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Aminoethylation of Thiopyrimidine Nucleosides

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ABSTRACT: Thiopyrimidine nucleosides can be readily alkylated by ethylenimine at pH 8. The reaction of 4-thiouridine, 2-thiouridine, 2-thiocytidine, and 2,4-dithiouridine has been studied. In contrast to cyanoethylation with acrylonitrile the 2-thionucleosides could be aminoethylated at readily detectable rates although proceeding at approximately one-fourth the rate of the 4-thionucleosides. 2,4-Dithiouridine showed both the rapid rate characteristic of 4-thionucleosides and the slower rate characteristic of 2-thionucleosides. Spectral analysis of the 4-thiouridine reaction product yields a typical cytidine derivative spectrum. Investigation of this reaction with ^{14}C-labeled ethylenimine, coupled with the pH dependence of the product spectrum, indicates that the final product of the reaction is N^4-β-thioethyl-*S*-β-aminoethylcytidine which is produced by a side-chain rearrangement reaction. The pH dependence of the reaction rate showed a discrete optimum at pH 8.1 and indicates the reactive species as being the thionucleoside anion and the ethylenimmonium cation. Pseudo-first-order rate constants at 25° with 0.19 M ethylenimine at pH 8 are reported as well as the second-order rate constant for the ethylenimmonium–thionucleoside anion reaction.

In the last few years thiopyrimidines have been shown to occur as minor constituents of bacterial tRNAs (Lipsett, 1965; Goehler and Doi, 1968; Carbon *et al.*, 1968). A variety of compounds containing suitably activated electrophilic double bonds have been found to alkylate certain nucleophilic thiopyrimidines—for instance, *N*-ethylmaleimide reacts with 4-thiouridine to form the *S*-NEM-4-thioU[1] adduct (Carbon and David, 1968). Similarly a detailed study of the reaction of acrylonitrile with 4-thiouridine demonstrated the formation of *S*-cyanoethyl-4-thioU (Ofengand, 1967). The pH dependence of the latter reaction showed a classical sigmoid rate curve with inflection at the pK_a of 4-thioU indicating that the thiopyrimidine anion was the reactive species. Both of the above reactions are Michael-type reactions and, as expected, the alkylated adduct was alkaline labile undergoing a reverse Michael reaction at high pH. The above two Michael reagents readily reacted with 4-thiouridine but did not react with 2-thiopyrimidine nucleosides (Ofengand, 1967; Carbon and David, 1968).

While investigating an unrelated problem I noted the facile aminoethylation of 4-thioU in *E. coli* tRNA by ethylenimine at pH 8 (Reid, 1968). The purpose of this communication is to report on the reaction of ethylenimine with a variety of thiopyrimidine nucleosides and to compare this aminoethylation reaction with the cyanoethylation of thiopyrimidine nucleosides reported by Ofengand.

Materials and Methods

Thionucleosides. 4-Thiouridine disulfide (lot R-5433) was purchased from Cyclo Chemical Corp., Los Angeles, and was reduced to 4-thiouridine with 1 mM dithiothreitol prior to use. 2-Thiocytidine and 2,4-dithiouridine were the kind gifts of Dr. Tohru Ueda, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan. 2-Thiouridine was the kind gift of Dr. John Carbon, Department of Biological Sciences, University of California, Santa Barbara, and had been originally obtained by him from Dr. Mitsuji Sano, Analytical Division, Central Research Laboratory, Daiichi Seiyaku Co. Ltd., Edogawa-Ku, Tokyo, Japan. Ethylenimine was

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[1] Abbreviations used are: 4-thioU, 4-thiouridine, 2-thioU, 2-thiouridine, 2-thioC, 2-thiocytidine.

purchased from Matheson, Coleman and Bell, East Rutherford, N. J.

