Preparation and Reactivity of Model Compounds Related to Oligomers from Thiamin. A Mechanism for Oligomerization

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Abstract: Compounds were synthesized having a pyrimidinylmethyl group as found in oligomers formed from thiamin. These compounds contain two such groups and a thiazole, phenylthio, or sulfonato substituent at the terminal methylene position and undergo nucleophilic substitution with aqueous sulfite ion. Comparison of the rate constants for reaction with sulfite ion involving a bis(pyrimidine) and 1′-methylthiaminium ion, both having the same thiazole leaving group, reveals that an *N*-pyrimidinylmethyl substituent increases reactivity over that by an *N*-methyl group by a factor of 19. Other comparisons show that a thiazole is replaced about 1.9 times faster than a pyrimidine ring by the action of sulfite ion. Additives can increase or decrease the induction period for the oligomerization of thiamin chloride in methanol. A mechanism similar to that for sulfite ion substitution is suggested for oligomerization; a key feature is the conversion of thiamin to a more reactive oligomer by quaternization which introduces a pyrimidinylmethyl unit.

Protonated thiamin (vitamin B_1) is stable in solution at ambient temperatures.[1] But thiamin base (Ia) can spontaneously decompose. Massive decomposition of a 0.1 M methanolic solution of the base at room temperature occurs over a few hours, for example.[2]

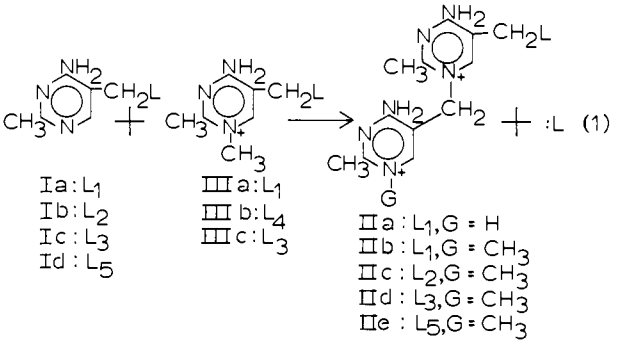
Only recently have we begun to understand the nature of the spontaneous decomposition of Ia. The thiazole portion is liberated as its base. The pyrimidine ring appears as a more complex product in which pyrimidine rings are linked together by a bond between the methylene group at the 5-position of one ring and annular nitrogen atom 1 of another to give a polycation. Structure IIa shows one such oligomer having just two pyrimidine rings; the terminal methylene contains a thiazole ring as found in thiamin. An oligomer containing five pyrimidine rings has been isolated from a mixture produced by the decomposition of thiamin in methanol.[3,4] We call this self-destructure reaction an oligomerization.

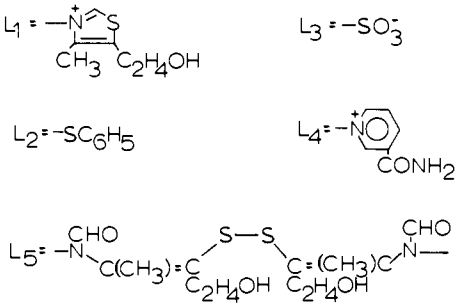
We have prepared the first simple oligomers of thiamin (IIb) and some of its analogues IIc–IIe. All the model compounds contain two pyrimidine rings substituted as in thiamin; the pyrimidine terminus is quaternized by a methyl group. Subsequent reaction of these model substances with sulfite ion, the classic nucleophile which cleaves thiamin, liberating its thiazole ring,[5] provides significant insight into differences in reactivity toward substitution between thiamin and its polycationic derivatives and analogues. Insight gained from kinetic studies employing sulfite ion and also from experiments involving the use of additives which either promote or inhibit oligomerization allows us to propose for the first time a mechanism of oligomerization.

Results

Syntheses. Thiamin is known to undergo nucleophilic substitution at its methylene side chain when the pyrimidine ring is made more electrophilic by protonation or quaternization.[5,6] We therefore elected to employ 1′-methylthiaminium ion (IIIa)[7] as the electrophile and pyrimidines (I) as nucleophiles. This approach has the advantage that the identities of the electrophile and nucleophile are fixed, unlike one involving protonated thiamin as the potential electrophile. Deprotonation of this thiamin produces a nucleophile which could then compete with other pyrimidines, giving mixed products. The method is an extension of that employed so successfully in the synthesis of many thiamin analogues from IIIa.[8]

In methanol, Ib and Ic react cleanly with IIIa to liberate the thiazole ring from IIIa and to incorporate pyrimidine rings having groups L_2 and L_3 in its place (eq 1). In this way a bis(pyrimidine)





product is formed, having a phenylthio (IIc) or a sulfonato group (IId) attached to the methylene end of the oligomer.

With sparingly soluble nucleophile Ic it proved beneficial to employ 2,6-lutidine as a catalyst. The product first isolated is a mixed salt containing two equivalents of Ic, one covalently attached and the other as a counterion along with perchlorate ion. Recrystallization from water easily gives the perchlorate salt, anion Ic remaining in solution.

