Sodium Colistimethate I: Dissociations of Aminomethanesulfonates in Aqueous Solution

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Abstract □ Neutral solutions of aminomethanesulfonates are largely dissociated into hydroxymethylamino compounds and sulfite (and bisulfite) ions. Identical solutions are formed either by dissolving pure crystalline aminomethanesulfonic acids or by combining the amine, formaldehyde, and sulfite at appropriate pH. Solutions of aminomethanesulfonates can react with an additional molecule of formaldehyde bisulfite to give a disubstituted amine, which in turn dissociates to give more sulfite ion. Neutral solutions of sodium colistimethate comprise complex equilibria in which individual molecules are substituted by varying numbers of methanesulfonate or hydroxymethyl groups, possibly as many as 10, but with three or four of these species more highly favored than the rest (possibly for steric as well as statistical reasons). When more than three molecules each of formaldehyde and sulfite are available for reaction with colistin, no evidence for the existence of free colistin is found. Furthermore, the likelihood for the simultaneous existence of five unreacted amino groups in one molecule is very small.

Keyphrases □ Colistimethate sodium solutions—equilibria phenomena □ *n*-Butylaminomethanesulfonic acid—synthesis, model solutions □ Equilibrium constants—*n*-butylaminomethanesulfonic acid solutions □ Iodine titration—*n*-butylaminomethanesulfonic acid, colistimethate solutions □ IR spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure □ Electrophoresis—analysis

Methanesulfonation of free amino groups of pharmaceuticals is a technique which has been used for many years in attempts to improve such properties as toxicity (or more precisely, therapeutic ratio), compatibility, and solubility, and to eliminate undesired side effects such as pain on injection. A review of many of the classes of drugs subjected to methanesulfonation was published by Logeman and Miori (1). Throughout the years it seems to have been more or less tacitly assumed that these methanesulfonates were physiologically active by virtue of slow "hydrolysis" to give back the parent drug. However, this hydrolysis has not been adequately demonstrated.

Sodium colistimethate[1] is a methanesulfonate[2] derivative of colistin, a basic polypeptide antibiotic having five free amino groups (2). This derivative has the sought-for advantages of reduced toxicity (higher therapeutic ratio) and elimination of pain on injection. Two studies have been reported in which attempts were made to ascertain whether or not the methanesulfonate is hydrolyzed in human serum, and, if so, to what extent and in what form it is excreted in the urine (3, 4). In these studies the investigators succeeded in demonstrating some changes in the form of the drug, but the complexity of the problem precluded an exact description of these changes.

The purpose of the present investigation was to lay some groundwork for a study of the transformations of sodium colistimethate *in vivo* by exploring aqueous solutions of the material.

In order to obviate complications resulting from the presence of five reactive sites in the sodium colistimethate molecule, the authors first studied solutions of a simple model compound, *n*-butylaminomethanesulfonic acid. This was isolated for identification, both as the pure zwitterion $n\text{-BuN}\overset{+}{\text{H}}_2\text{CH}_2\text{SO}_3^-$ and the crude sodium salt, $n\text{-BuNHCH}_2\text{SO}_3\text{Na}$. It could be dissolved in water or prepared *in situ* in order to study its properties in solution. Some studies were also carried out with phenethylaminomethanesulfonic acid.

EXPERIMENTAL[3]

A. Materials—The zwitterion, $n\text{-BuN}\overset{+}{\text{H}}_2\text{CH}_2\text{SO}_3^-$, was prepared by gradual addition of one molecular equivalent of butylamine to a formalin[4] solution (gives two phases) followed by addition of sulfur dioxide. As the sulfur dioxide was added, the two liquid phases became one and crystals began to form. The exothermic reaction was allowed to reach 65°. When nearly a molecular equivalent of the gas had been added, the reaction had become quite thick. The suspension was chilled, filtered, washed with isopropanol and ether, and recrystallized from 90% isopropanol. The product melted at 135–137°; lit. m.p. 136–139° (5).

Anal.—Calcd. for $C_5H_{13}NO_3S$: C, 35.91; H, 7.84; N, 8.38. Found: C, 36.04; H, 7.96; N, 8.68.

IR and NMR spectra were consistent with the structure, and there was only one NMR singlet (at 4.16 p.p.m.) when the NMR was run within 0.5 hr. of dissolving.

The sodium salt, $n\text{-BuNHCH}_2\text{SO}_3\text{Na}$, was difficult to obtain pure because of its lability and water solubility. The zwitterion was dissolved in hot methanol, and this solution was added to a molecular equivalent of sodium hydroxide in methanol. Ether was added to precipitate the salt which was filtered. The analysis of this material indicated considerable inorganic contamination.

Anal.—Calcd. for $C_5H_{12}NNaO_3S$: C, 31.74; H, 6.39; N, 7.40; Na, 12.09. Found: C, 26.42; H, 5.67; N, 6.18; Na, 12.54.

There were two singlets in the NMR at 4.12 and 3.78 p.p.m., representing not the material as is, but the equilibrium mixture in D_2O (see below).

The cyclic trimer of formaldehyde and butylamine, 1,3,5-tri-*n*-butylhexahydro-*s*-triazine (HHT) was obtained from the 1 *N* *n*-butylaminomethanesulfonate solutions described in Part D of *Experimental*. The supernatant oils which separated at high pH were isolated. One sample was distilled and boiled at 95° at 0.14 mm.; lit. b.p. 132–134° at 4–5 mm. (6). Their IR spectra were

[1] Coly-Mycin Injectable brand of sodium colistimethate is manufactured by Warner-Chilcott Laboratories, Morris Plains, N. J.

[2] By the term aminomethanesulfonates is meant $>\text{NCH}_2\text{SO}_3^-$ or $>\overset{+}{\text{N}}\text{HCH}_2\text{SO}_3^-$.

[3] Boiling and melting points are uncorrected. NMR spectra were determined in D_2O solution using a Varian A60 unless otherwise specified. Delta values, rather than tau, are quoted. All analyses and spectral determinations carried out in the Analytical and Physical Chemistry Dept. of the Warner-Lambert Research Institute under the direction of Mr. A. D. Lewis. Special acknowledgement to Mrs. Unni Zeek for elemental analyses and to Dr. C. Greenough for spectra. Biogram determinations were carried out under the direction of Dr. Samuel Ringel of the Warner-Lambert Research Institute.

[4] Formaldehyde and sulfite may be added simultaneously in the form of sodium formaldehyde bisulfite. Throughout this paper the term "sulfite" is used to cover the gamut of forms from sulfite at high pH through bisulfite to sulfurous acid and sulfur dioxide at low pH.

similar to each other and to the distilled sample. The NMR had a singlet at 3.25 p.p.m. in $CDCl_3$.

Anal.—Calcd. for $C_{15}H_{33}N_3$: C, 70.53; H, 13.02; N, 16.45. Found: C, 70.25; H, 12.80; N, 16.36.

The specific gravity was determined to be 0.87. Identical material was obtained from butylamine-formaldehyde solutions in the absence of sulfite.

B. NMR Measurements—A 2 *N* stock solution was prepared from equimolar amounts of *n*-butylamine hydrochloride and sodium formaldehyde bisulfite in deuterium oxide. To aliquots of this solution were added various amounts of sodium hydroxide and these solutions were diluted to 1 *N*, allowed to equilibrate to constant pH, and the NMR spectra run. Various singlets were found in the spectra which represent the protons of $-CH_2-$ between two heteroatoms. The ratio of the integral of these singlets to the total integral was calculated for each solution. If only one species containing CH_2 between heteroatoms were present, the ratio of its singlet integral to the total integral should be 2/11 or 0.18. The observed singlet ratio divided by 0.18, times 100, is therefore the percent of theory for each species represented by a singlet. The latter figure is plotted against pH in Fig. 1. Table I summarizes the data obtained.

