{RSH} MV + k_w(1 - MV)}{(1 + K_{Bzm\text{-}Co\text{-}OH_2} M)(1 + K_{RSH} M)} \qquad (8)$$

where k_m and k_w are rate constants in the micellar and aqueous bulk phases, $P_{Bzm\text{-}Co\text{-}OH_2}$ and P_{RSH} are partition coefficients of vitamin B_{12a} and the thiol between the micellar pseudophase and water, $K_{Bzm\text{-}Co\text{-}OH_2}$ and K_{RSH} are binding constants of vitamin B_{12a} and the thiol to the micelle, M is the concentration of the micellar surfactant, and V is the molar volume of the surfactant.

The utility of eq 8 has been tested for the interaction of vitamin B_{12a} with NOC in aqueous micellar CTABr (see Table II). Since vitamin B_{12a} does not bind to CTABr (Table IV) $P_{Bzm\text{-}Co\text{-}OH_2}$ and $K_{Bzm\text{-}Co\text{-}OH_2}$ can be equated to zero. Thus eq 8 simplifies to:

$$k_1^{app} = \frac{k_w(1 - MV)}{1 + K_{RSH} M} \qquad (9)$$

Further, at low micelle concentrations the micellar volume is negligible compared to the volume of water (i.e., $(1 - MV) \simeq 1$). Hence, eq 9 reduces to:

$$k_1^{app} = \frac{k_w}{1 + K_{RSH} M} \qquad (10)$$

Figure 5 illustrates the satisfactory agreement between experimental k_1^{app} values and those calculated by eq 10 using

Table VII. Reversed Micellar Effects on Kinetic and Thermodynamic Parameter for the Interaction of Thiols with Vitamin B_{12a}

	L-Cysteine	NOC	NDC
$\frac{k_1^{app} \text{ in DAP/benzene/H}_2\text{O}}{k_1^{app} \text{ in H}_2\text{O}}$	470	258	236
$\frac{k_{-1}^{app} \text{ in DAP/benzene/H}_2\text{O}}{k_{-1}^{app} \text{ in H}_2\text{O}}$	4789	3289	3030
$\frac{K^{app} \text{ in DAP/benzene/H}_2\text{O}}{K^{app} \text{ in H}_2\text{O}}$	0.10	0.08	0.08

$K_{RSH} = 830\ M^{-1}$ and $k_w = 46\ M^{-1}\ s^{-1}$. The discrepancy between the theoretical and experimentally determined binding constant is the expected result of the deviation from ideal behavior of CTABr on the Sephadex gel.[27] Additionally, differences in the NOC concentrations between the kinetic and gel filtration experiments may alter the aggregation behavior of CTABr.

Interactions in Reversed Micelles. Although completely insoluble in benzene, vitamin B_{12a} is solubilized by dodecylammonium propionate aggregates.[11] In this environment, one cobalamin molecule, dissolved in a 0.55 M water pool, is surrounded by some 300 surfactant molecules. The apparent microscopic environment of vitamin B_{12a} in the water pool is only slightly less polar than that in water.[11] Unfortunately, solubility problems precluded the investigation of the effects of changes of cosolubilized water concentration on the rates of anation or aquation. Under our experimental conditions, there is, in fact, less than one molecule of vitamin B_{12a} per surfactant aggregate.

The most striking observation is the substantial difference between the reactivities of vitamin B_{12a} and vitamin B_{12b} and their thiol complexes (Tables V and VI). Rate constants for the anation of vitamin B_{12a} in this reversed micelle by NOC and by NDC are some 1100- and 1400-fold faster than those of vitamin B_{12b}. Conversely, L-cysteine reacts with vitamin B_{12a} only some eightfold faster than with vitamin B_{12b}.[18] Similar differences exist for the aquation rates. Lack of information on K_{11} in the reverse system does not allow, however, numerical assessment of the differential reactivities of Bzm-Co-RSH and Bzm-Co-RS$^-$ complexes.