Unfortunately, Ia did not react with IIIa under similar conditions to form trication IIb exlusively. Analysis by NMR indicates the presence of not only the expected free thiazole but also several pyrimidine rings. Apparently a mixture of oligomers, possibly including IIb, is formed.

Consequently, attempts were made to synthesize IIb by a route long employed to prepare thiamin analogues.[9] Sulfite ion in

(1) Windheuser, J. J.; Higuchi, T. *J. Pharm. Sci.* **1962**, *51*, 354.
(2) Shimahara, N.; Nakajima, N.; Hirano, H. *Chem. Pharm Bull.* **1974**, *22*, 2081.
(3) Shimahara, N.; Asakawa, H.; Kawamatsu, Y.; Hirano, H. *Chem. Pharm. Bull.* **1974**, *22*, 2086.
(4) The nature of the group bonded to the terminal methylene group of pentamer was not specified. The elemental analysis suggests to us that it may be a hydroxy group. A methoxy substituent also is possible since similar compounds incorporate water to make the empirical formula variable. Pure product was prepared by recrystallization from aqueous acid.[3]
(5) Williams, R. R. *J. Am. Chem. Soc.* **1935**, *57*, 229.
(6) Zoltewicz, J. A.; Kauffman, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 3134.
(7) Zoltewicz, J. A.; Baugh, T. D. *Synthesis* **1980**, 217.
(8) Zoltewicz, J. A. *Synthesis* **1980**, 218.
(9) Matsukawa, T.; Yurugi, S. *Yakugaku Zasshi* **1951**, *71*, 1423, 1450.

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Table I. Conditions and Rate Constants for the Reaction of Sulfite Ion with 1'-Methylthiaminium Ion (IIIa) and Oligomers II in Aqueous Phosphate Buffer at 1.0 M Ionic Strength and 25.0 °C[a]

compd	pH	$10^2 \times [SO_3^{2-}]_{free}$,[b] M		$10^2 k_2$, M^{-1} s^{-1} [c]
IIIa	6.27	0.905		4.13
	6.38	1.2		4.27
	6.50	0.140		3.80
	6.51	2.17		4.26
	6.55	0.476		4.61
	6.86	3.15		3.98
	7.34	2.10		3.80
	7.52	4.38		4.01
			av	4.10 ± 0.21
IId	6.50	4.34		2.22
IIc	6.50	3.56		2.02
IIb	6.50	0.730		70.9, 2.11[d]
	6.50	3.56		83.1, 1.97[d]
			av	77.0, 2.04

[a] [Compound], $3-8 \times 10^{-5}$ M. Ionic strength maintained with KCl. [b] Calculated using $pK_a = 6.59$. [c] $k_2 = k_\psi/[SO_3^{2-}]_{free}$, where k_ψ is the pseudo-first-order rate constant. [d] Larger value pertains to substitution of the thiazole ring to give IId, while the smaller refers to the subsequent reaction of IId.

aqueous solution serves as a catalyst for the nucleophilic substitution of thiamin[6,9] and its derivatives.[9,10] We utilized the sparingly soluble salt calcium sulfite as a source of a low, roughly constant concentration of free sulfite ion in solution. Substrate IIIb, which has nicotinamide as a leaving group, was chosen in place of IIIa because it is more reactive.[11] Although a product having a dipyrimidinylmethyl unit as in II was isolated, it lacks a thiazole ring, having a sulfonato group instead. It is IId and not IIb. Our subsequent studies indicate the two failures to prepare IIb are to be expected from a knowledge of reactivities. The studies illustrate problems likely to be encountered in the preparation of other oligomers.

Thiamin oligomer IIb finally was synthesized by a simple two-step sequence, taking advantage of two facts. (1) Bis(pyrimidine) oligomers (II) having a poor leaving group may be less reactive than IIa toward substitution. Successful preparations of IIc and IId serve as examples. (2) The thiazolium ring of thiamin easily may be converted to a derivative which is a poor leaving group by the action of aqueous alkali. This ring-cleaved material converted to its disulfide, thiamin disulfide (Id), contains a masked and protected thiazolium ring.

In methanol, disulfide Id and IIIb cleanly give IIe, a disulfide that is formed by reaction at each of the pyrimidine rings in Id. An aqueous suspension of this material when treated with an equivalent of dithiothreitol at room temperature rapidly cleaves to its component thiol in a disulfide interchange reaction. Thiol then promptly cyclizes to regenerate a thiazolium ring in acidic solution, giving the desired oligomer IIb in high yield.

Sulfite Ion Kinetics. Rates of reaction of bis(pyrimidines) II toward aqueous sulfite ion were obtained in order to gain some insight into the reactivity of each of the two pyrimidine rings toward nucleophilic substitution. The reactivity of monopyrimidine IIIa also was determined because it is a logical reference to which the reactivities of the bis(pyrimidines) may be compared. Rate constants were obtained by standard spectrophotometric methods.