As Table I shows, three singlets were involved. One was present only below pH 7.9. Its position was constant with pH at 4.4 p.p.m., the same as that of formaldehyde bisulfite, $DOCH_2SO_3Na$. Its constancy in position between pH 3.2 and 6.8 is reasonable since a proton from the strong acid, formaldehyde bisulfite, should be completely dissociated in this range. The 4.4 signal was therefore assigned to $HOCH_2SO_3^-$.

The second singlet was nearly constant with pH at 4.16 to 4.23 p.p.m. It was assigned to *n*-$BuND_2CH_2OD$ because of the parallel between the free sulfite-bisulfite curve of Fig. 3 and the curve for this singlet in Fig. 1. Its constancy with pH and estimated pKa of roughly 8 to 9 (see *Discussion*) indicate that only the acidic form is important.

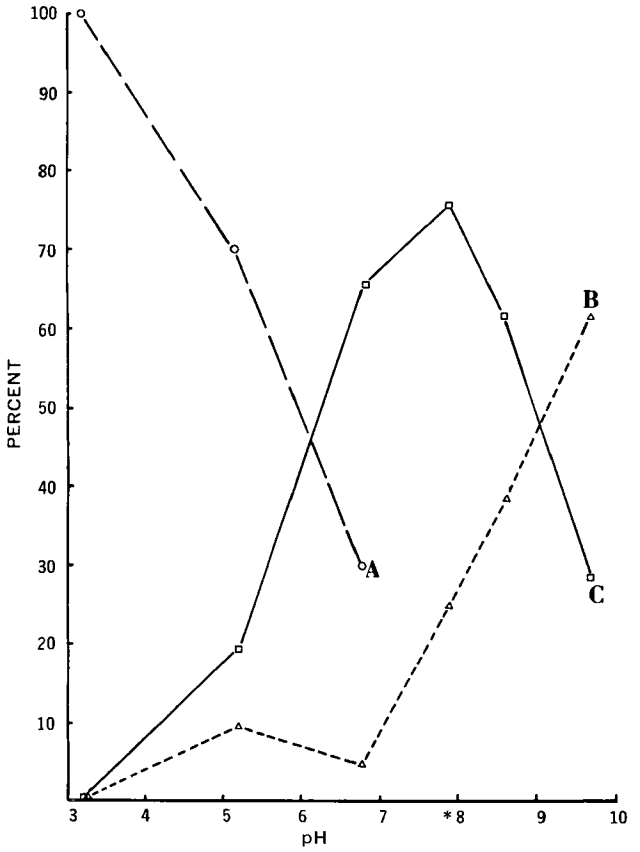


Figure 1—*NMR studies. Percent compositions in 1* N n-*butylaminomethanesulfonate solutions. Key: *, $HOCH_2SO_3^-$ signal missing at pH 7.9 and higher. A, $HOCH_2SO_3^-$; B, n-$BuNH_2^+CH_2SO_3^-$ ⇌ n-$BuNHCH_2SO_3^-$ ⇌; C, n-$BuNH_2^+CH_2OH$.*

Table I—NMR Data for 1 *N* *n*-Butylaminomethanesulfonate Solutions at Varying pH

Equivalents[a] NaOH	pH	Singlets: Position (p.p.m.)	% of Total Integral[b]	% of Theory
0.00	3.2	4.40 (O—CH_2—S)	18.0	100
0.05	5.2	4.43 (O—CH_2—S)	12.9	71.5
—	—	4.23 (N—CH_2—O)	3.5	19.5
—	—	4.18 (N—CH_2—S)	1.7	9.5
0.40	6.8	4.43 (O—CH_2—S)	5.3	29.6
—	—	4.18 (N—CH_2—O)	11.8	65.5
—	—	4.03 (N—CH_2—S)	0.9	4.9
0.50	7.9	4.16 (N—CH_2—O)	13.6	75.6
—	—	3.82 (N—CH_2—S)	4.4	24.4
0.70	8.6	4.18 (N—CH_2—O)	11.1	61.5
—	—	3.75 (N—CH_2—S)	6.9	38.5
1.00	9.7	4.17 (N—CH_2—O)	5.8	32.2[c]
—	—	3.78 (N—CH_2—S)	12.2	68.0[c]

[a] Equivalents relative to amine and formaldehyde bisulfite. [b] Integrals are accurate to ±2% for higher values, ±5% for lower values. [c] These figures become 28.8 and 61.2% when corrected for HHT if deuterium isotope effects are ignored. See Part D of *Experimental* for discussion of this correction.

The third singlet varied in position from 4.28 p.p.m. at pH 5.2 to 3.78 p.p.m. at pH 9.7. Since neither S, O, or $-SO_3^-$ should protonate in this range, one of the heteroatoms must be nitrogen. The variation in the average degree of protonation on nitrogen in *n*-$BuNDCH_2SO_3^-$ accounts for the chemical shift. If the other heteroatom were also nitrogen, however, the unprotonated species would be strongly basic while the doubly protonated species would be strongly acidic. Yet, the shift occurs essentially between pH 5.2 and 8.6. Thus, the third singlet is assigned to the remaining possibility, *n*-$BuND_2^+CH_2SO_3^-$ ⇌ *n*-$BuNDCH_2SO_3^-$. This assignment is confirmed by the fact that the pure solid zwitterion, *n*-$BuND_2^+$-$CH_2SO_3^-$, gives a singlet at 4.16 when dissolved in D_2O and run within 30 min., while the crude solid Na salt gives signals at 4.12 and 3.78 p.p.m. of the same relative intensities as those of the pH 9.7 solution of Fig. 1.

Figure 2 is a plot of chemical shift *versus* pH for the N—CH_2—S species from the data of Table I. From the inflection point of this graph, the pKa is estimated to be roughly 7.1.

The following is a calculation of K_1 for the overall reaction: $RNHCH_2SO_3^- + H_2O \overset{K_1}{\rightleftharpoons} RNH_2^+CH_2OH + SO_3^=$.

$$K_1 = \frac{[RNH_2^+CH_2OH]\,[SO_3^=]}{[RNHCH_2SO_3^-]}$$

where $[RNH_2^+CH_2OH]$ = the observed value from Table I.

$$[SO_3^=] = \frac{[HSO_3^-] + [SO_3^=]}{1 + H^+/K_2}$$

where $K_2 = [SO_3^=]/[HSO_3^-] = 1.1 \times 10^{-7}$ (from literature values) and $[HSO_3^-] + [SO_3^=]$ is assumed equal to $[RNH_2^+CH_2OH] + 3[HHT]$ (from Table VI below).

$$[RNHCH_2SO_3^-] = \frac{[RNHCH_2SO_3^-] + [RNH_2^+CH_2SO_3^-]}{1 + H^+/K_3}$$

where $K_3 = [RNHCH_2SO_3^-]/[RNH_2CH_2SO_3^-] = 8 \times 10^{-8}$ (based on the pKa determination of Fig. 2) and $[RNHCH_2SO_3^-] + [RNH_2^+CH_2SO_3^-]$ is observed value from Table I.