Table VII summarizes the reactivities of vitamin B_{12a} and its thiol complexes in the DAP entrapped water pool relative to those in bulk water. It should be pointed out that rate constants presented in Tables V and VI and hence the relative rates in Table VII had been calculated by using the stoichiometric concentration of thiols. If it is assumed that all the thiols are localized in the surfactant entrapped water pool, their effective concentration increases by 100-fold. Consequently, k_1^{app} for L-cysteine, NOC, and NDC are only factors of 4.7, 2.6, and 2.4 greater than those in bulk water (Table VII). Considerably larger effects had previously been observed in *smaller* water pools for the interaction of glycine with vitamin B_{12a}.[11] Such small water pools unfortunately could not be employed in the present study (vide supra).

Decomposition of the vitamin B_{12} thiol complexes is a unimolecular process. Assuming that the relatively high concentration of the uncomplexed thiol does not exert too great a salt effect, there are enhancements of k_{-1}^{app} in the reversed micellar environment in excess of 3000-fold (Table VII). Here, of course, no concentration effects need to be considered. it is more difficult to comment on the effects of reversed micelles on stability constants for the vitamin B_{12} thiol complex formation, since they are influenced by composite sets of parameters.

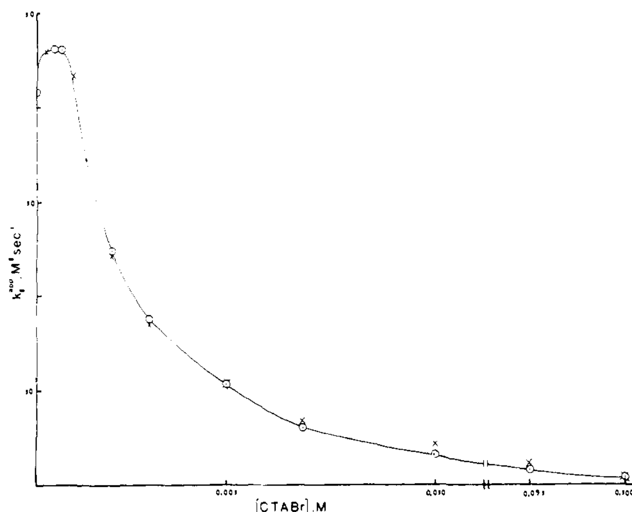


Figure 5. A plot of second-order rate constants for the anation of vitamin B_{12a} by NOC as a function of CTABr concentration. Experimental values are indicated by ⊙ and those calculated from eq 10 are given by ×.

Effects of reversed micelles on these reactions originate in possible alterations of the ionization equilibria of the different species, hydrogen bonding, dipole-dipole interactions, and changes in the effective concentration and activities of the reactants.

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Synthesis of Specific Deuterium Labeled Tyrosine and Phenylalanine Derivatives and Their Use in the Total Synthesis of [8-Arginine]vasopressin Derivatives: The Separation of Diastereomeric [8-Arginine]vasopressin Derivatives by Partition Chromatography[1,2]

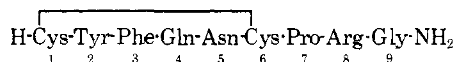
Diane M. Yamamoto,[3] Donald A. Upson,[4] David K. Linn, and Victor J. Hruby*

Contribution from the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received August 23, 1976*