As the data in Table I show, the reactivity of IIIa is first order in free sulfite ion over a 31-fold variation in concentration that spans the range 0.0014–0.044 M. There is no evidence for second-order kinetics in sulfite ion over this interval.[10,12] No pH dependence, after correcting for the protonation of sulfite ion, is found over the region 6.3–7.5. At higher pH hydrolytic cleavage of the thiazolium ring takes place.[13]

Significantly, the second-order rate constant 4.10×10^{-2} M^{-1} s^{-1} for 1'-methylated derivative IIIa of thiamin in H_2O is similar to the value 1.92×10^{-1} M^{-1} s^{-1} (D_2O) for the conjugate acid of thiamin under similar conditions. This agreement supports our suggestion that thiamin first undergoes protonation at N-1' before it reacts with sulfite ion.[6]

Substitution of the bis oligomers could take place by reactions involving one or both of the quaternized pyrimidine rings. But only a single spectroscopic change was observed for IIc and IId. Replacement of a sulfonato group in IId by a reaction with sulfite ion is degenerate, starting material and product being the same. Hence, the observed change with IId must reflect the formation of two sulfonic acids, Ic and IIIc, associated with cleavage of a methylene-pyrimidine bond.

Comparison of the rate constants for IIc and IId (Table I) suggests that phenylthio substrate IIc also reacts with methylene–pyrimidine bond cleavage to give pyrimidines Ib and IIIc. The identity of group L in II does not exert much of an effect on reactivity because L is so far from the reaction site; therefore, rate constants for IIc and IId are essentially the same.

By contrast, oligomerized thiamin IIb shows two spectroscopic changes indicating stepwise nucleophilic substitution. The slower process has a rate constant (Table I) that is not significantly different from that for IId. Therefore, the thiazole ring of IIb is replaced first, giving rise to IId which then reacts. Substitution of the thiazole ring is about 38 times faster than cleavage of the methylene–pyrimidine bond. Confirmation of this order of substitution is obtained by carrying out the reaction in an NMR tube, the spectrum of IId and free thiazole being generated from IIb in the first reaction.

Highly instructive is a comparison of the reactivities of thiamin IIIa and its bis oligomer IIb, both pertaining to substitution of the thiazole ring by sulfite ion. The bis substrate is 19 times more reactive. Clearly quaternization of thiamin by the pyrimidinylmethyl group, itself quaternized, enhances reactivity toward substitution considerably more than by N-methylation or by N-protonation.[14]

The effect of leaving groups on reactivity is revealed by a comparison between IIIa and either IIc or IId. Loss of the thiazole group from IIIa takes place about 1.9 times faster than removal of a pyrimidine ring from IIc or IId. Similarly, the pyridine derivative of thiamin is 2.0 times more reactive than thiamin, activation of the pyrimidine ring being by protonation rather than by methylation.[6]

Oligomerization. High concentrations of thiamin free base, 0.1–0.2 M, give rise to ready oligomerization. Following an induction period, oligomer begins to precipitate from solution; eventually a sample becomes semisolid with oligomer. Analysis by NMR reveals that a small amount, <10%, of free thiazole is present in methanolic solutions of Ia (chloride) at the onset of precipitation. Formation of the fine particles of precipitate produces a cloudiness which is readily apparent. Continued NMR analysis of this mixture shows that massive amounts of free thiazole then are produced rapidly. For example, 25 min after the induction period about 70% of free thiazole is present when a 0.10 M sample is kept in an NMR probe at about 30 °C. We arbitrarily took the onset of precipitation as the end of the induction period in studies designed to reveal the influence of additives on the length of this period.

In Table II are presented the results of some semiquantitative but highly informative experiments pertaining to the influence of various additives on the induction period. Our visual method of analysis is not capable of detecting small changes in reactivity; thus, the induction period for 0.10 and 0.20 M samples in methanol is essentially the same, being about 41–43 min.

Oligomerization may be initiated by IIIa or IIIb. Either additive present only to the extent of 1.6 mol % decreases the induction period for an 0.10 M solution of substrate to about 29 min.

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(11) Unpublished results of G. M. Kauffman.
(12) Doerge, D. R.; Ingraham, L. L. *J. Am. Chem. Soc.* **1980**, *102*, 4828.
(13) Zoltewicz, J. A.; Uray, G. *J. Org. Chem.* **1980**, *45*, 2104.