The value of the equilibrium constant for the overall reaction, $RNH_2^+CH_2OH + SO_3^= \overset{K_4}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$, is calculated as follows:

$$K_4 = \frac{[RNH_2]\,[HOCH_2SO_3^-]}{[RNH_2^+CH_2OH]\,[SO_3^=]}$$

where

$$[RNH_2] = \frac{[RNH_3^+] + [RNH_2]}{1 + H^+/K_5}$$

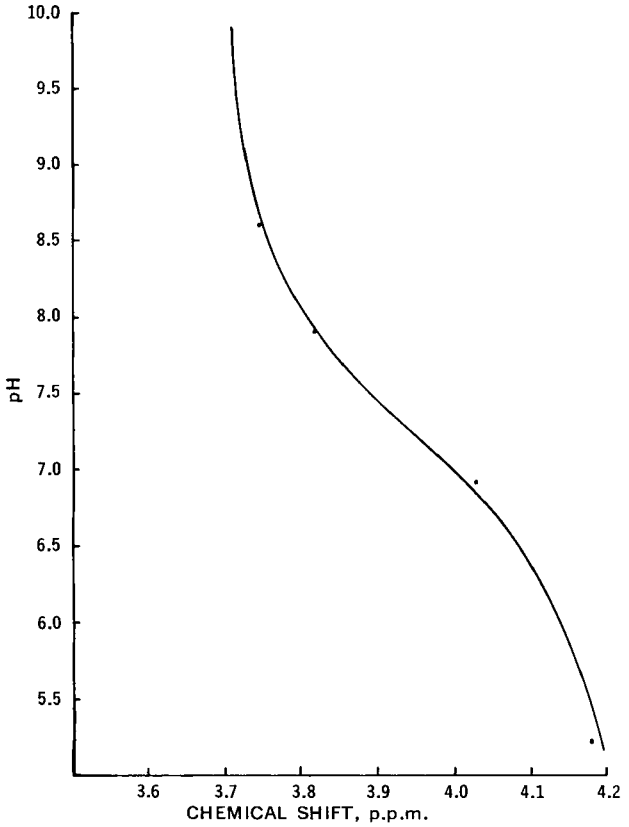


Figure 2—*Determination of the pKa of* $RNH_2^+CH_2SO_3^-$.

where $[RNH_3^+] + [RNH_2]$ is assumed equal to the observed value for $[HOCH_2SO_3^-]$ from Table I, and $K_5 = [RNH_2]/[RNH_3^+] = 2 \times 10^{-11}$ (from literature values). $[HOCH_2SO_3^-]$ = the observed value from Table I. $[RNH_2^+CH_2OH]$ = the observed value from Table I. $[SO_3^=]$ = the calculated value from Table II.

The equilibrium constant for the reaction, $RNHCH_2SO_3^- + H_2O \underset{K_6}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$, is calculated as follows:

$$K_6 = \frac{[RNH_2]\,[HOCH_2SO_3^-]}{[RNHCH_2SO_3^-]}$$

$[RNH_2]$ = the calculated value from Table III; $[HOCH_2SO_3^-]$ = the observed value from Table I; $[RNHCH_2SO_3^-]$ = the calculated value from Table II. Numerical values for K_1, K_4 and K_6 are listed in Tables II, III and IV.

The accuracy of these equilibrium constants is limited by the accuracy of the pH and NMR integral determinations, the validity of transposing data for the concentration of HHT from an aqueous to a deuterium oxide system, and the accuracy of the pKa determination for methanesulfonate from Fig. 2. Considering these factors, the equilibrium constants are reasonably consistent.

C. Iodine Titrations: The Technique—In all of the iodine titrations, the technique was patterned after Stewart and Donnally (7) who quenched benzaldehyde bisulfite solutions in acid, converting free sulfite and bisulfite to sulfurous acid. In acid solution,

Table II—Equilibrium Constant for the Reaction $RNHCH_2SO_3^- + H_2O \underset{K_1}{\rightleftharpoons} RNH_2^+CH_2OH + SO_3^=$[a]

pH	$(RNH_2^+CH_2OH)$	$(SO_3^=)$	$(RNHCH_2SO_3^-)$	K_1[a]
5.2	1.95×10^{-1}	3.34×10^{-3}	1.18×10^{-3}	0.55
6.8	6.55×10^{-1}	2.67×10^{-1}	1.63×10^{-2}	1.07
7.9	7.56×10^{-1}	6.78×10^{-1}	2.10×10^{-1}	2.44
8.6	6.15×10^{-1}	6.01×10^{-1}	3.73×10^{-1}	0.99
9.7	2.88×10^{-1}	4.54×10^{-1}	6.10×10^{-1}	2.13

[a] The average for $K_1 = 1.46$.

Table III—Equilibrium Constant for the Reaction $RNH_2^+CH_2OH + SO_3^= \underset{K_4}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$[a]

pH	(RNH_2)	$(HOCH_2SO_3^-)$	$(RNH_2^+-CH_2OH)$	$(SO_3^=)$	K_4
5.2	2.27×10^{-6}	7.15×10^{-1}	1.95×10^{-1}	3.34×10^{-3}	2.49×10^{-3}
6.8	3.71×10^{-5}	2.96×10^{-1}	6.55×10^{-1}	2.67×10^{-1}	6.31×10^{-3}

[a] Average $K_4 = 4.40 \times 10^{-3}$.

subsequent reactions to form or eliminate sulfurous acid were very slow. The sulfite and bisulfite in the original solution were then determined by titrating with standard iodine. Stewart and Bradley (8) and Le Henaff (9) have shown that reactions involving sulfurous acid are slow at low pH in solutions containing dialkylaminomethanesulfonates and methylaminomethanesulfonate. These data for *n*-butylaminomethanesulfonate solutions consist of three parts, each showing that very little sulfurous acid is either formed or consumed at low pH.

1. Zwitterion Dissociation—The pure zwitterion, *n*-$BuNH_2^+CH_2SO_3^-$, was dissolved in water to make a 1 *N* solution. When solution was complete, 0.1 *N* standard iodine solution was added to an end point at pH 1.9. This titration consumed iodine equivalent to only a very small percentage of the potential SO_2 present. After 5 min. the solution was titrated to a new end point. Enough iodine was decolorized during the latter titration to account for 0.4% of the sulfur present. Another sample was dissolved in aqueous HCl and titrated to an end point at pH 0.8. After 5 min., enough iodine was decolorized to account for 0.2% of the sulfur present.

2. Formaldehyde Bisulfite Dissociation—In a similar manner, 1 *N* solutions of formaldehyde bisulfite and butylamine hydrochloride were prepared and titrated to an end point at pH's 2.0 and 1.1. Very little iodine was consumed. After 5 min. the solutions were again brought to end points. Enough iodine was consumed in the latter titrations to account for 0.2% and less than 0.1% of the sulfur present, respectively.

3. Sulfite-Carbinol Amine Recombination—A solution was prepared which was 1 *N* in starting butylamine hydrochloride and formaldehyde bisulfite and contained enough sodium hydroxide so that the pH came to 7 after equilibrating overnight. Five-milliliter aliquots were quenched in acid to various pH's and titrated both immediately and after several minutes. The data are summarized in Table V. Note that the difference between the iodine consumed immediately and after several minutes is a rough measure of the reaction of carbinolamine with sulfurous acid at low pH. This is because the original solution at pH 7 contains a good deal of carbinolamine (see Fig. 1) and carbinolamine is the only species present which does not already contain $-SO_3^-$. Table V also gives an indication of the reproducibility.

All of the iodine titrations mentioned below were carried out by quenching solutions between pH 1 and 2 with 1.0 to 0.1 *N* HCl and titrating immediately with 0.1 *N* iodine solution. Quenching was accomplished by running the unknown solution into a stirring solution of the HCl at room temperature.