Conditions of Aminoethylation. All reactions were carried out at 25° and 0.19 M ethylenimine (10 μl/ml of reaction). The various thionucleosides were dissolved in 1 mM dithiothreitol solutions in distilled water to give an absorbance at the λ_{max} of 1 to 1.5. This solution (2.5 ml) was mixed with 2.5 ml of 0.4 M K-Tris-phosphate, pH 6.8 (0.4 M KH_2PO_4 titrated to pH 6.8 with saturated Tris solution), and the reaction was started by addition of 50 μl of ethylenimine. This buffer (0.2 M K-Tris-phosphate, pH 6.8) was chosen because the pH after addition of 10 μl/ml of ethylenimine is routinely 8 to 8.1 and also because of the broad range buffering capacity of this mixed buffer. For spectral analysis of the reaction at pH 8, 3-ml samples were placed in cuvets and the complete ultraviolet spectrum was taken in a Cary Model 15 spectrophotometer equipped with a repetitive scan accessory. Spectra were taken usually every 5 min for the first 1 or 2 hr then less frequently for the remainder of the reaction up to 3 or 4 hr.

pH Dependence of the Reaction Rate. Aliquots (5 ml) of 4-thioU in the same buffer as above were reacted in a thermostatted pH-Stat (radiometer PHM26-TT11) with 50 μl of ethylenimine followed by immediate titration with either 12 N HCl or 10 N KOH from a microburet to give the indicated pH values (the maximum dilution was 2% and was neglected). Aliquots (3 ml) of each reaction were placed in cuvets and monitored at 322 mμ in a thermostatted Gilford Model 240 spectrophotometer equipped with an automatic sample changer. Usually four reactions at four different pH values were monitored simultaneously and could all be set up and transferred into cuvets within 5 to 10 min of the start of the reaction. The absorbance changes were determined every 10 min or by continuous recording with a Honeywell Electronik 194 recorder.

Determination of pK_a Values. The pK_a of ethylenimine was determined by direct titration of an 0.19 M solution in distilled water with 1 N HCl using a Radiometer PHM26-TT11-ABU1b titration apparatus. For the spectrophotometric titrations 2-thioU and 4-thioU were dissolved in 0.1 M Tris–1 mM dithiothreitol to give an absorbance at the λ_{max} of 0.5 to 1.0. This solution (40 ml) was placed in the titration vessel and titrated to lower pH values in roughly 0.4 pH unit increments with glacial acetic acid. At each pH an aliquot was removed into a cuvet and the spectrum determined in a Cary Model 15 spectrophotometer. The maximum dilution during this procedure was less than 1% and was neglected.

Results and Discussion

The reaction of 2-thioU, 4-thioU, 2-thioC, and 2,4-dithioU with 0.19 M ethylenimine at 25° was first investigated at a constant pH of 8.0. Initially the reaction was monitored by taking the complete ultraviolet spectrum of the reaction at regular time intervals in order to evaluate the best wavelength for single wavelength monitoring in each case and also to establish that a simple single transformation from unalkylated to alkylated product was taking place. Figure 1a–d shows the spectral shift during the course of aminoethylation of the four thionucleosides. The aminoethylation of 4-thioU causes a progressive loss of the 320-mμ absorption peak with a discrete isosbestic point at 291 mμ and the generation of a new peak at 271 mμ. Similarly the aminoethylation of 2-thioU shows an isosbestic point at 237 mμ. Thus both of these reactions represent apparently simple molecular transformations with hypsochromic shifts of approximately 50 mμ upon aminoethylation. By choosing a suitable wavelength at which the reaction approaches zero absorbance (*i.e.*, negligible product absorbance) one can determine the apparent first-order constant for the reaction under these conditions. In all of these experiments the total concentration of ethylenimine is at least three orders of magnitude greater than that of the thionucleoside making the reactions pseudo first order. Thus at 330 mμ one obtains a linear semilog plot for 4-thioU aminoethylation yielding a pseudo-first-order rate constant of 0.012 min^{-1} and at 280 mμ one obtains a pseudo first-order rate constant of 0.0034 min^{-1} for 2-thioU aminoethylation. Actually this latter number may be slightly low since rate plots at the less sensitive wavelengths of 290 and 300 mμ give rate constants about 12% higher than this value. Nevertheless the reaction of ethylenimine with 2-thioU is easily detectable, although proceeding at one-third to one-quarter the rate of 4-thioU under these conditions, and hence differs from the Michael reaction of *N*-ethylmaleimide and acrylonitrile which react with 4-thioU but not with 2-thioU (Ofengand, 1967; Carbon and David, 1968). At the end of the aminoethylation of 4-thioU the 271-mμ-absorbing product was titrated to pH 12.3 and the complete spectrum monitored every 10 min. The aminoethyl product was found to be completely alkali stable with no detectable breakdown over 4 hr at room temperature. This is in contrast to the alkali lability of *S*-cyanoethyl-4-thioU which breaks down at pH 12 with a half-life of 38 min (Ofengand, 1967) and presumably reflects the absence of the cyano function which facilitates the alkyl elimination under alkaline conditions. Similarly the NEM-4-thioU adduct undergoes a reverse Michael addition under strong alkaline conditions (Carbon and David, 1968).