(14) The unquaternized pyrimidinylmethyl group also is more electron withdrawing than a methyl substituent.[15]

Table II. Influence of Additives on the Length of the Induction Period Associated with the Formation of Oligomers from Thiamin Chloride at 25 ± 1 °C in Absolute Methanol

[Ia,Cl], M	additive, M	min[a]	no. of of trials
0.20		43 ± 3	11
0.10		41 ± 3	7
0.10	IIIa, 1.6×10^{-3}	29 ± 2	3
0.10	IIIb, 1.6×10^{-3}	28 ± 1	2
0.10	B,2Cl,[b] 1.0×10^{-2}	~1430	1
0.10	DMAP,[c] 4.6×10^{-4}	132 ± 9	3
0.10	Ib, 1.0×10^{-2}	73 ± 19	7
0.10	NaN_3, 8.5×10^{-4}	>1220[d]	1
0.10	AP,[e] 9.5×10^{-4}	320	1
0.10	AP,[e] 9.5×10^{-3}	>3440[d]	1
0.19	AP,[e] 1.0×10^{-2}; APH,[f] 9.9×10^{-3}	>4230[d]	1

[a] Onset of precipitation produces a cloudy state, which is taken to be the end of the induction period. [b] Thiamin chloride hydrochloride. [c] 2,6-Dimethyl-4-aminopyridine. [d] Observations terminated at indicated time before the end of the induction period. [e] 4-Aminopyridine. [f] Conjugate acid of 4-aminopyridine.

Inhibition by low levels of additives is observed as well (Table II). Thus, the hydrochloride of thiamin (B,2Cl), 2,6-dimethyl-4-aminopyrimidine (DMAP), Ib, sodium azide, and 4-aminopyridine (AP), some present only to the extent of about 1 mol %, all serve as inhibitors of oligomerization. Inhibition periods as much as 10–100 times longer than the usual induction period are found.

By contrast, pyrimidine Ib, which differs from Ia in the identity of the leaving group, does not undergo oligomerization. A 0.23 M solution in methanol even after being heated at 100 °C for 5 days shows no decomposition on NMR analysis. The nature of the leaving group plays a significant role on reactivity for oligomerization.

Discussion

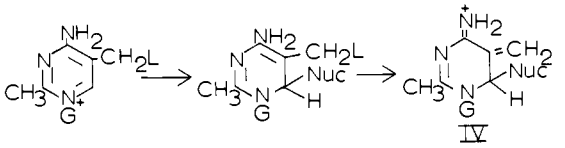
Decomposition of thiamin as its free base in methanol to give a thiazole and oligomers of the pyrimidine ring having structures such as IIa demonstrates the characteristics of a chain reaction. Following an induction period, products form quickly.[2,3] Moreover, additives present in small amounts may decrease or increase the length of the induction period.

A key feature of oligomerization is activation for substitution by quaternization of the pyrimidine ring. This is evidenced (a) by the formation of oligomers rather than simple substitution products, (b) by a shortening of the induction period due to the addition of quaternized pyrimidines IIIa and IIIb, and (c) by sulfite ion kinetic studies which show that *N*-pyrimidinylmethyl substrate IIb is 19 times more reactive than *N*-methyl ion IIIa. Thus, attachment of a pyrimidinylmethyl group to the annular nitrogen atom of another pyrimidine ring as in quaternized oligomer IIa gives a material having greater reactivity than starting material.

Two mechanisms by which the activating pyrimidinylmethyl group becomes bonded to another pyrimidine ring may be considered. First, bis(pyrimidine) IIa might form by an S_N2 reaction.[3] The pyrimidine ring attacks the methylene carbon of another Ia, probably protonated, to displace the thiazole ring. Our synthesis of IIIa from thiamin and dimethyl sulfate is an example of an S_N2 reaction in which a pyrimidine nitrogen atom serves as a nucleophile.[7] But it is not apparent why the addition of the conjugate acid of thiamin to a solution of thiamin free base (Table II) slows reaction as indicated by an increase in the induction period. By contrast, addition of N-methylated thiamin IIIa, which also has a positively charge ring, shortens the induction period. Hence, we disfavor this mechanism.

More likely is a reaction scheme which is a variation of that observed for sulfite ion reacting with thiamin[6,12] and thiamin analogues[10] and for hydroxide ion and thiamin analogues[16] in aqueous solution. In this scheme a pyrimidinylmethyl group becomes attached to another pyrimidine ring by a multistep process that generates nonaromatic electrophile IV.

Scheme I



IV

Electrophile IV may form by the sequence given in Scheme I. Thiamin first undergoes protonation (G = H), the conjugate acid adds a nucleophile and the resultant σ complex then eliminates the thiazole leaving group to yield electrophile. In related reactions the nucleophile is sulfite[6,12] or hydroxide ion.[16] Here it may be methoxide ion. For steric reasons it is not likely to be thiamin.

Following these initiation steps electrophile IV may be trapped by thiamin nucleophile. Rearomatization of trapped electrophile gives quaternized material IIa having a pyrimidinylmethyl group bonded to nitrogen. Scheme I may be repeated; now G is the activating pyrimidinylmethyl unit and the newly generated electrophile may be trapped either by thiamin or the free pyrimidine end of oligomer to give higher molecular weight oligomers in propagation steps.

Our syntheses of IIc–IIe from IIIa and IIIb and nucleophiles Ib–Id probably proceed by a similar mechanism. Because a poor leaving group is bonded to the methylene end of the bis(pyrimidine) products, further substitution is prevented.