D. Iodine Titrations: Determination of Sulfite Content of n-$BuNHCH_2SO_3^-$ Solutions at Varying pH—A series of solutions were prepared as for the NMR measurements except that H_2O was used in place of D_2O. Thus, the solutions were 1 *N* in starting butylamine hydrochloride and formaldehyde bisulfite and varied in sodium hydroxide. These solutions were equilibrated overnight at room temperature although equilibration was essentially complete within 2–3 hr. In those solutions whose pH's were 9.2 and above, a supernatant oil had appeared which was identified (Part A of *Experimental*) as the hexahydrotriazine (HHT). Five-milliliter samples of each of the solutions were quenched to between pH 1

Table IV—Equilibrium Constant for the Reaction $RNHCH_2SO_3^- + H_2O \underset{K_6}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$[a]

pH	(RNH_2)	$(HOCH_2SO_3^-)$	$(RNHCH_2SO_3^-)$	K_6
5.2	2.27×10^{-6}	7.15×10^{-1}	1.18×10^{-3}	1.38×10^{-3}
6.8	3.71×10^{-5}	2.96×10^{-1}	1.63×10^{-2}	0.68×10^{-3}

[a] Average $K_6 = 1.03 \times 10^{-3}$.

Table V—Effect of Time and pH on Quenched Carbinolamine-Sulfite Solutions

—Quench Soln.— Vol., ml.	Concn., HCl	pH after Quenching	Time before Titration, min.	I_2 Consumed (% of theory for Total S)	Approx. pH after Titration
3.5	0.1 *N*	4.1	<1	76.4	1.2
3.5	0.1 *N*	4.1	5	74.0	1.2
5.0	0.1 *N*	3.1	<1	76.2	1.2
5.0	0.1 *N*	3.1	7	73.2	1.2
1.7	0.5 *N*	2.0	<1	76.0	1.2
1.7	0.5 *N*	2.0	9	69.4	1.2
2.0	1.0 *N*	1.0	<1	76.4	0.6
2.0	1.0 *N*	1.0	11	67.0	0.6
10.0	1.0 *N*	0.2	<1	75.2	?

and 2 and titrated immediately with 0.1 *N* iodine. In those solutions which had a supernatant oil, the sample to be quenched was drawn from the aqueous layer. The supernatant oils were then separated and weighed. The percent of theory for HHT was calculated. The titers for the high pH samples were then corrected for the volume (weight divided by specific gravity) of HHT not represented. The data are summarized in Table VI and plotted in Fig. 3.

E. Effect of pH on the Reaction of Formaldehyde with n-Butylamine and Sulfite—A series of solutions were prepared 0.1 *N* in *n*-butylamine hydrochloride and sulfur dioxide, and up to 0.12 *N* in sodium hydroxide. The pH of each was determined and one molecular equivalent of formalin was added. The immediate pH change was noted and an aliquot was quenched and titrated with 0.1 *N* iodine. The titration was complete within 5 min. of formalin addition. After 1.5 hr., the pH was redetermined and another aliquot similarly titrated. The data are summarized in Table VII and plotted in Fig. 4.

F. Reaction Rate at Constant pH—A series of solutions were prepared 1 *N* in butylamine hydrochloride and sodium formaldehyde bisulfite. These were adjusted at room temperature to a given pH with 1 *N* sodium hydroxide from a buret. The pH was continuously monitored and 1 *N* NaOH was added as necessary to maintain a constant pH. Examples were also studied at greater dilution, higher temperature, and with incorporation of an additional molecule of sodium formaldehyde bisulfite. The pH's were maintained essentially within the limits indicated in Fig. 5, except in the pH = 9 case where the reaction was very rapid and close pH control was impossible. The irregularity of the lines reflect the inconstancy of pH. The data are plotted in Fig. 5

G. Iodine Titration: Effect of Varying Ratio of Reactants—Three stock solutions were prepared: (*a*) 1 *N* in *n*-butylamine hydrochloride and 1 *N* in sodium formaldehyde bisulfite; (*b*) 1 *N* in *n*-butylamine hydrochloride and 1 *N* in sodium formaldehyde bisulfite with the addition of one equivalent of formaldehyde; (*c*) 1 *N* in *n*-butylamine hydrochloride and 2 *N* in sodium formaldehyde bisulfite. Samples of each of these solutions were treated with varying amounts of 1 *N* sodium hydroxide solution. Aliquots of these solutions were periodically quenched in acid and titrated with

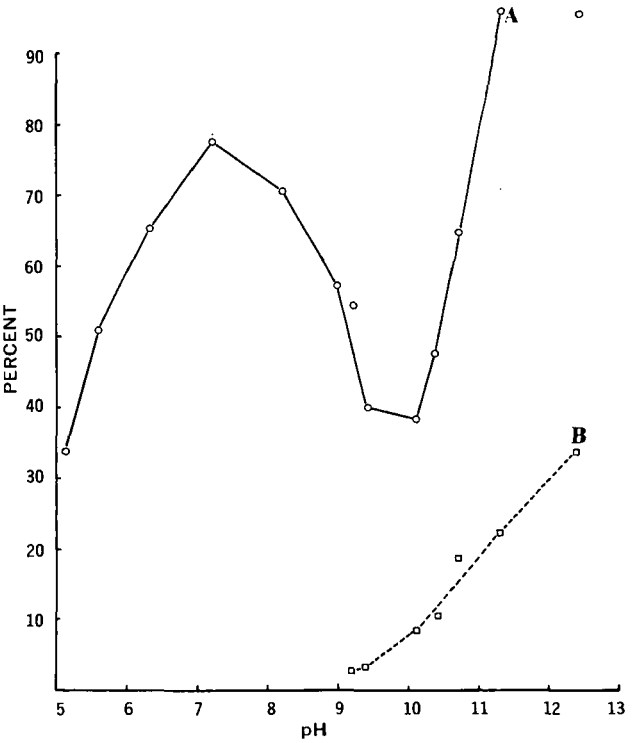


Figure 3—*Iodine titrations. Percent of theory for sulfite-bisulfite concentration in 1* N *n-butylaminomethanesulfonate solutions and percent of theory for HHT. Key: A, $SO_3^=$ and HSO_3^-; B, HHT.*

iodine until constant titers indicated equilibrium. Table VIII records the data obtained at the various pH values and Fig. 6 gives a graphic presentation of iodine consumed *versus* pH.

H. Iodine Titration of Colistin methanesulfonate Solutions—A series of solutions were prepared which were 0.35 *M* in colistin sulfate and contained from one to twenty molecular equivalents of sodium formaldehyde bisulfite. Enough sodium hydroxide was incorporated to bring the pH at equilibrium to 7.0. Each of these solutions was divided into three parts which were treated as follows: (*a*) allowed to stand at room temperature for 20 hr.; (*b*) allowed to stand at 5° for 20 hr.; (*c*) diluted to 0.047 *M* with water and allowed to stand at room temperature for 20 hr. Following each treatment, the samples were quenched in acid and titrated with iodine. The data so obtained are shown in Table IX and are presented in graphic form in Fig. 7.

