It would be worthwhile to isolate the pure compounds from the crude fractions; strong acids, weak acids, and alkaloids and then test them on tumor-bearing mice, because many compounds in the pure state have been reported to be highly active against certain types of leukemias, but were devoid of activity in crude fractions. For example, Svoboda (11) reported the alkaloids, leurocristine and leurosidine, were very active against P-1534 leukemia, but were relatively inactive in crude fractions from which they were isolated. A similar situation was observed by Farnsworth *et al.* (12).

SUMMARY

1. Pharmacologically active compounds present in black walnut were evaluated for antitumor activity on spontaneous and transplanted tumors in mice.

2. Ellagic acid depressed the tumor growth in both spontaneous and transplanted tumor. This effect was more apparent on the spontaneous tumors than on transplanted tumors. Ellagic acid increased the weight of mice with spontaneous tumors but decreased the weight in animals with transplanted tumors.

3. Juglone decreased the tumor growth rate and weight of the spontaneous tumor mice.

4. Strong acids slightly decreased the rate of tumor growth (spontaneous), but weak acids (spontaneous) and alkaloids (transplanted) did not have any significant effect. Furthermore, the weight of the mice was not affected by any of these three groups.

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Keyphrases

Antitumor compounds—*Juglans nigra*
Acidic components, *Juglans nigra*—antitumor activity
Ellagic acid—antitumor activity
Juglone—antitumor activity
Alkaloids, *Juglans nigra*—antitumor activity

Complex Formation Influence on Reaction Rate IV

Studies on the Kinetic Behavior of 3-Carbomethoxy-1-methylpyridinium Cation

By DANA BROOKE* and DAVID E. GUTTMAN†

The kinetics of reaction of the ester, 3-carbomethoxy-1-methylpyridinium cation (NME), were investigated in the pH region 8.0–9.8, in the absence and presence of the electron donor, 8-chlorotheophyllinate anion (CT). Hydrolysis of the ester was shown to be first order with respect to hydroxide ion in this region. The rate of hydrolysis was significantly decreased in the presence of CT. Spectral studies demonstrated the formation of a complex between the ester and CT. The rate of reaction of the complexed ester was about one-fifteenth that of the free ester. Both glycine and TRIS buffers were shown to contribute to the rate of reaction. The contribution due to glycine appeared to be the result of glycinate anion functioning as a general base catalyst. The mechanism of the TRIS reaction appeared to be different from that of glycine. The data suggested that neutral TRIS and the ester reacted by two different pathways to form an unstable ester or a stable amide.

N[1]-ALKYLPYRIDINIUM cations have been extensively used as model compounds in assessing the chemical and physical-chemical properties of pyridine coenzymes. Investigations of the kinetic and complexometric behavior of such cations are, therefore, of general interest. The present investigation was initiated to study the possible effects of complex formation upon the kinetic behavior of the ester, 3-carbomethoxy-1-

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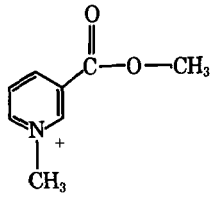
methylpyridinium cation (I). The fact that this ester undergoes hydrolysis in weakly alkaline solutions at conveniently measurable rates made NME an attractive candidate for study from an



I

experimental point of view. Moreover, this ester has been shown to form charge transfer complexes with important biological materials such as reduced flavin mononucleotide (1). Others have shown that this ester forms a cyanide-addition compound in weakly alkaline solutions of potassium cyanide (2). However, it was reported that as the ester was hydrolyzed the cyanide-addition compound was destroyed.

As will be seen, NME undergoes hydrolysis in unbuffered solutions or in solutions buffered with glycine. When tris-(hydroxymethyl)aminomethane (TRIS) was employed as a buffer, the ester underwent specific base-catalyzed hydrolysis, aminolysis due to neutral TRIS, and esterolysis due to neutral TRIS and hydroxide ion. In each reaction, the reactivity of the ester was significantly reduced by complex formation with CT. The complexed ester was found to be approximately 5% as reactive as the ester.

EXPERIMENTAL

Materials—The iodide salt of NME, m.p. 124–125° (uncorr.) and the chloride salt of N^1-methylnicotinic acid (NM acid), m.p. 244–245° (uncorr.) were prepared by established methods (3, 4). 8-Chlorotheophylline was obtained from the Aldrich Chemical Co. Solutions of this compound as the anion were prepared by dissolving appropriate amounts in water and using sufficient sodium hydroxide to adjust the pH to values of 8.0 or greater. All other chemicals used were of reagent grade.