Abstract: Derivatives of tyrosine specifically deuterated at the α carbon ([α-$^2H_1$]tyrosine) and at both the α and β carbons ([α,β,β-$^2H_3$]tyrosine) and a derivative of phenylalanine specifically deuterated at the α carbon ([α-$^2H_1$]phenylalanine) have been synthesized in high yield. These labeled compounds have been resolved enzymatically, and the enantiomers and racemates have been converted to *N-tert*-butyloxycarbonyl derivatives. The deuterium labels were not exchanged under the conditions of the syntheses. The protected derivatives as well as specifically deuterated derivatives of *S*-benzylcysteine and of glycine were used to prepare specifically deuterated analogues of [8-arginine]vasopressin using solid phase peptide procedures. The use of improved synthetic procedures resulted in considerable improvements in the yields of [8-arginine]vasopressin compared with previous reports. In addition, new solvent systems for partition chromatography purification of [8-arginine]vasopressin on Sephadex were developed which allowed a facile one-step separation of diastereomers of [8-arginine]vasopressin containing a racemic amino acid at either the 1-hemicystine or the 2-tyrosine positions of the hormone. The following specifically deuterated hormone derivatives were synthesized: [9-[α,α-$^2H_2$]glycinamide,8-arginine]vasopressin (**15**), [1-hemi-[α-$^2H_1$]cystine,8-arginine]vasopressin (**21**), [1-hemi[β,β-$^2H_2$]cystine,8-arginine]vasopressin (**17**), [2-[α-$^2H_1$]tyrosine,8-arginine]vasopressin (**19a**), [2-[α,β,β-$^2H_3$]tyrosine,8-arginine]vasopressin (**20**), [3-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin (**18a**), [1-hemi-D-[α-$^2H_1$]cystine,8-arginine]vasopressin (**16**), [2-D-[α-$^2H_1$]tyrosine,8-arginine]vasopressin (**19b**), [1-hemi-D-cystine,3-[α-$^2H_1$]phenylalanine,8-arginine]vasopressin (**18b**).

The use of fully deuterated and specifically deuterated amino acids, peptides, and proteins for a variety of physical-chemical[5-14] and biological[15-17] studies has become increasingly prevalent during the past several years. However, the limited availability of these compounds is still a serious limitation to their use. The principal source of deuterated amino acids has been proteins obtained from algae grown in D_2O solutions.[6a,b,e] In general, the amino acids obtained from these sources are perdeuterated in all nonexchangeable positions. Furthermore a number of amino acids are not obtained or are obtained in small quantities. In addition, for many purposes it is necessary to have available specific partially deuterated derivatives. For the latter purposes, simple synthetic methods in which the appropriate deuterium label is retained throughout to give the desired labeled derivative are needed. Some examples of the latter have appeared in the literature[15-19] but much remains to be done.

In this paper we report simple, high-yield syntheses of [α-$^2H_1$]tyrosine, [α,β,β-$^2H_3$]tyrosine, and [α-$^2H_1$]phenylalanine, resolution of the labeled enantiomers, and incorporation of these derivatives and specific deuterium labeled *S*-benzylcysteine and glycine into [8-arginine]vasopressin. In addition, we report the development of a solvent system for partition chromatography which permits separation of [8-arginine]-vasopressin (AVP) diastereomers by partition chromatography on Sephadex and the use of recent improvements in synthetic peptide chemistry which substantially improve the overall yields of AVP from those previously reported in the literature.



The syntheses of DL-[α-$^2H_1$]tyrosine (**5**) and DL-[α,β,β-$^2H_3$]tyrosine (**6**) were accomplished by the procedures outlined in Scheme I. To obtain **5**, methyl *p*-anisate (**1**) was treated with lithium aluminum hydride,[20] and the alcohol **2** was converted to **3** with hydrobromic acid. The benzyl bromide **3** was reacted with sodium diethyl acetamidomalonate in ethanol to give the condensation product **4** which was hydrolyzed with deuterium bromide to give DL-[α-$^2H_1$]tyrosine (**5**). To obtain **6**, lithium aluminum deuteride was used in the first step of the scheme. The overall yield of **5** and **6** from **1** was about 65%.

The deuterated DL-tyrosine derivatives **5** and **6** were readily resolved into their enantiomers without loss of label by *N*-trifluoroacetylation,[21a] followed by reaction with carboxypeptidase A-DFP.[21b] The resulting deuterated L-amino acids (or the D- or DL-amino acids) could be readily converted to *N-tert*-butyloxycarbonyl (*N*-Boc) derivatives using the general method employing *tert*-butyl azidoformate.[22] No detectable loss of label occurred during either the resolution or the preparation of the *N*-Boc derivatives.