I. Electrophoresis of Colistin methanesulfonate Solutions—A series of solutions were prepared which were 0.35 *M* in colistin sulfate and contained from 0 to 10 molecular equivalents of sodium formaldehyde bisulfite. Enough sodium hydroxide was incorporated to bring the pH to 7.0 after several days equilibration. One lambda of each solution was spotted on strips of Eastman cellulose-coated

Table VI—Determination of Sulfite Content of *n*-$BuNHCH_2SO_3^-$ Solutions at Varying pH

Moles NaOH Added	pH after 20 hr.	Equivalents I_2 Consumed	Percentage S Present as $SO_3^=$	Percentage HHT Present
0.2	5.1	0.34	33.8	—
0.3	5.6	0.51	51.0	—
0.4	6.3	0.66	65.8	—
0.5	7.2	0.78	77.8	—
0.6	8.2	0.71	70.8	—
0.7	9.0	0.57	57.4	—
0.8	9.2	0.56	54.5	2.8
0.9	9.4	0.42	40.1	3.5
1.0	10.1	0.43	38.8	8.7
1.2	10.4	0.54	47.8	10.6
1.4	10.7	0.83	64.9	18.8
1.6	11.3	1.30	96.3	22.6
2.0	12.4	1.58	96.0	34.1

Table VII—pH and Iodine Titration Changes in Reaction of Formaldehyde with *n*-Butylamine and Sulfite

Normality in NaOH	pH before Addition of HCHO	pH just after Addition of HCHO	Equivalents I_2 Consumed (Initially)	Final pH	Equivalents I_2 Consumed (Finally)
0.000	3.8	4.0	0.57	3.1	0.14
0.0025	3.8	4.0	0.53	3.0	0.06
0.005	4.6	4.7	0.69	3.3	0.06
0.01	5.5	5.7	0.55	6.2	0.05
0.02	6.1	6.9	0.25	6.6	0.18
0.04	6.4	7.2	0.10	7.2	0.40
0.06	6.9	8.4	0.12	8.0	0.48
0.08	7.0	8.6	0.30	8.7	0.45
0.10	7.5	9.2	0.16	9.7	0.24
0.12	9.0	10.4	0.35	10.2	0.19

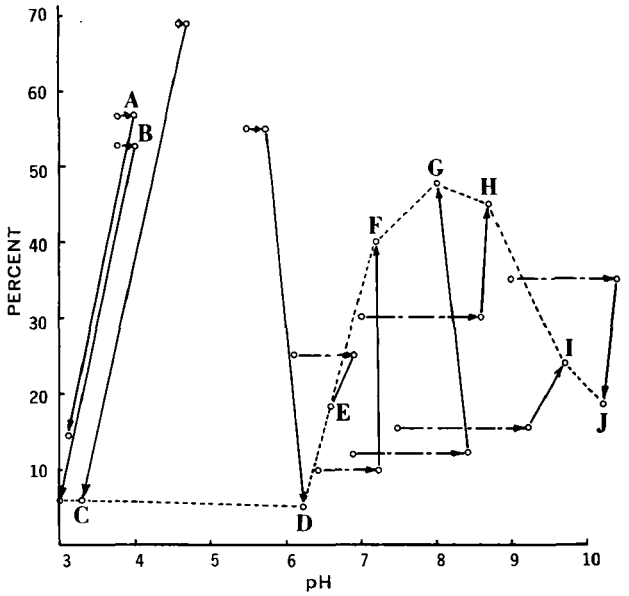


Figure 4—*Iodine titrations. Kinetic effects of adding formalin to 0.1 N $BuNH_2$-SO_2-NaOH. Changes in pH and percent of theory for sulfite. Key: ⟶ pH and sulfite change during 1.5 hr.; — — — ⟶ initial pH change; - - - - - - - - - equilibrium curve. A, 0.000 N in NaOH; B, 0.0025 N in NaOH; C, 0.0050 N in NaOH; D, 0.0100 N in NaOH; E, 0.0200 N in NaOH; F, 0.0400 N in NaOH; G, 0.0600 N in NaOH; H, 0.0800 N in NaOH; I, 0.1000 N in NaOH; J, 0.1200 N in NaOH.*

chromatogram sheet which had been prewetted with pH 7 phosphate buffer and subjected to electrophoresis in a pressure pad apparatus (E-C Corp.) with pH 7 buffer for 1 hr. and 20 min. at 300 v. (16.5 ma.). The electrophoresis was carried out in a cold room at 5°. Immediately after electrophoresis, the strips were dried in an oven at 80° and sprayed with ninhydrin. The results are shown in Fig. 8.

J. Electrophoresis of Radioactive Colistin methanesulfonate Solution—A 0.35 *N* solution of sodium colistimethate (prepared using radioactive formaldehyde) was stored 9 days at room temperature and then 2 days at 5°. One lambda was spotted on a strip of 3 MM paper which had been prewetted in pH 7 buffer and subjected to electrophoresis in a pressure pad apparatus in a cold room at 5° using pH 7 buffer. The electrophoresis was run for 4 hr. at 500 v. (22 ma.). The paper was immediately dried in an oven at 80°. The radioscan is shown in Fig. 9. In similar electrophoreses, all of the peaks except the one indicated by an arrow were ninhydrin positive and all but this one and the well-separated ones at the end were bio-active. Furthermore, a set of electrophoreses were run in which one strip was spotted with radioactive formaldehyde bisulfite, one with the radioactive sodium colistimethate, and a third with the one spotted on top of the other. In this way it was demonstrated that the peak indicated by an arrow is formaldehyde bisulfite. The integral of this peak is only about 5% of the total integral. Hence, the likelihood of the presence of free colistin in which none of the amino groups are substituted is statistically extremely small.

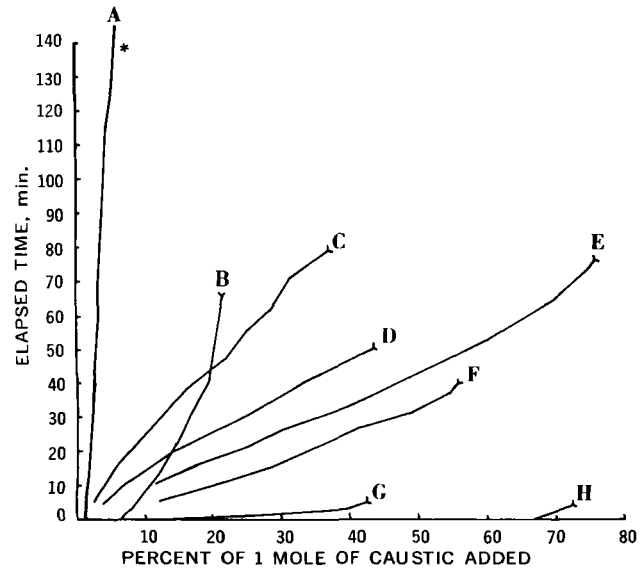


Figure 5—*Potentiometric titration. Equilibration rate studies. Caustic was added to $BuNH_3Cl$-$HOCH_2SO_3Na$ solutions at controlled rate. pH maintained as indicated. Ambient temperature, equimolar reactants, and 1 N initial concentration, except as indicated. Key: *, equilibrium reached at constant rate in 5 hr. and 20 min. A, 5.8–6.0; B, 7.0–7.2, 0.1 N; C, 6.3–6.5; D, 7.0–7.2; E, 7.0–7.2 extra mole $HOCH_2SO_3Na$; F, 8.0–8.2; G, 7.0–7.2, 54–57°; H, about 9.0.*

Table VIII—Change in Composition of *n*-Butylaminomethanesulfonate Solutions with Change in pH and Reactant Ratio

Molar Ratio	Equiv. NaOH Added	pH Attained at Equilibrium	Equiv. I_2 Consumed
Equimolecular amounts of amine hydrochloride and sodium formaldehyde bisulfite	0.10	5.1	0.17
	0.20	5.6	0.36
	0.30	6.1	0.56
	0.40	6.6	0.72
	0.50	7.6	0.77
	0.60	8.2	0.73
	0.70	8.6	0.76
	0.80	9.0	0.61
	0.90	9.7	0.28
Equimolecular amounts of amine hydrochloride and formaldehyde bisulfite with addition of 1 mole of formaldehyde	0.20	5.3	0.06
	0.80	6.8	0.19
	1.00	7.4	0.32
	1.10	8.9	0.37
	1.50	10.6	0.08
1 mole of amine hydrochloride with 2 moles of sodium formaldehyde bisulfite	0.20	5.3	0.25
	0.80	6.8	1.20
	1.00	7.8	1.40
	1.10	9.1	1.32
	1.50	10.7	0.70

DISCUSSION

Two early observations led the authors to suspect that aminomethane sulfonate solutions were involved in complex equilibria. When the crude sodium salt, *n*-$BuNHCH_2SO_3Na$, was dissolved in water (giving a pH of about 9.7), an insoluble oil soon separated which proved to be the cyclic hexahydrotriazine (HHT) derived from three moles each of the amine and formaldehyde. It was also noted that when a sample of the zwitterion was dissolved in deuterium oxide (giving a pH of about 3.2), the NMR singlet characteristic of the protons of the methylene group between N and S slowly decreased and a new signal appeared corresponding to the protons of the methylene group of $DOCH_2SO_3^-$. These transformations evidently involved more than simple hydrolysis to the parent amine.