Spectral Studies of Complex Formation—Solutions of CT were found to exhibit large increases in absorbance in the visible region of the spectrum on the addition of NME, a species with no visible spectrum. This effect, which is indicative of complex formation, is shown in Fig. 1. Studies of this phenomenon were conducted at 30° using a Beckman, model DB, spectrophotometer equipped with a thermostated cell compartment. Ionic strengths of solutions were brought to desired values with sodium chloride. Absorbance measurements were made as soon as possible after the solutions were prepared to avoid loss of NME due to hydrolysis. In all solutions the total NME concentration $(NME)_t$, was negligibly small compared to the CT concentration. The absorbance value of each solution was determined at a wavelength of 380 mμ and corrected for the contribution of CT to yield the absorbance, A_c, due to complex. Values of $(NME)_t/A_c$ at a particular CT concentration were calculated from the slopes of plots of A_c against $(NME)_t$, all of which were linear and passed through the origin. Plots of $(NME)_t/A_c$ against the reciprocal of CT concentration, the well known Benesi-Hildebrand treatment (5), were used to estimate complex stability constants.

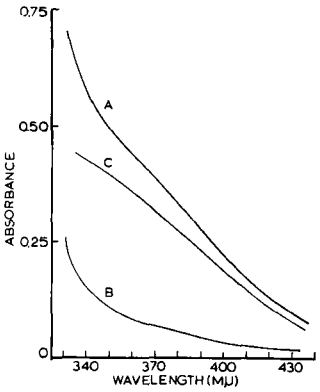


Fig. 1—Spectrum of a solution containing 0.002 M NME and 0.1 M CT (Curve A) compared to that of a solution containing only 0.1 M CT (Curve B). Curve C is the difference spectrum of the two solutions.

Fluorometric Assay for NME—An assay procedure for NME was based on the conversion of ester to N^1-methylnicotinamide (NMN) by means of an ammonolysis reaction. The NMN thus formed was determined by a fluorometric procedure (6, 7). Under the conditions of the assay it was found that about 5% of the ester was converted to NMN and the remaining ester was hydrolyzed. The ammonolysis reaction was found to be complete after 2 hr. and the NMN produced was found to be stable under the reaction conditions. Solutions containing 1×10^{-5} to 7×10^{-5} M NME were assayed by placing 3-ml. samples into 50-ml. volumetric flasks containing 5 ml. of a stock solution of 0.5 M $(NH_4)_2SO_4$ which had been adjusted to a pH of 8.97. The flasks were stoppered and allowed to stand to permit conversion of ester to NMN. After 4 hr., 5 ml. of redistilled acetone containing 2×10^{-4} M manganous chloride was added to each flask, followed by 3 ml. of 3 M sodium hydroxide solution. The flasks were allowed to stand for 30 min. Then 3.5 ml. of 4 N HCl was added to each flask and the flasks were allowed to stand 30 min. Finally, the flasks were made to volume with 2.5% monobasic potassium phosphate. Blanks were prepared in exactly the same manner but contained no NME. The fluorescence of the resulting solutions was measured using a Turner, model 110, fluorometer equipped with a 365-mμ excitation filter and a 436-mμ emission filter. It was found that NM acid, introduced into the samples, did not influence the fluorescence readings of the resulting solutions. Similarly, TRIS and neutral salts used in reaction solutions did not affect the assay. The total fluorescence, F_t, of known solutions was found to be linear with NME concentration and passed through the fluorescence reading of the blank, F_b.

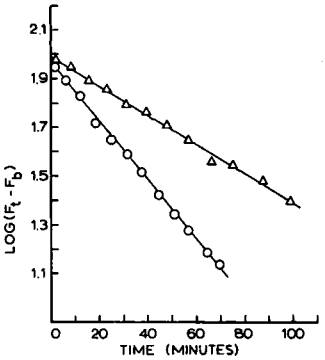


Fig. 2—Example kinetic plots obtained by fluorometrically determining residual NME in solutions buffered with 0.03 M TRIS to pH values of 8.71 (○) or 8.52 (△). Initial concentration of NME was 7.0 × 10^{-5} M and ionic strength was adjusted to 0.13 with addition of sodium perchlorate.

Kinetic Studies—The kinetics of disappearance of NME from solutions maintained at constant pH, constant temperature, and 0.03 *M* TRIS was followed by determining the residual concentration of NME in 3-ml. samples of the reaction solutions at various times. Reaction solutions adjusted to an ionic strength of 0.13 with sodium perchlorate or sodium chloride were prepared in 100-ml. volumetric flasks and kept at 30° in an oil bath. The pH values of reaction solutions were measured during the course of the reaction and were found not to change significantly. A Leeds and Northrup 7401 pH meter was used to make all pH measurements. The residual concentrations of NME in samples of the reaction solutions were determined fluorometrically. Since (F_t − F_b) was proportional to NME concentration, the logarithm of this term was plotted against time to determine apparent first-order rate constants for the disappearance of ester from reaction mixtures. Sample kinetic plots are shown in Fig. 2.

The rates of reaction of ester in the absence or presence of CT in unbuffered solutions at constant pH and 30° were determined by measuring the rate of acid formation on a Radiometer TTT-1 titrator-pH stat equipped with SBR-2, SBU-1 recorder. Ionic strength was brought to desired values with the addition of sodium perchlorate or sodium chloride. The molar amount of sodium hydroxide required to maintain pH of the reaction mixtures during the course of reaction correlated with the molar amount of NME in the original solutions.