Figure 1 also shows the spectral shift accompanying the aminoethylation of 2-thioC. Although not as discrete as in the previous cases, an isosbestic point in the vicinity of 235–240 mμ is observed with the expected hypsochromic shift on aminoethylation. As before, the rate plot at 280 mμ yields a pseudo-first-order process with a rate constant of 0.0023 min^{-1}.

Thus under these constant reaction conditions 4-thioU is aminoethylated at a rate 3.5 to 5 times faster than 2-thiopyrimidine nucleosides. The reason for the lowered reactivity of 2-thiopyrimidine nucleosides toward ethylenimine (and toward acrylonitrile) is not immediately obvious. It is apparently not due to intramolecular hydrogen bonding with ribose hydroxyl groups since this should be reflected in a similarly decreased reactivity of 2-thiopyrimidines toward both ethylenimine and acrylonitrile whereas the 4-thioU:2-thioU reactivity ratio is approximately 4 for ethylenimine at pH 8 and greater than 460 for acrylonitrile at pH 10 (Ofengand, 1967). It is also difficult to invoke steric limitations due to the proximity of the ribose ring to the sulfur at position 2 since the difference in bulkiness of ethylenimine and acrylonitrile seems hardly great enough to explain reactivity differences of more than two orders of magnitude. Thus the differences probably reflect an inherently greater nucleophilicity of sulfur atoms (and sulfide

