Effect of Methylamine on Periodate-Oxidized Adenosine 5′-Phosphate*

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ABSTRACT: In the pH range between 4 and 10, the methylamine-catalyzed elimination of the phosphoryl residue from periodate-oxidized adenosine 5′-phosphate (5′-AMP) (II) followed an optimum curve with a maximum around neutrality. At neutral pH, under conditions permitting the complete conversion of the aldehyde groups into their carbinolamine derivatives (III), phosphate release was still increased by increasing the concentration of methylamine. Inhibition of the elimination reaction became apparent when methylamine reached a 100–125-fold molar excess over periodate-oxidized 5′-AMP. The release of the phosphoryl residue was accompanied by the formation of a chromophore considered to represent a C=C—C=N grouping in the sugar moiety. Upon transfer into aqueous mixtures containing 0–90% (v/v) dimethyl sulfoxide, dimethylformamide, or acetonitrile, phosphate liberation followed an optimum curve with respect to solvent composition. Addition of 50% (v/v) of either organic solvent at pH 4–10 enhanced the release of the phosphoryl residue primarily on the acid side of the pH optimum. The results are interpreted in terms of a scheme whereby the formation initially of the carbinolamine derivative of periodate-oxidized 5′-AMP (III) is followed by acid-catalyzed dehydration to form the corresponding Schiff base (IV). The stage is thus set for attack by another methylamine molecule (V) which, acting as a base, will abstract the proton from C-4′ to permit the release of inorganic phosphate *via* a classical base-catalyzed β-elimination mechanism. A shift in the rate-limiting step may be brought about by manipulation of the pH, the concentration of methylamine, and the organic solvent content if present.

Sequential removal of single nucleotide residues from the 3′-OH-bearing terminus has been successfully employed in studies on the structure and function of the terminal nucleotide sequences of tRNA (Neu and Heppel, 1964; Khym and Uziel, 1968; Uziel and Khym, 1969) and of viral RNA (Whitfeld, 1965; Steinschneider and Fraenkel-Conrat, 1966; Weith and Gilham, 1967; Kamen, 1969). The procedures used included a step in which a primary amine catalyzed the removal of a periodate-oxidized terminal nucleoside residue. Studies on the model reaction in which methylamine catalyzed the release of the phosphoryl residue from periodate-oxidized adenosine 5′-phosphate (5′-AMP) (II) showed that the initial step in this reaction was the formation, preferentially at pH 9, of a carbinolamine adduct (III) with the aldehyde groups (Khym and Cohn, 1961). The appearance of P_i, however, depended upon subsequent acidification to pH 7 suggesting (Cohn and Khym, 1962) that the carbinolamine adduct (III) cleaved in an acid-catalyzed, hitherto unknown reaction rather than *via* a β-elimination mechanism in which methylamine acted as a base catalyst. Yet, a similar pH dependence may have been also observed if a slow acid-catalyzed reaction of the carbinolamine derivative was rate limiting. Subsequently, rapid attack by a second methylamine molecule abstracting the proton from C-4′ would then initiate the release of the phosphoryl residue *via* a classical β-elimination mechanism.

This study was thus prompted by the desire to examine whether a new reaction for the stepwise degradation of RNA may have been found as suggested by the studies of Khym and Cohn (1961) and Cohn and Khym (1962). The experiments reported below were performed in aqueous solvents as well as in aqueous mixtures of dimethyl sulfoxide, dimethylformamide, and acetonitrile. Due to the multitude of interactions presumably involved, they will be evaluated qualitatively only and a suggestion will be made as to the sequence of the reactions which take place following the addition of methylamine to periodate-oxidized 5′-AMP leading eventually to the release of the phosphoryl residue.

Materials and Methods

Sodium metaperiodate was purchased from Mallinckrodt Chemical Works Ltd., St. Louis, Mo. Stock solutions (10^{-1} M) were made up in double-distilled water (which was used throughout this investigation) and stored in dark bottles in the refrigerator. *Adenosine 5′-phosphate* (Purissimum) was from Fluka, AG, Bucks SG, Switzerland. *Methylamine hydrochloride* obtained from the British Drug Houses Ltd., Poole, England, was twice recrystallized from ethanol and then dried under reduced pressure. Stock solutions (2.5 M) were prepared in water and adjusted to the desired pH with minimal amounts of either HCl or NaOH. *Dimethyl sulfoxide* and *dimethylformamide* were purchased, respectively, from Fluka AG, Buchs SG, and from the British Drug Houses Ltd., Poole, England. They were distilled under vacuum and kept over Molecular Sieve Type 4A in dark bottles. *Acetonitrile* (Reagent grade) was from Fisher Scientific Co., Fairlawn, N. J., and was used without further purification.