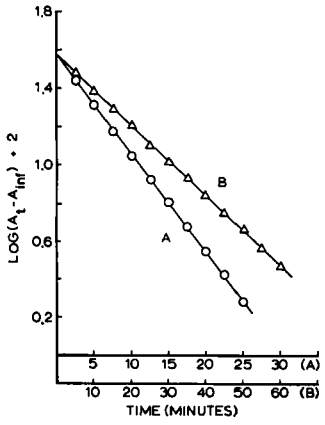


Fig. 3—Kinetic plots prepared by following the absorbance at 380 mμ of reaction solutions prepared to contain 0.005 M NME and 0.1 M CT. Conditions of pH and buffer were: 0.4 M TRIS, pH 9.18 (Curve A), and 0.26 M TRIS, pH 8.63 (Curve B).

The rates of loss of total ester from solutions containing CT, and which were buffered to constant pH with TRIS or glycine buffers of moderate concentration were studied by following the disappearance of absorbance at 380 mμ with time. Solutions at 30° were prepared in 50-ml. volumetric flasks to contain 0.005 *M* NME and appropriate concentrations of buffer and CT. In most cases, ionic strength was controlled by the concentration of CT in the reaction solutions. A portion of the reaction solution was placed in 1-cm. quartz cells and the absorbance at 380 mμ was followed with time using water as a blank. The pH values of reaction mixtures were found not to change significantly during the course of reaction. Representative kinetic plots are shown in Fig. 3.

pKa Values—The values for pKa which were used in calculations were: 8-chlorotheophylline, 5.5 (8); TRIS, 8.10 (9); glycine, 9.78 (10).

RESULTS AND DISCUSSION

Spectral Studies—Spectrophotometric examination of solutions containing both NME and CT suggested that formation of a complex occurred. Figure 1 illustrates the spectrum of a solution of 0.1 *M* CT, the spectrum of a solution containing 0.1 *M* CT and 0.002 *M* NME, and the difference spectrum. Since the ester has no visible spectrum, the large increase in absorbance values of a solution of CT caused by the addition of NME was indicative of complex formation. That a new, broad absorption band appeared at longer wavelengths than the spectrum of either interactant suggests that a charge-transfer complex was formed. This is not surprising since pyridinium cations are well known for their ability to act as acceptors in charge-transfer interactions. Spectral data were analyzed by the method of Benesi and Hildebrand (5) to yield complex stability constants at several ionic strengths. The stability constant at an ionic strength of 0.1 was estimated to be 4.7 M^{-1}. The stability constant at an ionic strength of 0.2 was 4.3 M^{-1}, and at 0.3 was 3.9 M^{-1}.

Complex Formation Influence on Kinetic Behavior—In order to survey the possible effects of complex formation upon the kinetics of hydrolysis of NME, it was first necessary to study these kinetics in the absence of CT. This was done by utilizing two different experimental approaches. In the pH region from 8.0 to 8.8, loss of ester from solutions buffered to a constant pH with 0.03 *M* TRIS was followed by fluorometrically determining the residual concentration of NME as a function of time. In the pH region from 8.6 to 9.6, the rate of acid production in reaction solutions was determined by means of a pH-stat technique. The rate constants obtained by these two methods are presented in Fig. 4 in the form of a pH-rate constant profile. The good correlation between the apparent rate constants for the disappearance of ester and corresponding constants

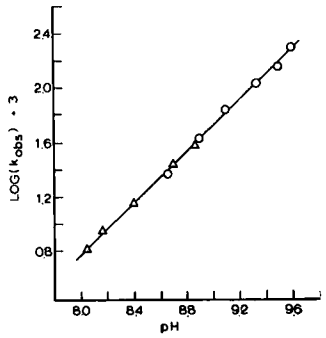


Fig. 4—A plot illustrating the influence of pH on the apparent rate constant for loss of NME from solutions of 0.03 M TRIS (△), and on the apparent rate constant for the production of acid in unbuffered solutions of NME (○).

for the appearance of acid showed that the reaction was solely one of hydrolysis. The slope of the profile in Fig. 4 was 0.97 which indicated that the hydrolysis, under these conditions, was specific base catalyzed. The linearity of the profile suggested that TRIS, in 0.03 *M* concentration, did not contribute significantly to the rates of reaction. The specific base catalytic constant was calculated to be $0.54 \times 10^4\ M^{-1}$ min.$^{-1}$.

The kinetics of ester hydrolysis were also studied in solutions containing 0.1 *M* CT by the pH-stat method. A pH-rate constant profile representing these studies is presented in Fig. 5. That this profile fell below that determined in the absence of CT showed that complex formation did significantly decrease the reactivity of the ester toward hydrolysis. The slope of the profile was found to be 1.05 indicating that the hydrolysis of the ester in solutions containing CT was also specific base catalyzed. That no irreversible reaction occurred between CT and NME was reflected by the fact that the molar quantity of base required to maintain constant pH during the course of reaction corresponded to the initial molar quantity of ester in the reaction solution. The fact that the slope of the profile was not less than 1.0 provided evidence that CT did not act as a general base catalyst.