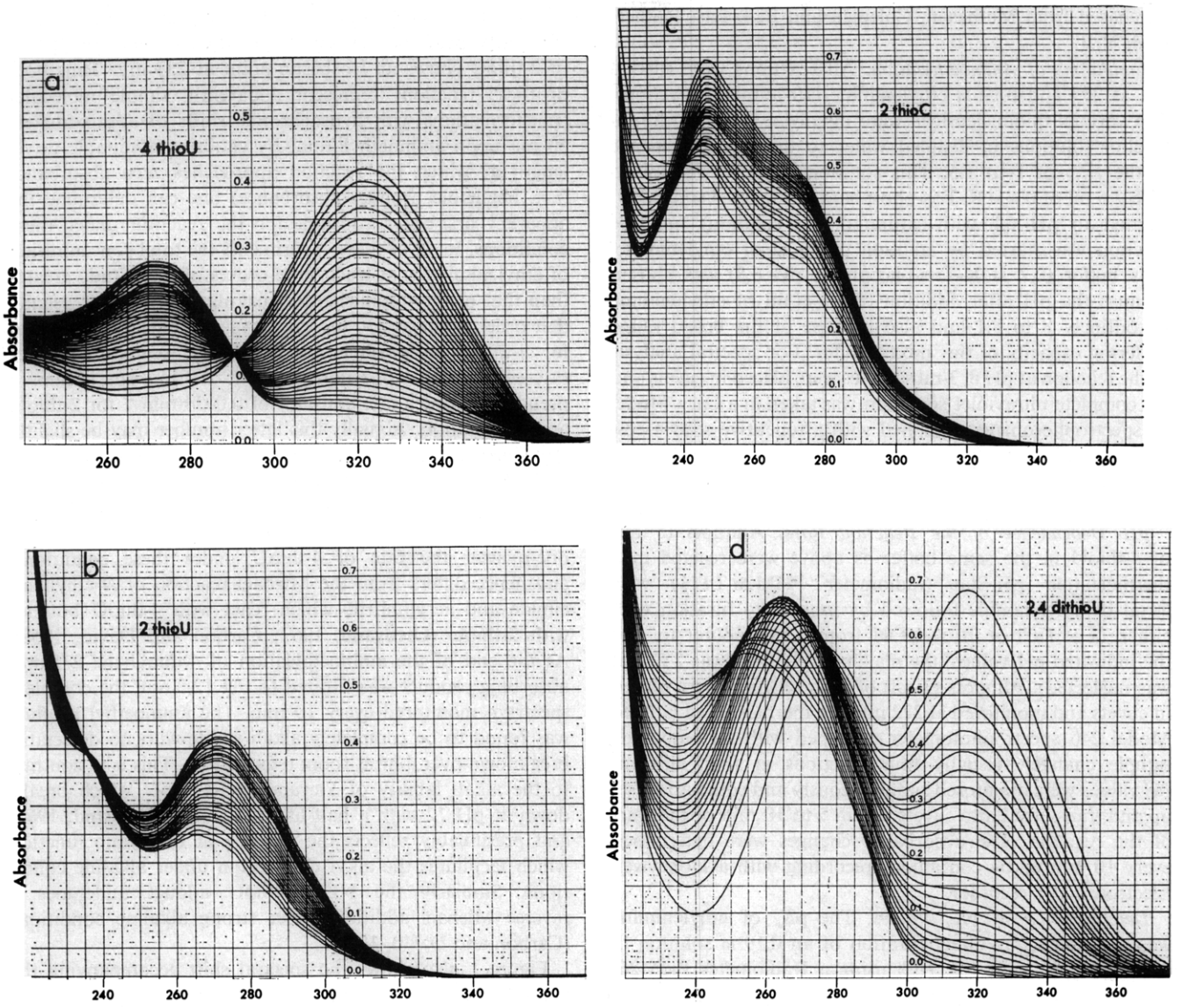

FIGURE 1: Spectral change during the aminoethylation of thiopyrimidine nucleosides at pH 8. The thionucleosides were dissolved in buffer as described in the Methods section to give absorbance readings of 0.4–0.7. The samples were kept at 25°, titrated to pH 8.0 with ethylenimine (10 μl/ml = 0.19 M), and the spectrum determined as soon as possible in a Cary 15 spectrophotometer maintained at 25°. All reactions were monitored for 3–4 hr. Spectra were taken at the following time intervals: (a) 4-thioU, every 5 min up to 2 hr, then every 10 min for the 3rd hr; (b) 2-thioU, every 5 min up to 1 hr, every 10 min up to 3 hr, then every 20 min up to 4 hr; (c) 2-thioC, every 5 min up to 1 hr, every 10 min for the 2nd hr, and every 20 min for the 3rd hr; (d) 2,4-dithioU, every 5 min up to 1 hr, every 10 min for the 3 hr; (d) 2,4-dithioU, every 5 min up to 1 hr, every 10 min for the 2nd hr, then every 20 min up to 4 hr.

ions) at the 4 position of pyrimidine rings than at the 2 position.

In view of the lowered reactivity of 2-thiopyrimidines *vs.* 4-thiopyrimidines it was of interest to investigate the reactivity of 2,4-dithioU which contains both types of sulfur on the same ring. Figure 1 shows the spectral change accompanying the aminoethylation of 2,4-dithiouridine under the same conditions as described previously. Initially one observes a rapid decrease in absorbance at 320 mμ and, for the first hour the spectra faithfully pass through an isosbestic point at 278 mμ and show an increasing absorbance at 265 mμ. This reflects the transition from unalkylated to monoalkylated nucleoside and the aminoethylation is presumably at the 4 position. After longer reaction times the spectra gradually depart from this isosbestic point, start to decrease at 265 mμ, and establish a new isosbestic point at approximately 252 mμ. This second and much slower reaction reflects the transition from monoalkylated to dialkylated product and presumably occurs at the 2 position. Although this could perhaps reflect the 2 alkylation of unalkylated material this is unlikely since, after 2 hr of reaction, the concentration of unalkylated material is approximately 10% of that of the monoalkylated derivative. The pseudo-first-order rate constant determined at 340 mμ obviously reflects the first alkylation reaction at the 4 position and has a value of 0.020 min^{-1} which is somewhat more rapid than the reaction with 4-thioU itself. Since the unalkylated and monoalkylated material both have the same absorbance at 278