The sulfite ion kinetic studies suggest that oligomer may undergo substitution not only at the methylene end but also at interior positions, breaking a bond to a pyrimidine ring. Sulfite ion substitution at the methylene position of IIIa having a thiazole leaving group is only about 1.9 times faster than substitution at a methylene site of IIc and IId having a pyrimidine ring as a leaving group. Moreover, for higher oligomers there is a statistical factor favoring reaction at the more prevalent interior positions over the end group. If a similar reactivity pattern holds for oligomerization, high-molecular-weight oligomers will not form. Insolubility is also a factor limiting chain length in methanol.

An interesting facet of the mechanism is the contrasting reactivity of pyrimidine and methoxide ion nucleophiles toward two different electrophiles. Methoxide ion rather than pyrimidine adds to aromatic pyrimidinium ion in Scheme I. But the order is reversed when they compete for electrophile IV. Similar contrasts have been observed with other pairs of nucleophiles and thiamin analogues.[10,16]

Rate retardation by added thiamin hydrochloride now becomes understandable. Addition of this acid lowers pH and decreases the concentration of methoxide ion and the rate of formation of electrophile.

Additives IIIa and IIIb promote reactivity by readily being converted to electrophile according to Scheme I. Because they already contain an activating methyl group, they need not undergo protonation as does Ia and so they are much more reactive.

Other additives may inhibit oligomerization by increasing pH and/or by trapping electrophile IV in a chain-breaking process. The strong but sterically hindered base 2,6-dimethyl-4-aminopyridine probably inhibits reaction by raising solution pH. Methoxide ion at high pH may compete with pyrimidine nucleophile for IV and break growing chains. Pyrimidine Ib, which has essentially the same basicity and steric requirements as Ia, may on the other hand inhibit by trapping IV, giving rise to material having a poor leaving group. Sodium azide[17] and 4-aminopyridine both are moderately strong bases and good nucleophiles in methanol. Their inhibition may be associated with a pH increase and nucleophilic trapping.

Given the sensitivity of the rate of oligomerization to inhibiting additives and the slow rate in aqueous solution,[1,2] it is no surprise

(15) Breslow, R.; McNelis, E. *J. Am. Chem. Soc.* **1959**, *81*, 3080.

(16) Zoltewicz, J. A.; Uray, G. *J. Am. Chem. Soc.* **1981**, *103*, 683.

(17) Ritchie, C. D.; Skinner, G. A.; Badding, V. G. *J. Am. Chem. Soc.* **1967**, *89*, 2063.

that the oligomerization of thiamin was discovered only recently.

We anticipate that some analogues of thiamin also will undergo oligomerization. As our synthetic and sulfite ion kinetic studies suggest, the choice of a leaving group is important. Analogues having good leaving groups such as a pyridine ring may undergo oligomerization.

The general features of the polyelectrolyte-forming reaction now seem established. Further studies are required to increase our level of understanding and to explain, for example, why oligomerization occurs so much faster in methanol than in water.

Experimental Section

Preparation of 4-Amino-1-[(4-amino-1,2-dimethyl-5-pyrimidinio)-methyl]-2-methyl-5-((thiophenoxy)methyl)pyrimidinium Diperchlorate (IIc). A mixture of 1.0 g (2.1 mmol) of 1′-methylthiaminium diperchlorate[7] and 1.0 g (4.1 mmol) of 2-methyl-4-amino-5-((phenylthio)-methyl)pyrimidine[18] suspended in 20 mL of methanol was stirred at room temperature for 36 h until homogeneous. Solvent was removed under reduced pressure and a small amount of dichloromethane was added to aid crystallization, giving 1.15 g of product. Recyrstallization from 0.1 M perchloric acid affords 1.0 g (86%) of product as a hemihydrate, mp 159–161 °C. Anal. Calcd for $C_{19}H_{24}Cl_2N_6O_8S\cdot0.5H_2O$: C, 39.56; H, 4.36; N, 14.56. Found: C, 39.59; H, 4.37; N, 14.58. The material crystallized from methanol contains no water, mp 218 °C dec. Anal. Calcd for $C_{19}H_{24}Cl_2N_6O_8S$: C, 40.22; H, 4.26; N, 14.81. Found: C, 40.19; H, 4.29; N, 14,80. NMR (Me_2SO-d_6) δ 9.49, 9.25, 8.83, 8.58 ($2NH_2$), 7.89, 7.85 (pyrimidine), 7.31 (m, 5 H), 5.02 (CH_2N), 4.08 (CH_2S), 3.74 (NCH_3), 2.64, 2.55 ($2CH_3$).

Preparation of 4-Amino-1-[(4-amino-1,2-dimethyl-5-pyrimidinio)-methyl]-2-methyl-5-(sulfonatomethyl)pyrimidinium Perchlorate (IId). Method A. A suspension of 1.50 g (6.68 mmol) of sodium (4-amino-2-methyl-5-pyrimidinyl)methylsulfonate (Ic),[5] 4.00 g (8.35 mmol) of 1′-methylthiaminium diperchlorate,[7] 1 mL of 2,6-lutidine, and 60 mL of methanol was heated at reflux for 48 h. The hot solution was filtered and the cake was washed with hot methanol to give 2.40 g of product, mp >325 °C.