It was observed, in the course of these studies, that the characteristic yellow color of freshly prepared solutions of CT and NME faded considerably during the course of reaction. Spectral studies showed that the hydrolysis product of NME, N^1-methylnicotinate, also interacted with CT. However, the absorbance at 380 mμ due to this interaction was relatively small compared to that found with a corresponding ester–CT system. The fading, therefore, undoubtedly resulted as a consequence of ester hydrolysis. Kinetic studies were then conducted which took advantage of this effect. Here, the loss of absorbance at 380 mμ of solutions containing NME and CT was used as a reaction parameter to reflect the loss of total NME. The kinetics of loss of NME from solutions containing CT were studied under a variety of conditions by this method. In most cases, TRIS buffers of moderate concentration were used to maintain constant pH. Figure 3 illustrates kinetic plots obtained by this procedure. It can be seen that these plots are linear for at least 3 to 4 half-lives. It was found, however, that under these conditions, where buffer concentrations were much higher than those employed previously, a buffer contribution to rates of reaction was apparent. Figure 6 illustrates the effect on the apparent rate constant of varying the total TRIS concentration under the conditions of constant pH and CT concentration. The value for the apparent rate constant at zero TRIS concentration was interpolated from pH-stat studies on buffer-free solutions (Fig. 5). The apparent rate constants were found to be linear with TRIS concentration indicating that the buffer reaction was first order in TRIS. As will be seen, the TRIS buffer reaction was found to be considerably more complex than one of general base catalysis, and involved a reaction between ester and neutral TRIS, and one between ester, neutral TRIS, and hydroxide ion.

The influence of varying the concentration of CT on the apparent rate constants for disappearance of NME from solutions buffered to a pH of 8.60 with 0.26 *M* TRIS was evaluated. Since CT, in moderate concentrations, was easily salted out of solution, no attempt was made to maintain ionic strength at a constant value in these experiments. The results are summarized in Fig. 7. A modified form of an equation derived by Connors and Mollica (11) was

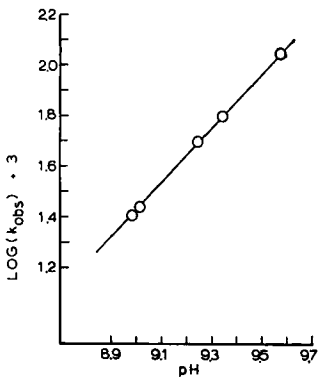


Fig. 5—Illustration of the influence of pH on the apparent rate constant for production of acid in unbuffered solutions of NME containing 0.1 M CT.

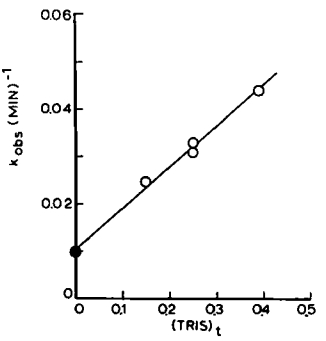


Fig. 6—Plot depicting the influence of total TRIS buffer concentration on the apparent rate constant for loss of NME from solutions of pH 8.60 which contained 0.1 M CT. The open symbols refer to spectral determinations of apparent rate constant. The solid symbol is the apparent hydrolytic rate constant for NME at this pH and CT concentration as interpolated from the data of Fig. 5.

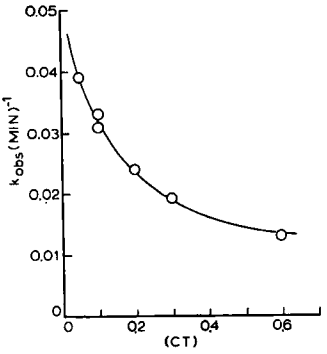


Fig. 7—Illustration of the influence of CT concentration on the apparent rate constant for the loss of NME from solutions buffered to pH 8.60 with 0.26 M TRIS.

used to evaluate these data to determine if complexed ester underwent reaction, and to determine, kinetically, the complex stability constant. It can be shown that

$$\frac{k'(CT') - k^*(CT^*)}{(CT') - (CT^*)} = k_c + \frac{k^* - k'}{K[(CT') - (CT^*)]} \quad \text{(Eq. 1)}$$

where k' = apparent rate constant determined when the CT concentration was (CT'), k^* = apparent rate constant determined when the CT concentration was (CT*), k_c = apparent rate constant for loss of complexed ester, K = complex stability constant.