mμ (first isosbestic point) then the rate at this wavelength should reflect the monoalkylated–dialkylated transition. However the 2,4-dialkylated product must have significant absorption at 278 mμ since the absorbance at this wavelength does not approach zero and we have made no attempt to abstract the rate constant for the slower second reaction from the time-dependent spectral changes of this compound. Furthermore the kinetic interpretation is complicated by the need to make assumptions concerning the effect (or lack of effect) of the first aminoethylation on the rate of the second aminoethylation.

Characterization of the Product and Site of Alkylation. The product of the reaction of 4-thioU with ethylenimine was studied in some detail to investigate more fully the nature of the compound formed in this reaction. Although the product was expected to be *S*-aminoethyl-4-thioU it can be seen from Figure 1 that the compound formed in this reaction has a single peak ultraviolet spectrum with a maximal absorption at 271 mμ and shows similarities to cytidine-derivative spectra. The suspicion that the product is not *S*-aminoethyl-4-thioU is derived from the lack of spectral similarity with *S*-cyanoethyl-4-thioU—the latter showing an absorption maximum at 301 mμ with a pronounced shoulder at 265 mμ (Ofengand, 1967).

The product from an exhaustive aminoethylation reaction was chromatographed on Dowex 50 columns and the purified material subjected to mass spectrometry (Hitachi-Perkin Elmer Model RMU-7). Although the compound was poorly volatile, spectra were obtained at 260° using maximum sensitivity. At 30 eV no parent peaks were obtained due to apparent side-chain fragmentation; however peaks were obtained at *m/e* values of 136 and 111. The absence of peaks greater than 150 and the presence of the above peaks in the 100–150 mass range show similarities with the mass spectra of N^4-substituted cytidines reported by Hecht *et al.* (1969)—the latter 111 peak being due to cytosine itself.

To investigate the extent of aminoethylation, isotopic labeling experiments were carried out. Reduced 4-thioU was reacted at 35° and pH 8.2 with 0.19 M [^{14}C]ethylenimine (59,000 cpm/μmole). When the reaction was spectrophotometrically complete (2 hr) the excess volatile ethylenimine was removed by low-pressure evaporation to dryness at pH 11 into an acid trap. The reaction product was dissolved in water, adjusted to pH 5.5, and applied to a Dowex 50 column equilibrated with water. Elution was carried out with a linear gradient from 0 to 2 N NH_4OH and yielded a peak eluting at 0.1 N NH_4OH containing 47% of the 270-mμ-absorbing material (compound C) and a second peak eluting at 0.9 N NH_4OH (compound D) which accounted for 53% of the material. Neutralized aliquots were counted and showed compound C to contain 5200 cpm/A_{271} unit and compound D to contain 10,420 cpm/A_{271} unit. To convert this into molar stoichiometry we have made use of the fact that the ϵ_{271} of the product is 67% of the ϵ_{322} of 4-thioU which we determined to be 15.7×10^3 (the ϵ_{322} of 4-thioU is a useful parameter since this is the isosbestic wavelength for the transition between undissociated 4-thioU and the sulfide ion and hence is pH independent). Thus the ϵ_{271} of the product was determined to be 10.6×10^3. Using this factor the extent of aminoethylation of compounds C and D was found to be 0.93 mole/mole of nucleoside and 1.87 moles/mole of nucleoside, respectively. Since compound D had incorporated