In view of these observations methods of distinguishing several species without disturbing the equilibria were sought. Fortunately, several of these species have methylene groups between two heteroatoms which are seen as singlets in the NMR. The chemical shifts of these singlets serve to distinguish one species from another. Their integrals are a measure of concentration. Therefore, NMR's were run on a set of equilibrated 1 *N* *n*-butylaminomethanesulfonate solutions in which three such species were identified, as described in the *Experimental* section, Part B.

The data from these NMR studies are presented graphically as Fig. 1. This graph shows the change in relative magnitude of these species with pH. These magnitudes are reasonable for the following reasons:

1. Formaldehyde bisulfite should be a more stable species than carbinolamine at low pH because free amine concentration is much

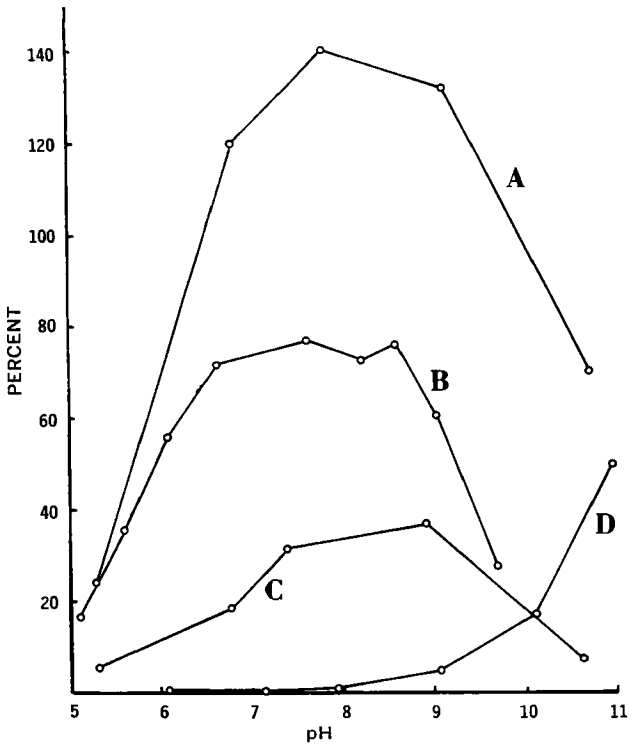
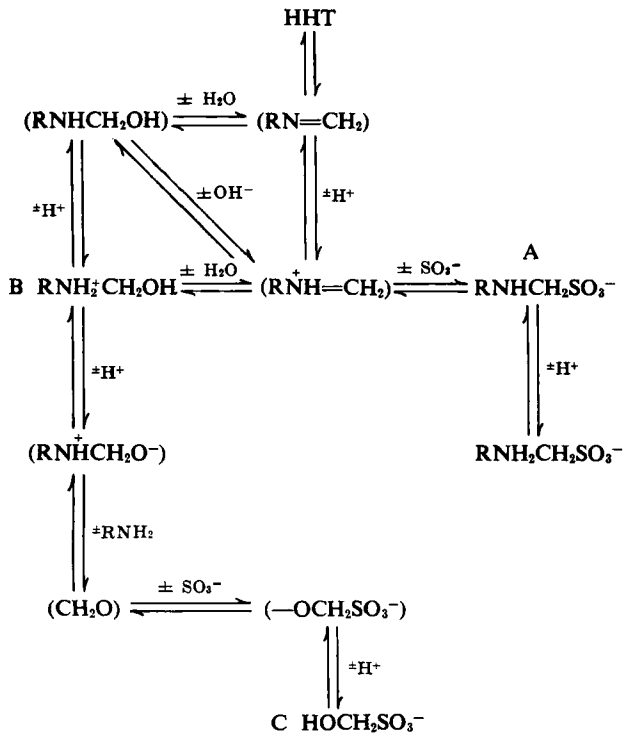


Figure 6—*Iodine titrations. Effect of reactant ratio on sulfite concentration in 1* N $HOCH_2SO_3^-$, *1* N n-$BuNHCH_2SO_3^-$, *and 1* N n-$BuNHCH_2SO_3^-$ *plus molecular equivalent HCHO or* $HOCH_2SO_3^-$. *Key: A, Sulfite from* n-$BuNHCH_2SO_3^-$ + $HOCH_2SO_3^-$; *B, Sulfite from* n-$BuNHCH_2SO_3^-$; *C, Sulfite from* n-$BuNHCH_2SO_3^-$ + *HCHO; D, Sulfite from* $HOCH_2SO_3^-$.

lower than sulfite concentration. Both of these species are in competition for free formaldehyde (see Scheme I).



Scheme I

2. The maximum for carbinolamine at pH 8 is reasonable since this is roughly the pKa of carbinolamine. Thus, Kallen and Jencks (10) have found that primary carbinolamines have pKa's roughly 2 to 3 units lower than the parent amine (10.7 for *n*-butylamine). The data of Table I show that the basic form of carbinolamine is negligible and is thus apparently less stable than HHT, which floats to the surface and is not seen in the NMR.

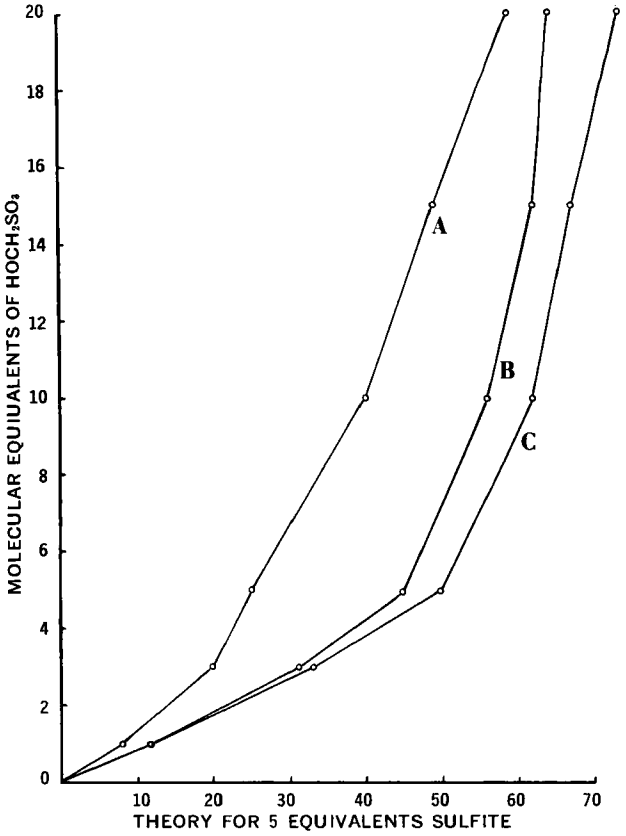


Figure 7—*Iodine titrations. Effect of reactant ratio on sulfite concentration in colistin methanesulfonate at pH 7.0. Key: A, R.T., 0.044* N *in colistin sulfate; B, R.T., 0.35* N; *C, 5°, 0.35* N *in colistin sulfate.*

3. The concentration of methanesulfonate should begin to rise around pH 7.1. As is shown in Fig. 2, 7.1 is roughly the pKa of the methanesulfonate. The data of Table I show that the acidic form of the methanesulfonate is minor.