A plot of the data according to Eq. 1, based on a reference (CT*) of 0.05 M, is given in Fig. 8. The intercept is k_c and the reciprocal of the slope is K. The value for the apparent rate constant for loss of free NME can be estimated by setting (CT') to zero and calculating k'. Here, the value for the apparent rate constant for loss of free ester was calculated to be 0.052 min.$^{-1}$ which does not agree with the apparent hydrolytic rate constant of 0.021 min.$^{-1}$ for free ester as interpolated from the pH-rate constant profile prepared in the absence of CT (Fig. 4). The difference between these two apparent rate constants will be shown to be due to the reaction of TRIS with free NME. The value for k_c was estimated to be 0.0035 min.$^{-1}$. Therefore, in reaction solutions containing CT and 0.26 M TRIS at a pH

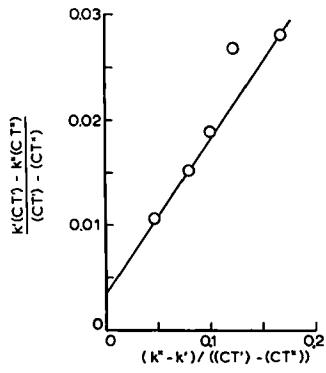


Fig. 8—Data of Fig. 7 plotted according to Eq. 1. See text.

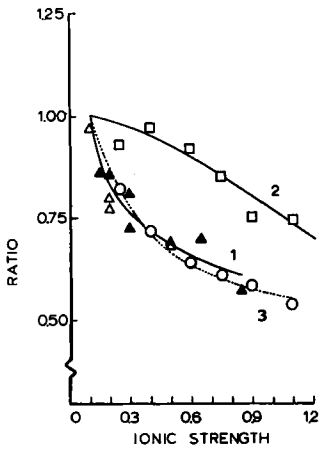


Fig. 9—Plot showing the influence of ionic strength on the apparent rate constant for loss of NME from solutions containing no buffer and no CT (Curve 1, △), on the rate constant for loss of NME from solutions containing 0.1 M CT and 0.26 M TRIS (Curve 2, □), and on the extent of complex formation between NME and CT (Curve 3, ○). The ordinate is the ratio of observed values at a given ionic strength to the observed value at an ionic strength of 0.1. Open symbols refer to the use of sodium perchlorate to adjust ionic strength, closed symbols refer to the use of sodium chloride to adjust ionic strength.

of 8.6, the free ester underwent reaction at about 15 times the rate of the complexed ester.

The value for the complex stability constant, K, was estimated from the kinetic data to be 6.5 M^{-1}. The discrepancy between this value and those estimated from spectral data can be rationalized on the basis that ionic strength effects, resulting from the variation of CT concentration, contributed to the overall effect of CT in the kinetic studies. It is informative, here, to consider the effects of ionic strength variation on the complexometric and kinetic behavior of NME.

The influence of ionic strength variation on the apparent rate constants for loss of NME from unbuffered solutions containing no CT, as determined by pH-stat studies at a pH of 9, and those for the loss of NME from solutions containing 0.1 M CT and 0.26 M TRIS, as determined spectrally, is illustrated in Fig. 9. Curve 1 shows the influence of ionic strength on the apparent rate constants determined in the absence of CT and buffer. Curve 2 shows the influence of ionic strength on the apparent rate constants determined in the presence of CT and buffer. Curve 3 of Fig. 9 reflects the influence of ionic strength on the extent of complex formation between CT and NME. This influence was evaluated by determining the absorbance due to the complex at zero time in those solutions containing CT and buffer. The influence of ionic strength on the apparent rate constants determined in the absence of CT and buffer, and on the extent of complex formation in solutions containing CT and buffer is seen to be qualitatively the same (Curves 1 and 3). That both Curve 1 and Curve 3 decrease and show approximately the same dependence on ionic strength is not unexpected since both curves describe the effect of ionic strength on the reaction of an anion with a cation. However, the dependence

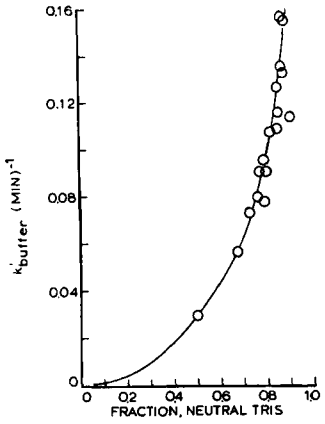


Fig. 10—Plot of the apparent TRIS buffer rate constant observed in solutions containing 0.1 M CT against the fraction of neutral TRIS.