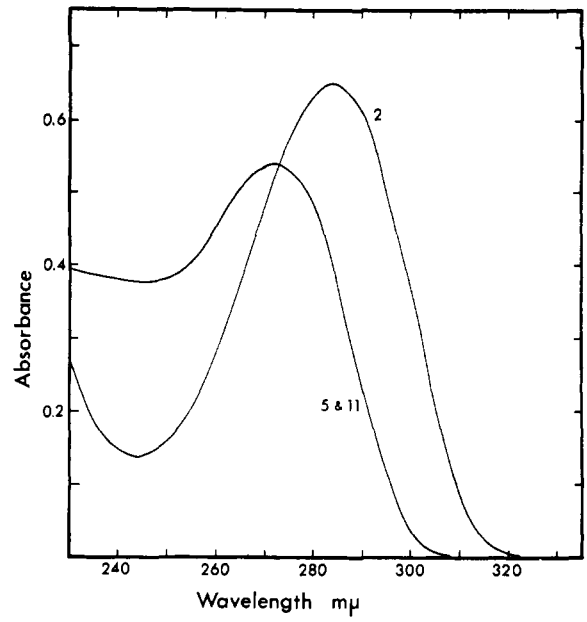

FIGURE 2. Ultraviolet spectrum of the dialkylated nucleoside (compound D). 4-ThioU was subjected to ^{14}C aminoethylation as described in the text and the reaction mixture chromatographed on Dowex 50. The material eluting in a symmetrical peak at 0.9 N NH_4OH (compound D) was found to have incorporated 2 moles of ethylenimine and was subjected to spectral analysis at the pH values indicated on the curves above.

2 moles of ethylenimine the possibility that the second aminoethylation had occurred on the N1 ring nitrogen was investigated by studying the pH-dependent spectrum of this compound. As seen in Figure 2 the dialkylated nucleoside still shows a typical cytidine bathochromic shift from 271 to 282 mμ upon protonation. This indicates that N1 has not been alkylated since N1,N3-dimethylcytosine exhibits the acid spectrum of cytidine at all pH values. It should be noted that the ultraviolet spectra of C and D are the same.

The only interpretation consistent with all of the above data is that C and D are in fact cytidine derivatives produced by rearrangement of the initially produced *S*-aminoethyl-4-thioU as shown in Figure 3. The identicality of the spectra of the monoalkylated and dialkylated compounds C and D coupled with their cytidine-characteristic bathochromic shifts upon protonation indicates that the second alkylation takes place on the rearranged side chain itself and has no effect on the chromophore. The possibility that the dialkylated sulfonium ion E is the species which rearranges can be discounted by the actual isolation of the monoalkylated intermediate C which already exhibits the cytidine-characteristic spectral properties. Furthermore we have observed that longer more exhaustive aminoethylation after the reaction is spectrophotometrically complete leads to the conversion of C into the dialkylated compound D at a relatively slow rate which is approximately equal to the rate of aminoethylation of simple alkyl thiols.

Essentially similar conclusions concerning the nature of the final reaction product were reported during the revision of this manuscript by Scheit (1969) using independent methods. An interesting aspect of the above reaction is the observation that, including the starting compound, four molecular

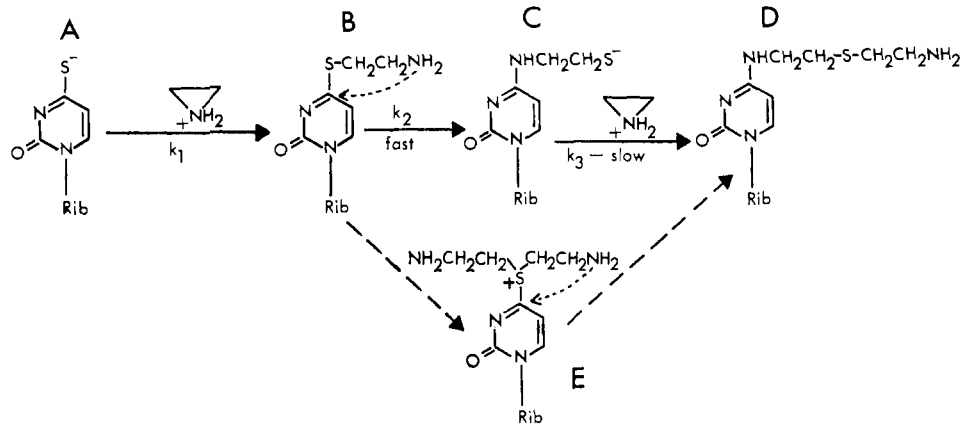

FIGURE 3: Mechanism of reaction of 4-thioU with ethylenimine to produce N^4-β-thioethyl-S-β-aminoethylcytidine.

species are present during the course of the reaction despite the sharp isosbestic point seen in Figure 1a which is usually interpreted as indicating a simple molecular transformation of one compound into another. The only rationalization of the experimentally observed time-dependent spectral change is that the intermediate B is present in a very low and undetectable steady-state concentration during the reaction due to a very rapid rearrangement reaction presumably caused by the high effective local concentration of the amino group which anchimerically displaces the sulfur from the pyrimidine ring.