NMR analysis indicates this product to be a mixed salt having 1 equiv. of unquaternized sulfonic acid Ic as a counterion. Product was dissolved in 70 mL of boiling water. After cooling to room temperature, the precipate of 1.07 g of IId, mp >325 °C, was collected. Additional crops are contaminated with sulfonic acid Ic. The yield, corrected to reflect mixed salt formation, is 67%. Recrystallization from 0.1 M perchloric acid gives the analytical sample, dried at 100 °C. Anal. Calcd. for $C_{13}H_{19}ClN_6O_7S\cdot0.5H_2O$: C, 34.86; H, 4.50; N, 18.77. Found: C, 34.86; H, 4.51; H, 18.76. NMR (D_2O) δ 8.03, 7.83 (pyrimidine), 5.30 (CH_2N), 4.16 ($CH_2SO_3^-$), 3.80 (NCH_3), and 2.68 ($2CCH_3$).

Method B. To a solution of 0.30 g (1.0 mmol) of 1-[(4-amino-1,2-dimethyl-5-pyrimidinio)methyl]-3-carbamoylpyridinium diperchlorate (IIIb)[8] and 0.308 g (1.00 mmol) of thiamin chloride hydrochloride in 5 mL of water was added 0.10 g (0.64 mmol) of calcium sulfite dihydrate. The suspension was stirred at room temperature for 24 h. An NMR spectrum does not show a signal for CH_2N at δ 6.0, indicating that IIIb has reacted completely but 60% of the thiamin remained. Filtration gave back 0.015 g of the sulfite. During the course of the reaction the pH changed from 6.4 to 5.8. (A control having the same composition but without the sulfite shows no reaction.) Addition of excess solid sodium perchlorate leads to the coprecipitation of 0.14 g of thiamin diperchlorate and IId, estimated by NMR analysis to be in a 3:2 ratio. Recrystallization from 0.1 M perchloric acid gave 0.038 g of pure IId (8.7%). The yield becomes 22% if a correction is applied to reflect the incomplete conversion of thiamin.

Preparation of Disulfide IIe Tetraperchlorate. A suspension of 0.675 g (1.5 mmol) of 1-[4-amino-1,2-dimethyl-5-pyrimidinio)methyl]-3-carbamoylpyridinium diperchlorate (IIIb)[8] and thiamin disulfide (Id) in 25 mL of methanol was stirred at room temperature. After 2 days most of the suspension dissolved but a small amount of greasy residue was present; this was removed and manipulated twice with 5-mL portions of methanol to produce crystals. The entire mass then was transferred back to the reaction mixture. After 3 days the first colorless crystals began to form. After a total of 7 days, the crystals were then suspended in about 10 mL of hot methanol and collected to give 0.70 g of product, mp 184–188 °C. Recrystallization from 0.1 M $HClO_4$ afforded 0.50 g (54%) of the analytical sample, which now has mp 250–255 °C. Anal. Calcd for $C_{38}H_{56}Cl_4N_{14}O_{20}S_2$: C, 36.96; H, 4.57; N, 15.88. Found: C, 36.85; H, 4.71; N, 15.84. NMR (Me_2SO-d_6) δ 9.60, 9.33, 8.61, 8.48 ($2NH_2$), 8.05, 7.97, 7.82 (CH), 5.12 (CH_2N), 4.62 (OH), 4.41 (CH_2N), 3.74 (NCH_3), 3.40 (CH_2OH), 2.65 (CH_2), 2.63, 2.59, 1.97 (CH_3). Minor signals are found at 8.07, 8.30, 1.97, 1.79, possibly due to a minor conformational isomer.

Preparation of 4-Amino-1-[(4-amino-1,2-dimethyl-5-pyrimidinio)-methyl]-5-{[5-(2-hydroxyethyl)-4-methyl-3-thiazolinio]methyl}-2-methylpyrimidinium Triperchlorate (IIb). To a suspension of 0.450 g (0.364 mmol) of disulfide IIe in 8 mL of water containing a pH electrode was slowly added with stirring a solution of 0.56 g (0.36 mmol) of dithiothreitol in 5 mL of water. The pH was kept between 3 and 6 by the addition of 0.1 M $HClO_4$, the final value being 5.5. The solution was evaporated to dryness under reduced pressure while keeping the temperature below 40 °C. Two 10-mL portions of methanol then were added and removed under reduced pressure. Addition of ethyl acetate to the residue gave 0.400 g of crystals. The crude product was dissolved in about 10 mL of methanol at room temperature, leaving a small amount of dark residue. After some time at 0 °C, 0.31 g (61%) of colorless, hygroscopic crystals of product, mp 173–179 °C dec, was collected. Anal. Calcd for $C_{19}H_{28}Cl_3N_7O_{13}S$; C, 32.56; H, 4.03; N, 13.98. Found: C, 32.33; H, 4.07; N, 13.90. NMR (D_2O) δ 9.49 (thiazole CH), 7.92 (CH), 5.53, 5.32 (CH_2N), 3.89 (CH_2OH), 3.85 (NCH_3), 3.18 (CH_2), 2.77, 2.66, 2.55 (CH_3).