Periodate Oxidation and Methylamine Treatment. 5′-AMP (10 mg/ml, approximately 30 μmoles/ml, in H_2O) was treated with sodium periodate (0.1 M, 0.5 ml/1 ml of 5′-AMP) for 30 min in the dark at room temperature (15–22°). To terminate the reaction, ethylene glycol (1 μl, 18 μmoles/1 mg of 5′-AMP) was added and allowed to react at 25° for 15–20 min. Of this solution, 0.05–0.10 ml (0.9–1.8 μmoles of periodate-oxidized 5′-AMP) was added to reaction mixtures preequilibrated at 25° for 15–30 min, containing 0.1 ml of buffer $\Gamma/2 = 0.1$, 0.1 ml of a 2.5 M methylamine hydrochloride stock solution adjusted to the desired pH, organic solvent if present,

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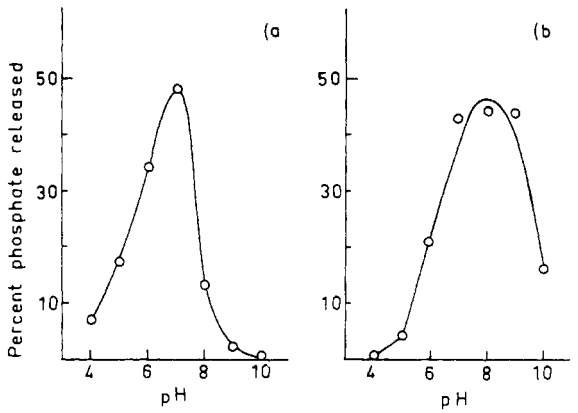

FIGURE 1: Effect of pH on P_i liberation. (a) Reaction mixtures contained 0.9 mM periodate-oxidized 5′-AMP and 250 mM methylamine at the desired pH, as described in Materials and Methods. The reaction was allowed to proceed for 30 min at 25° prior to reduction with sodium borohydride. Buffers used were pH 4.0, 5.0, sodium acetate; pH 6.0, 7.0, sodium cacodylate; pH 8.0, 9.0, sodium barbital (freshly prepared, 0.2 ml of 50 mM); pH 9.0, 10.0, sodium bicarbonate. Values at pH 9.0 represent the average in both buffers. (b) Same as in part a but using 10 mM methylamine. Samples withdrawn after 90 min at 25°. The amounts of P_i present in the absence of methylamine (usually within 3–4% in part a, 5–6% in part b) have been determined separately under the conditions of each experiment and were duly subtracted. Average of two experiments, each determined in duplicate.

and water to a final volume of 1.0 ml; 0.1-ml aliquots (0.09–0.18 μmole of 5′-AMP derivatives) were withdrawn after indicated times at 25° and immediately reduced with 1–2 mg (25–50 μmoles) of crystalline $NaBH_4$ (Khym and Cohn, 1961) for at least 30 min at 25°. Reduction under these conditions was apparently instantaneous. No error due to the instability in this assay of incompletely reduced 5′-AMP derivatives (Khym and Cohn, 1961) or due to the presence of organic solvents was apparent. pH designations of solutions containing organic solvents refer to the buffers and stock solutions employed only. No claim is made here as to either the actual pH values of these solutions or the significance of this concept in the present context.

TABLE I: Effect of Methylamine Concentration on Phosphate Release.[a]

Methylamine pH 7.6[b]		Methylamine pH 7.0[c]	
Molar Excess	% P Released	Molar Excess	% P Released
		5	15
30	24	25	34
60	33	50	52
125	40	75	60
250	29	100	67
300	19	125	66

[a] Reaction mixtures prepared as described in Materials and Methods containing the appropriate dilutions of methylamine hydrochloride were allowed to react for 30 min at 25°. Buffer: sodium cacodylate. [b] 0.8 mM periodate-oxidized 5′-AMP, 25–250 mM methylamine. [c] 2 mM periodate-oxidized 5′-AMP, 10–250 mM methylamine.

P_i was determined according to Chen *et al.* (1956). Organic solvents in the amounts present did not interfere with this assay.