Due to the limited amounts of material available we have not investigated fully the nature of the products from the aminoethylation of 2-thiopyrimidine nucleosides—however, one would predict that, once the initial S^2-aminoethylation has taken place, the rearrangement reaction would also take place in this compound at rates equal to or greater than those for S^4-aminoethylthiouridine.

pH Dependence and Ionic Form of the Reactive Species. Since the thionucleosides can exist either in the neutral or anionic form and ethylenimine itself can exist as the protonated cation or the free base, there are four possible reaction mechanisms theoretically possible. In light of the sigmoid-shaped pH dependence of the rate of cyanoethylation of 4-thioU it was of interest to determine the pK_a's of reagent and reactant and the pH dependence of the rate of aminoethylation in order to determine the ionic form of the reactive species. Direct titrimetric determination of dilute aqueous solutions of ethylenimine indicated a pK_a for protonation of the base of 8.05. The pK_a of 4-thioU was determined

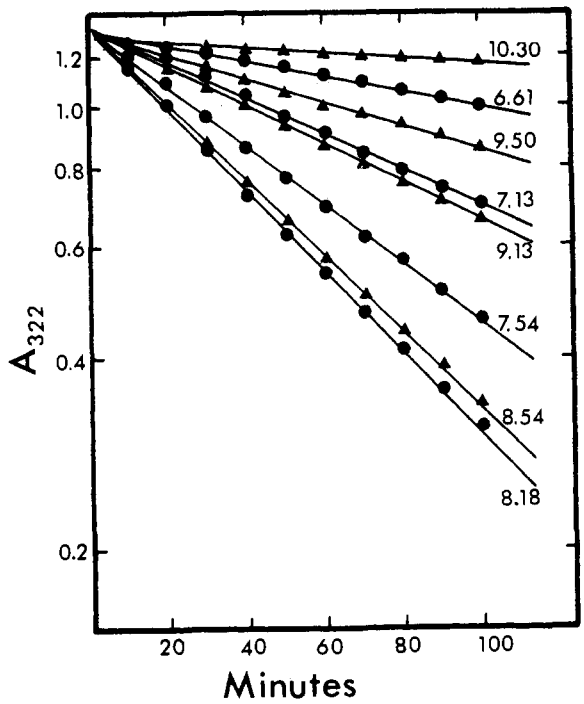

FIGURE 4: Rate of aminoethylation of 4-thioU as a function of pH. All reactions were performed at 25° with a total ethylenimine concentration of 1.9×10^{-1} M and a total 4-thioU concentration of 8.3×10^{-5} M. Reactions were adjusted to the indicated pH values as described in the Methods section and monitored at the ionization isosbestic wavelength of 322 mμ.

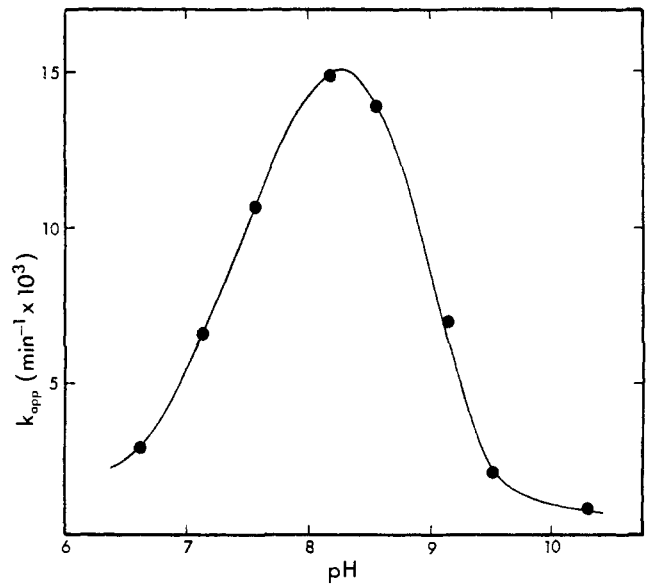

FIGURE 5: Reaction rate–pH profile of 4-thioU aminoethylation. The psuedo-first-order rate constants were derived from the rate plots in Figure 4 and plotted against the pH for each reaction.