Finally, the data show that the protonated form of HHT is absent. Since it should be a strong base, it is reasonable that it should appear only as the pH becomes high.

Evidently, the species described above are interconverted by a set of equilibria. In Scheme I, an attempt is made to give a summary of the equilibria which may be involved. In doing so the authors invoke the carbonium-immonium intermediate postulated for other Mannich base-type reactions (11). Species which are present in concentrations too small to detect by NMR or iodine titration are represented in brackets. The letters A, B, and C identify the species for which the equilibrium constants of interconversion have been calculated.

Table IX—Iodine Titrations of Colistin Methanesulfonate Solutions

Equivalents $HOCH_2SO_3Na$ Added	Room Temp. 0.35 M^a	5°, 0.35 M^a	Room Temp. 0.047 M^a
1	12	12	8
3	31	33	20
5	45	50	25
10	56	62	40
15	62	67	49
20	64	73	59

[a] The figures are the iodine consumed calculated as a percentage of five equivalents.

The consistency of the equilibrium constants calculated in the *Experimental* section lends support to this scheme. Note, also, that since free energy is proportional to log K, the log of K_1 (for the reaction A → B) plus the log of K_4 (for the reaction B → C) should equal log K_6 (for the reaction A → C). Thus, $0.0164 + (-2.36) \cong -2.98$, in further support of Scheme I.

Besides NMR, these equilibria have been studied by means of acid quenching. The equilibrium is thus effectively frozen with respect to free sulfite and bisulfite, and these species can then be titrated with iodine. It has been established (*Experimental* section) that the amount of sulfite or bisulfite ion in any aminomethanesulfonate solution can be measured by iodine titration after quenching in 0.1 N hydrochloric acid. At this low pH, any reaction to form or eliminate sulfurous acid is slow.

In Fig. 3, this technique is used to further study the variation of 1 N butylaminomethanesulfonate equilibria with pH and is presented in defense of Scheme I. It is interesting that the amount of sulfite (or bisulfite) parallels the amount of $RNH_2^+CH_2OH$ measured by NMR (Fig. 1) and, in particular, reaches a maximum at about pH 8. This is because $RNH_2^+CH_2OH$ is the only species measured by the NMR which does not contain the $-SO_3^-$ moiety. This finding lends added credence to Scheme I.

At pH 9–10, the amount of free sulfite reaches a minimum but does not go to zero. At still higher pH, the value rises rapidly. Also in Fig. 3, the amount of supernatant HHT which appears at high pH is plotted. The rise in free sulfite at high pH is to be expected, since amine and formaldehyde are sequestered in the form of HHT. From the data of Fig. 3, Scheme I is further supported by presenting direct evidence of free sulfite and bisulfite and of HHT. These species could not be seen in the NMR spectra.

This article has thus far discussed the composition of aqueous *equimolar* aminomethanesulfonate solutions *at equilibrium* and its variation with pH. It was also felt useful to determine what happens as this equilibrium is displaced. The discussions on Figs. 4 and 5 which follow therefore relate to kinetics.

Figure 4 shows what happens *kinetically* when formalin is added to 0.1 N RNH_2-SO_2-NaOH mixtures at various pH's. The initial pH change and an iodine titer were determined within 5 min. of addition and repeated after 1.5 hr. At lower pH's (3 to 6) where the end products are RNH_3^+ and $HOCH_2SO_3^-$ (Fig. 1), the amount of bisulfite drops with time. This demonstrates that the reaction of formaldehyde with bisulfite (or sulfite[5]) is not complete before the first titration. In support of this is the relatively small initial rise in pH. The pH of formaldehyde bisulfite formation should rise according to the equation (7):

$$H_2O + CH_2O + SO_3^= \rightleftharpoons HOCH_2SO_3^- + OH^-$$

At higher pH's (6 to 10), the amount of sulfite rises with time. Here the concentration of sulfite is high and reaction of formaldehyde with sulfite is essentially complete within 5 min. Hence, the initial pH rise is high and the initial titer is low. The kinetically controlled reaction of formaldehyde with sulfite is then reversed[6] by the thermodynamically controlled reaction of formaldehyde with free amine. Hence, the final titer is high. This is reasonable since a) the concentration of free amine (pKa ~ 10.6) is much lower than that of sulfite (pKa ~ 6.9), b) free amine is less polarizable than sulfite, and c) free amine is nevertheless more basic than sulfite (13).

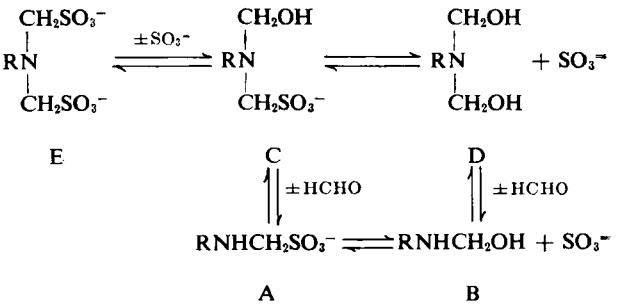
Above pH 10, sulfite concentration falls between titrations. Here, free amine is relatively concentrated and reacts rapidly with formaldehyde. The slow reaction here is apparently formation of the methanesulfonate from the carbinolamine, since HHT formation would *increase* sulfite.

The data plotted in Fig. 5 provide further kinetic information. A study was made of the time required for the reaction of butylamine with formaldehyde bisulfite to reach equilibrium at 25°C at various pH's. Here, the "kinetically controlled" reaction of formaldehyde with sulfite had already occurred and the reactions measured were the dissociation of $HOCH_2SO_3^-$ and subsequent formation of $RNH_2^+CH_2OH$ and $RNHCH_2SO_3^-$. An equimolar solution (approximately 1 N) of butylamine hydrochloride and sodium formaldehyde bisulfite was adjusted to a particular pH and then increments of sodium hydroxide required to maintain that pH were plotted against time. Each of the titrations ends at the end of the line on the graph; *i.e.*, further addition of caustic would cause pH to go up. It is obvious that at pH 6 the reaction is quite slow; at pH 7 it takes a little less than an hour; at pH 9 it is quite rapid. This is probably a reflection of both increase in free amine concentration and decrease in formaldehyde bisulfite stability with rising pH (see Fig. 6). Also plotted on the same graph are (*a*) an example of 0.1 the concentration, indicating that more time is required to reach equilibrium and less caustic is consumed to reach it; (*b*) an example at pH 7, but at 54–57°, indicating the reaction is more rapid; (*c*) an example showing that when 2 moles of sodium formaldehyde bisulfite are used at pH 7 and 25°, the rate is faster and about 50% more caustic is consumed than in the run with one mole of sodium formaldehyde bisulfite.

The composition at equilibrium of *equimolar* solutions and the mechanisms by which such equilibria are reached have been discussed but thus far the effects of varying molar ratios have not been considered. The extra consumption of caustic at equilibrium in Fig. 4, when the amount of formaldehyde bisulfite is doubled, is not due to mass action. This is because formaldehyde bisulfite does not dissociate at pH 7 (see Fig. 6 below) and because free amine or amine salt is essentially absent at this concentration as well (see Fig. 1). The extra consumption of caustic would result, however, as a consequence of diminished basicity if the amine became disubstituted.[7]

It thus appeared that a new equilibrium not included in Scheme I was at work in nonequimolar solutions. Figure 6 shows what happens to sulfite concentration at equilibrium when an extra mole of formaldehyde bisulfite is added to 1 N butylaminomethanesulfonate at various pH's. It is obvious that near neutrality the amount of free sulfite is cut approximately in half by the addition of 1 mole of formaldehyde and is approximately doubled by the addition of 1 mole of formaldehyde bisulfite. This is considered evidence for disubstitution on the amine.