Total phosphate was routinely determined spectrophotometrically (as 5′-AMP) prior to the addition of the other reagents.

Electrophoresis in the Presence of Bisulfite. Aliquots were applied to Whatman No. 3MM filter paper and subjected to electrophoresis for 60–120 min at 20 V/cm in an acetate–bisulfite buffer (Theander, 1957, diluted fivefold) in which compounds bearing free aldehyde groups acquire additional charges by complexing bisulfite ions. In a typical run following oxidation with periodate, 5′-AMP and adenosine moved 23 and 17 cm, respectively in 120 min while 2′,3′-AMP which is not oxidized and a picric acid marker moved 8.5 and 14 cm, respectively. The mobility of untreated 5′-AMP is equal to that of 2′,3′-AMP. All runs were performed using freshly prepared periodate-oxidized adenosine derivatives as markers. Spots were visualized under ultraviolet light. The presence of organic solvent did not noticeably affect the electrophoretic mobility of the above compounds.

Results

Effect of pH. Initially, the pH dependence of the methylamine (250 mM) catalyzed liberation of P_i from periodate-oxidized 5′-AMP (0.9 mM) will be considered. Confirming previous results (Khym and Cohn, 1961) the extent of P_i release increased with decreasing basicity in the range of pH 10–7. However, contrary to the expected behavior, increasing the acidity from pH 7 to 4 again decreased phosphate liberation (Figure 1a). Special precautions to keep the ionic strength constant were not taken since the concentration of methylamine ($pK = 10.6$), which was present mostly in the protonated form, was considered sufficiently high to provide for constant ionic strength over most of the pH range studied. In a similar experiment but using 10 mM methylamine a similar curve was observed except that the maximum now appeared closer to pH 8 (Figure 1b).

When periodate-oxidized 5′-AMP (1 mM) was treated with methylamine (250 mM) at pH 9 for 105 min at 25°, all ultraviolet light absorbing material migrated like periodate-oxidized 5′-AMP during electrophoresis in the presence of bisulfite (Theander, 1957), as described in Materials and Methods. The dialdehyde groups thus appeared to remain intact during treatment with methylamine at pH 9 indicating that little, if any, base-catalyzed disproportionation had occurred.

Effect of Methylamine Concentration. If the formation of the carbinolamine addition compound (III) is sufficient to permit subsequent release of the phosphoryl residue (Cohn and Khym, 1962), further increase in the methylamine concentration should not result in additional liberation of P_i. On the other hand, the observation that higher amounts of P_i were released may be taken as evidence that additional methylamine molecules participated in the reaction. Using a 25-fold molar excess of methylamine over periodate-oxidized cytidine 5′-phosphate, the formation at pH 7.6 of the addition compound corresponding to III was complete within 1 min (Cohn and Khym, 1962). Under similar conditions or at pH 7.0, the optimum at 0.25 M methylamine, phosphate release as measured after 30 min was enhanced by increasing amounts of methylamine until a 100–125-fold molar excess over periodate-oxidized 5′-AMP (Table I) or 5′-CMP was reached while further addition of methylamine gradually inhibited

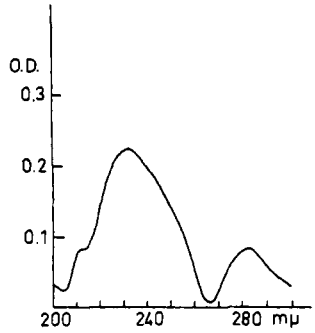

FIGURE 2: Difference spectrum of methylamine-treated periodate-oxidized 5′-AMP. 5′-AMP was oxidized with sodium periodate as above. Aliquots (1.8 μmoles, 0.1 ml) were transferred to reaction mixtures with sodium cacodylate (pH 7.0) containing or lacking 250 mM methylamine (pH 7.0), brought to a final volume of 1.0 ml with water, and kept for 60 min at 22°. Aliquots (50 μl) were transferred into quartz cuvets containing 3.0 ml of sodium cacodylate (pH 7.0) (Γ/2 = 0.1, diluted tenfold) and the difference spectrum of methylamine-treated *vs.* the nontreated compound was immediately measured in a Cary 14 differential spectrophotometer. A similar spectrum was observed after 60 min when 5 μl (0.09 μmole) of periodate-oxidized 5′-AMP was directly transferred to 3 ml of a similar reaction mixture containing 250 mM methylamine and read against a blank without methylamine.