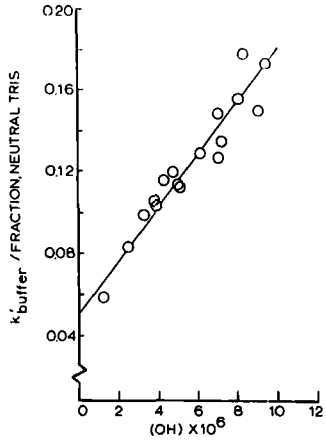


Fig. 11—Illustration of the influence of hydroxide ion concentration on the apparent rate constant for the reaction of neutral TRIS with NME observed in solutions containing 0.1 M CT.

of the apparent rate constants for loss of NME from solutions containing CT and TRIS is seen to be much smaller. This observation can reasonably be explained by considering that although the kinetic parameters for the reaction in these systems might be expected to be decreased by increasing ionic strength, the extent of complex formation would also be expected to be decreased resulting in higher concentrations of free NME. Moreover, the apparent rate constant here reflects, in part, a reaction between neutral TRIS and NME and this reaction would not be expected to be as sensitive to ionic strength variations as would the reaction of the ester with hydroxide ion. The indication from Curve 2 is that, within certain limits, strict control of ionic strength in reaction solutions containing CT and TRIS is not necessary, but that ionic strength variation over wide ranges does result in measurable decreases in apparent rate constants. These considerations also suggest that the observed influence of CT on reaction rate was due to both complex formation and an ionic strength effect and that the latter resulted in the generation of a slightly larger complex stability constant than was expected on the basis of spectral studies.

Buffer Reactions—The reaction of TRIS with NME was examined in solutions containing 0.1 *M* CT at various pH values and at different TRIS concentrations. The ionic strength of each solution was 0.1. The apparent buffer rate constants per mole of TRIS, k'_{buffer}, were calculated by the equation

$$k'_{buffer} = \frac{k_{obs.} - k_{hyd.}}{(buffer)_t} \qquad \text{(Eq. 2)}$$

where $k_{obs.}$ = experimentally determined apparent rate constant, $k_{hyd.}$ = apparent hydrolytic rate constant under the same conditions of pH and CT concentration, but in the absence of TRIS, as interpolated from the data of Fig. 8, $(buffer)_t$ = total buffer concentration.

It was found that a plot of k'_{buffer} against the fraction of neutral TRIS, as shown in Fig. 10, extrapolated to the origin but increased in slope as the fraction increased. Since the buffer reaction was shown to be first order in TRIS (Fig. 6), the data suggested that hydroxide ion was also involved in a buffer reaction. The data were found to give a straight line with positive slope and positive intercept when k'_{buffer} divided by fraction of neutral TRIS was plotted against hydroxide ion concentration. This plot is shown in Fig. 11. These considerations suggested that the TRIS buffer rate equation had terms in neutral TRIS and in netural TRIS and hydroxide ion. The kinetics of TRIS reaction with phenyl ester (9), and with δ-thiolvalerolactone (12) have been observed to give similar rate equations. Bruice and York (9) compared the reaction of TRIS with phenyl esters to that of pentaerythritol (which is essentially TRIS in which the $—NH_2$ group has been replaced by a $—CH_2OH$ group) with phenyl esters and concluded that the reaction of neutral TRIS with esters was one of aminolysis, and that of neutral TRIS and hydroxide ion with esters was one of esterolysis. They found that the rate equation for the reaction of pentaerythritol with esters involved only the term in pentaerythritol and hydroxide ion. This was taken as an indication that the similar term in the rate equation for the reaction of TRIS with esters described esterolysis. They concluded that hydroxide ion reacted with TRIS to form an alkoxide ion which then reacted with the ester.

It would appear from the present investigation that an aminolysis reaction between neutral TRIS and NME also occurred. This is suggested by a comparison of the spectrum of the final (time infinity) reaction mixture which had been prepared with 0.005 *M* NME, 0.1 *M* CT, and 0.4 *M* TRIS to the spectrum of a solution prepared with 0.005 *M* NM acid, 0.1 *M* CT, and 0.4 *M* TRIS. Such a comparison is shown in Fig. 12. That the final reaction solution had absorbance readings which were significantly higher than those of a solution which would be anticipated if all the NME had been converted to acid suggested that a relatively stable product had been formed and that this product interacted to a greater degree with CT than did NM acid. That this reaction product was not an ester such as that which would be expected from an esterolysis reaction was indicated by the stability of the spectrum of the final reaction solution over a period of 4 hr. In contrast to esters, an amide

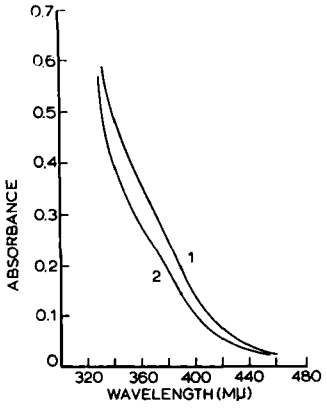


Fig. 12—Spectrum of a time infinity reaction solution which had been prepared to contain 0.005 M NME, 0.1 M CT, and 0.4 M TRIS at pH 9.18 (Curve 1), and that of a solution containing 0.005 M NM acid, 0.1 M CT, and 0.4 M TRIS at pH 9.18 (Curve 2).

formed by the reaction of neutral TRIS with NME would be quite stable under the conditions of the experiment. Such an amide would also be expected to interact with CT to a greater degree than NM acid which would be ionized at the carboxyl group under these conditions. The assumption of stability for the newly formed amide is based on studies of the kinetics of alkaline hydrolysis of NMN (13) in which it was shown that the specific base catalytic constant was 0.23 M^{-1} min.$^{-1}$ (ionic strength 1.0, temperature 30°) which is about 4 orders of magnitude smaller than the specific base catalytic constant for NME. The fact that ammonia reacts with NME to form NMN also supports the conclusion that an aminolytic reaction with TRIS did occur.