TABLE I: Concentration of Ionic Species and Rate Constants for 4-ThioU Aminoethylation at Various pH Values.

pH	First-Order k_{app} (min^{-1})	Concn (EI^+) M × 10^{-3}	Concn (4ThioU-S^-) M × 10^{-5}	Initial Rate M × 10^{-6} min^{-1}	(EI^+) × (4ThioU-S^-) M^2 × 10^{-8}	Second-Order[a] k M^{-1} min^{-1}
6.61	2.82×10^{-3}	181.64	0.365	0.1911	66.3	
7.13	6.52×10^{-3}	169.67	1.087	0.4778	184.4	0.259
7.54	10.65×10^{-3}	145.16	2.324	0.7835	337.4	0.232
8.18	14.84×10^{-3}	80.94	5.229	1.0128	423.2	0.239
8.54	13.86×10^{-3}	46.36	6.599	0.9236	305.9	0.301
9.13	6.97×10^{-3}	14.63	7.785	0.4778	113.9	
9.50	2.00×10^{-3}	6.46	8.076	0.3058	52.2	
10.30	0.84×10^{-3}	1.06	8.263	0.0637	8.8	

[a] The second-order rate constant is calculated from the equation $k = (-dC/dt)/[(EI^+)(4thioU\text{-}S^-)]$.

in a spectrophotometric titration by monitoring the disappearance of the anionic form at 310 mμ or the disappearance of the neutral form at 330 mμ and both methods yielded a pK_a of 7.95—in good agreement with the value of 8.0 reported by Ofengand. Similarly spectrophotometric titration of 2-thioU at 275 mμ indicated a pK_a of 7.5. Armed with these values the rate of aminoethylation of 4-thioU as a function of pH was investigated under conditions where the total concentration of ethylenimine (all species) was 0.19 M and the total concentration of 4-thioU (all species) was 8.3×10^{-5} M. These reactions were conveniently monitored at 322 mμ since it had previously been shown that regardless of pH the product has no absorbance at this wavelength. Furthermore 322 mμ is the isosbestic point for the dissociation of 4-thioU into the anion so that all reactions have the same initial absorbance at this wavelength regardless of the pH of the reaction. As shown in Figure 4 the rate of aminoethylation is slow at pH 6 and pH 10 and is more rapid around pH 8. This is shown more obviously in Figure 5 in which the apparent pseudo-first-order rate constant is plotted as a function of reaction pH. The only reaction mechanisms consistent with this rate profile are the thionucleoside anion–ethylenimmonium cation reaction or the neutral nucleoside–uncharged ethylenimine reaction. Unfortunately these are difficult to distinguish kinetically due to the similarity in pK_a of the reagent and reactant. However in view of the markedly greater nucleophilicity of aromatic sulfide anions over the corresponding sulfhydryl compounds (Pearson *et al.*, 1968) and the demonstration that the 4-thioU anion is the reactive species in cyanoethylation reactions (Ofengand, 1967) we feel that the aminoethylation reaction proceeds *via* the 4-thioU anion and the protonated ethylenimmonium ion as the reactive species. Thus it is apparent why, even at pH 10.3, the reaction is still pseudo first order since the ethylenimmonium ion concentration is still more than an order of magnitude greater than that of the 4-thioU anion. Using the pK_a of reactant and reagent and the standard Henderson–Hasselbalch equation, the concentration of thionucleoside anion and ethylenimmonium cation can be calculated at each of the reaction pH values used in Figure 4, as shown in Table I. From the pseudo-first-order rate constant and the actual concentrations of the reactive species the true second-order rate constant for the 4-thioU anion–ethylenimmonium ion reaction can be evaluated by this admittedly somewhat insensitive method. Nevertheless, within the range of one pH unit either side of the pK_a's, the values obtained for the second-order rate constant are in quite good agreement.

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