Identification of Products from Sulfite Ion Substitution. Compound IIb was added to a phosphate (H_2O) buffer containing excess sulfite ion at pH 6.8. The formation of free substituted thiazole (δ 8.75 and 2.37), sulfonate ion Ic, and its corresponding N-methylated derivative IIIc was confirmed by their characteristic chemical shifts associated with pyrimidine and methyl protons at δ 8.03 and 2.47 and δ 8.10 and 2.65, respectively.

Similarly, IIIa is cleanly cleaved to the free thiazole and the N-methylated derivative IIIc, which is sparingly soluble. This 4-amino-1,2-dimethyl-5-(sulfonatomethyl)pyrimidinium betaine has mp >300 °C. Anal. Calcd for $C_7H_{11}N_3O_3S$: C, 38.70; H, 5.10; N, 19.34. Found: C, 38.68; H, 5.10; N, 19.30.

When IIc was added to an aqueous solution of sulfite ion a precipitate of 4-amino-2-methyl-5-(phenylthio)methyl)pyrimidine[18] appeared immediately, the material being identified by its melting point. NMR analysis of the mother liquor showed the presence of the pyrimidinium betaine IIIc.

Oligomer produced in an experiment without additives was collected by filtration. This material gives a milky suspension in water. But if oligomer is added to a solution of sodium sulfite in D_2O (pD about 8.9), a clear solution results almost immediately. An NMR spectrum taken just after mixing shows that oligomer has been converted to a mixture of two sulfonate ions, Ic and IId lacking the NCH_3 group. Three pyrimidine protons, two $CH_2SO_3^-$, one CH_2N, and three CH_3 signals appear, each group having about the same intensity, which suggests the two products are present to about the same extent. After the solution stands overnight, only Ic is seen. Formation in the initial mixture of just two main sulfonate ion products is expected from our kinetic studies. Random cleavage of internal positions of oligomer produces an unquaternized pyrimidine ring which is much less reactive than a quaternized ring. The free ring then appears as a pyrimidinylmethyl group attached to a pyrimidinylmethylsulfonate ion, i.e., IId without its NCH_3 substituent.

Sulfite Ion Kinetics. Kinetic studies were made by using a Zeiss PMQ II spectrophotometer equipped with a digital readout and a thermostated cell block maintained at 25 ± 0.1 °C by circulating water from a Lauda K-2/R refrigerated constant temperature bath. pH measurements were made by using a Radiometer GK2321C combination electrode and a Radiometer PHM 64 pH meter. Preliminary kinetic runs for each substrate were made with a Cary 17D recording spectrophotometer in order to determine the optimum wavelength. Wavelengths for kinetic runs were 267 nm for IIIa and 270 nm for IIb–IId.

Small volumes of sodium sulfite stock solution were prepared frequently using boiled water containing phosphate buffer. Solutions were kept under argon or nitrogen. Ionic strength was maintained at 1.0 M with KCl. A few crystals of Na_2EDTA and/or small amounts of methanol were added to retard oxidaton. Sulfite ion was standardized in the usual way, using starch–iodine and thiosulfate.

For a kinetic run a 3-mL sample of buffered sulfite ion solution was added to reference and sample cuvettes either by pipet or by syringe with a Teflon needle. Transfer was made with a good flow of argon. Following thermal equilibration in the thermostated block, a few microliters of substrate stock solution was added. Absorbance changes were recorded for at least 10 half-lives. Plots of ln (ΔA) vs. time were linear for several half-lives; ΔA was calculated with respect to the absorbance at "infinity". The pH and sulfite ion concentrations of the reaction mixture were determined at the end of a run and compared with initial values. Runs in which sulfite ion changed significantly due to oxidation were discarded; pH changes were insignificant.

No problems were encountered except in the case of IIb, which undergoes a consecutive reaction. Although the difference in reactivity

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between the two stages of substitution is large enough for clear separation, it is difficult to estimate precisely the infinity absorbance for the first stage because the total change is small ($\Delta A \sim 0.060$). However, linearity over 2 half-lives suggests that the rate constant for the first stage is well estimated.

Influence of Additives of the Length of the Induction Period for the Oligomerization of Thiamin Chloride. A stock solution (0.20 M) of thiamin chloride[2] was prepared immediately before use. Solid substrate was dissolved in methanol over a 2-4-min period with the aid of a vibromixer. A stopwatch was activated when the methanol was added to solid. One-half milliliter then was added by syringe to a 5-mm NMR tube containing methanol or a methanolic solution of additive. After the sample was mixed, the total elapsed time was about 5-6 min. After a few trials to determine conditions, a set of samples was examined together; one member of the set always consisted of substrate without additive.