The overall reaction mechanism for NME in solutions containing TRIS and CT is quite complex. The fact that NME and its reaction products can possibly interact with CT to form complexes which contribute to the absorbance values of a reaction solution might be expected to make this approach to the determination of apparent rate constants for the loss of NME difficult. However, it can be shown that rate constants determined by following the time course of absorbance of solutions containing NME and CT do accurately reflect the rates of disappearance of NME. A possible overall mechanism describing the reactions of NME is presented by the following equations. Here, the possibility of the various complexes undergoing reaction is neglected since this has been shown to be small.

$$\text{NME} + \text{OH} \xrightarrow{k_1} \text{NM acid} \quad \text{(Eq. 3)}$$

$$\text{NME} + \text{T} \xrightarrow{k_2} \text{NM acid} \quad \text{(Eq. 4)}$$

$$\text{NME} + \text{T} \xrightarrow{k_3} \text{amide}' \quad \text{(Eq. 5)}$$

$$\text{NME} + \text{OH} + \text{T} \xrightarrow{k_4} \text{ester}' \quad \text{(Eq. 6)}$$

$$\text{ester}' + \text{OH} \xrightarrow{k_5} \text{NM acid} \quad \text{(Eq. 7)}$$

$$\text{ester}' + \text{T} \xrightarrow{k_6} \text{NM acid} \quad \text{(Eq. 8)}$$

$$\text{ester}' + \text{T} \xrightarrow{k_7} \text{amide}' \quad \text{(Eq. 9)}$$

where the various k_i's represent specific rate constants for the indicated rate processes, and amide′ = the amide formed by TRIS aminolysis of NME, ester′ = the ester formed by TRIS esterolysis of NME, T = neutral TRIS. Here, processes represented by Eqs. 3 and 7 refer to specific base catalysis, those by Eqs. 4 and 8 refer to possible general base catalysis, those by Eqs. 5 and 9 refer to aminolysis, and that represented by Eq. 6 refers to esterolysis. If it is assumed that all four of the pyridinium cations interact with *CT* to contribute to the absorbance of the reaction solution, A_t, and if it is assumed that only amide′ and NM acid remain in the reaction solution at infinite time, and that only these interact with CT to contribute to the final absorbance, $A_{\text{inf.}}$, then it can be shown that the function $(A_t - A_{\text{inf.}})$ is described by:

$$A_t - A_{\text{inf.}} = B_1 \exp.(-C_1 t) + B_2 \exp.(-C_2 t) \quad \text{(Eq. 10)}$$

where B_1 and B_2 are constants at constant pH, and constant CT and TRIS concentrations, and $C_1 = f_1[k_1(\text{OH}) + k_2(\text{T}) + k_3(\text{T}) + k_4(\text{T})(\text{OH})]$, $C_2 = f_2[k_5(\text{OH}) + k_6(\text{T}) + k_7(\text{T})]$, t = time. Here f_1 refers to the fraction of free NME in the reaction solution, and f_2 refers to the fraction of free ester′ in the reaction solution. From Eq. 10 it would appear that a plot of log $(A_t - A_{\text{inf.}})$ against time would exhibit curvature. However, plots of the experimental data, as shown in Fig. 3, were found to be linear for at least 3–4 half-lives. This indicates that either the C_1 term or the C_2 term of Eq. 10 is effectively zero. Since apparent rate constants determined from plots of log $(A_t - A_{\text{inf.}})$ against time were found to be dependent on (OH), (T), and (T) (OH), it is clear that the C_1 term cannot be zero. Thus, Eq. 10 can be approximated by:

$$A_t - A_{\text{inf.}} = B_1 \exp.(-C_1 t) \quad \text{(Eq. 11)}$$

Apparent rate constants evaluated from semilog plots of $(A_t - A_{\text{inf.}})$ *versus* time reflect, as can be seen from Eq. 11, the rate of loss of NME since C_1 is defined by processes which involve only NME. Therefore, conclusions drawn from experiments of this kind do not appear to be invalidated by the complexity of the reaction mechanism.