The curves in Fig. 6 can be adequately explained by the following equilibria shown as Scheme II.



Scheme II

Species A-E will also be in equilibrium with their protonated forms. Addition of an extra mole of formaldehyde to B and A provides Species D and C having an extra methylolamine moiety. These will react with the sulfite present, and the equilibrium will shift toward C and E. The net effect will be to reduce free sulfite. Addition of an extra mole of formaldehyde bisulfite to B and A will provide D and C, but accompanied this time with an extra mole of sulfite. Though D and C will then go toward C and E, the net result will be increased sulfite. That the free sulfite found is not due to the dissociation of formaldehyde bisulfite is also illustrated in Fig. 6. Its dissociation at pH's below about 9 is minimal.

Having thus investigated the methanesulfonates of simple amines, it was easier to approach colistin with its five primary amine groups. These studies of the methanesulfonates derived from colistin were carried out by means of iodine titration and by electrophoresis.

[5] While the amount of sulfite is low at pH 3–6, it is a strong nucleophile (specific reaction rate with benzaldehyde at 21° = 7.35×10^5 min.$^{-1}$). Bisulfite is a much weaker nucleophile (rate with benzaldehyde at 21° = 26 min.$^{-1}$) (7). The formaldehyde reaction is apparently slow because of the low concentration of the stronger nucleophile.

[6] That $HOCH_2SO_3^-$ does not react directly with amine and then dissociate was shown by Le Henaff (12). In addition, it has been found that at least at pH 5 and 6, $HOCH_2SO_3^-$ reacts much more slowly with amine than does CH_2O, followed by $NaHSO_3$. Thus, $HOCH_2SO_3^-$ dissociation is apparently rate-controlling.

[7] The possibility of dimethanesulfonation is well documented (12, 14)

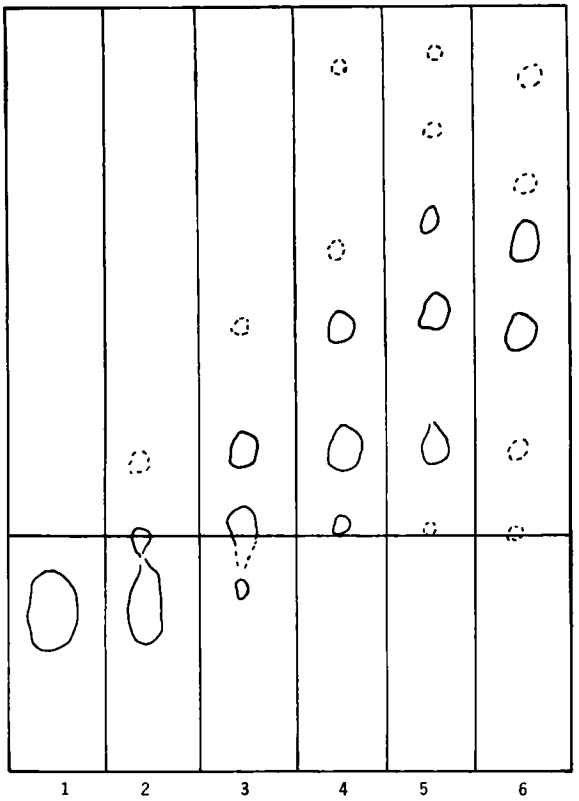


Figure 8—*Electrophoretograms. Effect of reactant ratios on colistin methanesulfonate electrophoretic patterns visualized with ninhydrin. Key: 1, colistin sulfate; 2, colistin sulfate plus 1 equiv. $HOCH_2SO_3Na$; 3, colistin sulfate plus 3 equiv. $HOCH_2SO_3Na$; 4, colistin sulfate plus 5 equiv. $HOCH_2SO_3Na$; 5, colistin sulfate plus 7 equiv. $HOCH_2SO_3Na$; 6, colistin sulfate plus 10 equiv. $HOCH_2SO_3Na$.*

Figure 7 shows the results from a series of iodine titrations of equilibrated solutions at pH 7 where the number of moles of sodium formaldehyde bisulfite per mole of colistin was varied from one to twenty, and also where temperature and concentration were varied. At a ratio of 5 to 1 (one equivalent of formaldehyde bisulfite per amino group) at 25°, nearly half of the formaldehyde bisulfite added is present in the solution as free sulfite (or bisulfite) indicating dissociation of aminomethanesulfonate groups to hydroxymethylamino groups and sulfite just as was found for the model compounds. As this ratio was increased, more sulfite was liberated but in decreasing increments, particularly after the 10 to 1 ratio was reached, indicating little additional reaction of the formaldehyde bisulfite. In view of our findings with model compounds, it is probable that the increase in sulfite between 5 and 10 moles indicates disubstitution on the amine group, in addition to reaction with remaining free amine groups. Lowering the temperature increased the amount of free sulfite and decreasing the concentration decreased the amount of free sulfite.

An aspect of the chemistry of sodium colistimethate which could not be studied in model compounds is the statistical factor introduced by the presence of five free amine sites per molecule. One would expect a spectrum of compounds in which the amines are substituted to varying degrees and in varying positions. Figures 8 and 9 present the electrophoresis patterns as illustrations of this statistical factor. It is uncertain which species are represented by which spots. It is apparent, however, that the more highly methanesulfonated molecules should move farthest. In Fig. 8 colistin itself moves slowly toward the cathode. Addition of 1 mole of formaldehyde bisulfite provides a small spot that stays about at the origin in addition to the colistin spot; addition of 3 moles of formaldehyde bisulfite provides a pattern with the colistin spot barely visible and

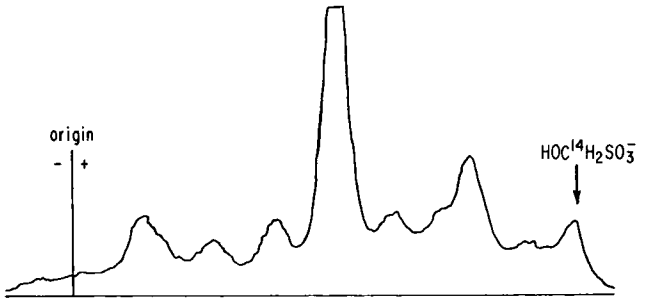


Figure 9—*Electrophoretogram. Radioscan pattern of a colistin methanesulfonate electrophoretogram.*

two spots moving toward the anode; addition of 5 moles of formaldehyde bisulfite gives a pattern with no colistin visible, a small spot moving slightly toward the anode, two larger well-separated spots moving toward anode, and several smaller spots moving even faster toward the anode; additions of 7 and 10 moles of formaldehyde bisulfite give patterns with increasing amounts of spots moving faster toward the anode. It may be noted here that the electrophoretic pattern of the solution made, using 5 moles of formaldehyde bisulfite, is very similar to that obtained from a commercial sample of sodium colistimethate.

Electrophoresis has been run on sodium colistimethate prepared using radioactive formaldehyde. Figure 9 is a reproduction of the radioscan of this separation. The peak identified as formaldehyde bisulfite contains roughly 5% of the total radioactivity. Hence, the rest of the formaldehyde is tied up with the amino groups in the colistin molecule, and the number of free amino groups per molecule is limited to a low figure. Thus, the number of free colistin molecules must be very low. The electrophoresis paper of the radioactive material was also subjected to a biogram determination, and all spots except formaldehyde bisulfite and one or two very fast-moving spots (not shown), which may be degradation products, showed antibacterial activity.

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