The tubes were inverted frequently and held to the light to facilitate detection of suspended particles; NMR tubes were advantageous in that the viewing area was maximized. The onset of precipitation (formation of a cloudy solution) was taken to be the end of the induction period. There was some day-to-day variation in the length of the induction period, especially with phenylthio compound Ib acting as inhibitor. But the order of inhibition or acceleration with respect to additive-free material was invariant.

After thiamin chloride stood for several weeks, the decomposition temperature of thiamin chloride decreased somewhat. The initial decomposition range, 165-169 °C (lit.[2] range 160-163 °C dec), could be restored by suspending finely powdered material in absolute ethanol for a few minutes prior to use. No difference could be detected in induction periods for old and new substrate.

The condition corresponding to the onset of precipitation was examined by NMR. Only small amounts (<10%) of free thiazole could be detected for the condition taken to be the end of the induction period as evidenced by the formation of precipitate. It is convenient to scan the aromatic protons at low field. The liberated thiazole signal for H-2 appears about midway between those for the thiazole and pyrimidine signals of thiamin. Even when the NMR tube contains massive amounts of precipitated pyrimidine oligomer, it still is possible to obtain good-quality signals for thiamin and free thiazole.

Curiously, one sample of 0.10 M thiamin chloride did not respond as usual, a faint cloudiness appearing only after about 2927 min. Other samples prepared from the same 0.20 M stock solution behaved normally. We have no explanation for this unexpected inhibition; an NMR spectrum obtained after 1220 min appeared to be normal.

Some samples containing inhibitor were examined by NMR to verify that little or no substitution took place at reaction times greater than that for the usual induction period. Free thiazole was detected when precipitate finally began to form, however.

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Synthesis and Characterization of a Novel Cholesterol Nitroxide Spin Label. Application to the Molecular Organization of Human High Density Lipoprotein

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Abstract: The purpose of this study was to synthesize a cholesterol nitroxide that closely mimics cholesterol in its physical and biological properties and to utilize this molecule to probe the nature of cholesterol-protein interactions in human high density lipoprotein particles. The rigidly labeled cholesterol nitroxide **15** (Δ^5-3β-hydroxy steroid) was prepared by addition of isohexylmagnesium bromide to nitrone **12**, a key intermediate readily obtained from dehydroisoandrosterone via intermediates **8** and **11**. Also formed from **12** in the Grignard reaction were hemiketal **23**, nitroxide **25**, and a tautomeric mixture of **21** and **21a**. This latter mixture was shown to serve as a precursor for **23**, and likely for **25**. Cu^{2+}-catalyzed air oxidation of **23** gave **27**. Formation of **21-21a** likely was initiated by attack of the α anion of nitrone **12** on the acetyl group of another molecule. The structure of **21-21a** was confirmed by an independent synthesis from **12** via intermediates **13**, **14**, and **32**. Nitroxide **15** was also converted into its oleate ester **16** and tritiated analogue **20** (sp act., 1.6 Ci/mMol). Nitroxide **15** both served as a substrate for cholesterol oxidase and also entered into the lecithin-cholesterol acyl transferase reaction, albeit with an efficiency less than that of cholesterol itself. The extent of hydrolysis of nitroxide oleate **16** by cholesterol esterase was about the same as that of cholesterol oleate, suggesting that toward this enzyme, **15** behaved like cholesterol. In dipalmitoylphosphatidyl choline (DPPC)-cholesterol vesicles at -196 °C, **15** showed $2A_{max}$ = 63.8 G (±0.5 G), similar to the value of 64.5 G at -196 °C for **15** in a mixed crystal with cholesterol. These values are consistent with the desired hydrophobic location of the nitroxide group in these systems. In the vesicle system at 25 °C, the ESR spectrum of **15** was similar to that of **2** (hindered rotation about the long molecular axis). At 45 °C the ESR spectral lines of **15** were much sharper than those of **2**. This is a consequence of the fact that, unlike **2**, the long molecular axis of **15** does not correspond to any of the principal axes of the nitroxide group. Thus, rotation about the long molecular axis partially averages all the x, y, and z components. Cholesterol nitroxide **15** was readily incoporated into human high density lipoproteins. Analysis of the ESR line shape showed the presence of cholesterol-lipid and cholesterol-protein contacts in HDL_3. These results are in contrast to the conclusion of other investigators, namely, that cholesterol is excluded from the immediate vicinity of a membrane protein that penetrates through cholesterol-containing phospholipid bilayers.

Cholesterol (**1**) is one of the principal lipids of mammalian membranes.[2] The nature of the molecular interactions of cholesterol with phospholipids,[2a-7] proteins,[8-11] and other molecules relevant to membrane biochemistry such as the polyene antibiotics[12-14] and certain toxins[15,16] has been investigated extensively,

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