Based on the above considerations, and with the assumption that reaction rates of complexes are insignificantly small, the rate equation which describes loss of total NME from buffered solutions containing CT and TRIS can be written:

$$-\frac{d(\text{NME})_t}{dt} = \frac{(\text{NME})_t}{1 + K(\text{CT})} \times [k_1(\text{OH}) + (k_2 + k_3)(\text{T}) + k_4(\text{T})(\text{OH})] \quad \text{(Eq. 12)}$$

Here the term $(\text{NME})_t/[1 + K(\text{CT})]$ is equivalent to $f_1(\text{NME})_t$, and is the concentration of free NME in the reaction solution. The values for the parameters of Eq. 12 are: $K = 4.7\ M^{-1}$, $k_1 = 0.54 \times 10^4\ M^{-1}$ min.$^{-1}$, $(k_2 + k_3) = 0.078\ M^{-1}$ min.$^{-1}$, $k_4 = 1.88 \times 10^4\ M^{-2}$ min.$^{-1}$. The value for the complex stability constant, K, is that obtained from spectral studies at an ionic strength of 0.1. The rate constant k_1 was calculated from the data of Fig. 4. The sum of the rate constants $(k_2 + k_3)$

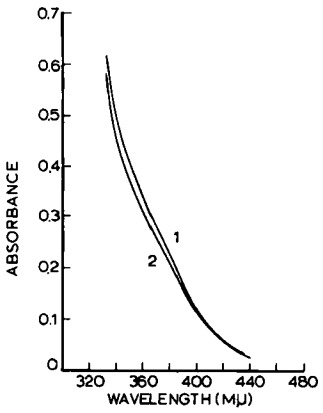


Fig. 13—Spectrum of a time infinity reaction solution which had been prepared to contain 0.005 M NME, 0.1 M CT, and 0.4 M glycine at pH 9.80 (Curve 1), and that of a solution which contained 0.005 M NM acid, 0.1 M CT, and 0.4 M glycine at pH 9.80 (Curve 2).

was calculated from the intercept of Fig. 11. Here, it will be recognized that the intercept was $f_1(k_2 + k_3)$. Similarly, the value for k_4 was calculated from the slope of Fig. 11, the slope being f_1k_4. With these values it is possible to predict the apparent rate constant, at 30°, for the disappearance of total NME at any concentration of hydroxide ion, CT, and neutral TRIS. Thus, in a system which contains no CT and which is maintained at a pH of 8.6 with 0.26 *M* TRIS buffer, the apparent rate constant is calculated to be 0.051 min.$^{-1}$. This is in excellent agreement with the value of 0.052 min.$^{-1}$ which was estimated from the Connors and Mollica treatment of data obtained under the same conditions of pH and TRIS concentration by extrapolating to zero CT concentration. This agreement supports the overall mechanism which has been proposed.

The reaction of NME in the presence of 0.1 *M* CT was also studied in glycine buffers by following the decrease of absorbance with time. Kinetic plots were linear for at least 3–4 half-lives. The results of these studies suggested that glycinate anion catalyzed the hydrolysis of NME. Buffer rate constants for various conditions of pH and glycine concentration were calculated from Eq. 2. A plot of k'_{buffer} against the fraction of glycinate anion was linear and passed through the origin indicating that the buffer reaction was first order with respect to glycinate anion. Figure 13 compares the spectrum of a final reaction solution to that which would be anticipated if all the NME had been hydrolyzed. It is seen that there was little difference. This suggests that glycinate anion did not participate to any significant extent, in an aminolysis reaction, but functioned as a general base that catalyzed the hydrolysis. The general base catalytic constant for glycinate anion was estimated to be 0.39 M^{-1} min.$^{-1}$. This value is 5–6 times larger than the rate constant for the reaction of neutral TRIS with NME ($k_2 + k_3$). Thus, if neutral TRIS functioned as a general base catalyst in the hydrolysis of NME (that is, if k_2 is not zero), its catalytic activity was small compared to that of glycinate anion. Presumably, the greater catalytic efficiency of glycinate anion is due to its greater basicity.

Conclusion—In the present investigation it has unequivocally been shown that complex formation between CT and NME reduced the reactivity of the ester toward specific base-catalyzed hydrolysis. Evidence was obtained to suggest that reactions involving NME and buffer species were similarly affected by complex formation. The reduced reactivity of complexed ester can be rationalized on the basis of at least two effects. Donation of charge by the complexing agent to the pyridinium ring undoubtedly reduces the electron withdrawing ability of the ring resulting in a much lower degree of "acyl activation" of the ester carbonyl. In addition, the attack of nucleophiles on the carbonyl function of complexed NME is almost certainly hindered, on a steric basis, by the presence of complexing agent. It has also been shown that when the manifestations of complex formation between reactant and complexing agent, and between products and complexing agent differ greatly, this difference can be conviently used to follow rates of reaction. Using this approach, the rather involved kinetics of reactions of NME in buffered aqueous media were investigated.

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Keyphrases

Complex formation—reaction rate influence
3-Carbomethoxy-1-methylpyridinium cation—reaction kinetics
8-Chlorotheophylline effect—3-carbomethoxy-1-methylpyridinium hydrolysis
Buffer systems—3-carbomethoxy-1-methylpyridinium hydrolysis
Colorimetric analysis—spectrophotometer
Fluorometry—analysis