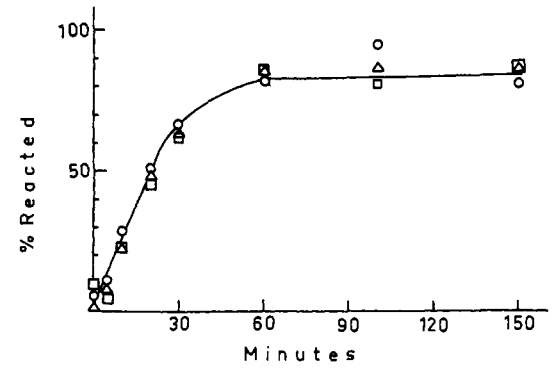

FIGURE 3: Kinetics of methylamine-catalyzed release of P_i, increase in OD_{230}, OD_{282}. The reaction mixture described in Materials and Methods (4 ml) (containing 0.9 mM periodate-oxidized 5′-AMP and sodium cacodylate (pH 7.0) with or without 250 mM methylamine) was kept in triplicate at 25°. At the indicated times, 0.1-ml aliquots were removed in duplicate from two of the methylamine-containing reaction mixtures for the determination of P_i and reduced with sodium borohydride as described previously. Simultaneously, 0.2-ml aliquots were removed from each reaction mixture and immediately added to 2.8 ml of pH 7.0 sodium cacodylate (Γ/2 = 0.1, diluted tenfold). The optical density was determined within 10 min and usually less. Phosphate liberation and OD_{230} were determined in one experiment while OD_{282} was measured separately. In constructing the curve it was assumed that P_i and optical density reached 82% of their maximum after 60 min. OD_{230} (○), OD_{282} (□), and P_i (△).

the appearance of P_i. In the absence of the attendant inhibition the dependence of phosphate liberation on the concentration of methylamine is likely to have been considerably more pronounced.

Spectrum of the Elimination Product. Phosphate release *via* a β-elimination mechanism should have been accompanied by the formation of a double bond between C-4′ and C-5′ of the sugar moiety. However, the existence of this bond could not be demonstrated directly since the elimination product, in line with previous observations on periodate-treated nucleoside derivatives (Khym and Cohn, 1960), was generally labile and could not be purified intact even under the mild conditions of chromatography on either Bio-Gel P-2 or on paper using water-saturated 1-butanol as the solvent.

Chemical considerations (see Discussion) suggested that the compound most likely to engage in a β-elimination reaction would be present in the form of a Schiff base (IV). The newly formed double bond should then, together with the Schiff base, generate a conjugated double-bond system with characteristic absorption in the ultraviolet region of the spectrum. In order to detect this chromophore as against the high-background absorption in this region of the adenine moiety, it was decided to measure the difference spectrum of periodate-oxidized 5′-AMP treated with methylamine *vs.* a blank lacking methylamine. As shown in Figure 2, two peaks were apparent in the difference spectrum absorbing at 230 and 282 mμ, respectively, while no such absorption was observed with similarly treated 3′-AMP. The small shoulder at approximately 210 mμ is not a characteristic of the difference spectrum and is due to the reaction with methylamine of the formaldehyde formed when the excess periodate was previously destroyed with ethylene glycol. The positions as well as the extinction coefficients of the observed peaks (ε 5800 and 1700, respectively, at pH 7) were within the range expected from measurements in related systems (Cavalieri *et al.*, 1948). It is unlikely that the changes expected to occur during phosphate elimination had a pronounced effect on the spectrum of the adenine moiety itself. Structural considerations indicate that a chromophore with similar properties would not have arisen except by the formation of a C-4′,C-5′ double bond.

A kinetic study (Figure 3) revealed that the absorption at both wavelengths of the difference spectrum followed a course parallel to that of phosphate liberation. Indeed, by assuming that the absorption at both wavelengths equaled 82% of the maximum after 60 min as determined for phosphate release, the rest of the points fall practically on the same curve.

In some of the controls, a difference spectrum similar to that shown in Figure 2 appeared when the above periodate oxidation and methylamine treatment were applied to adenosine. Apparently, the conditions employed for the elimination of the phosphoryl residue were conducive also to the elimination of the hydroxyl group from the periodate-oxidized nucleoside.

Effect of Dimethyl Sulfoxide, Dimethylformamide, and Acetonitrile on Phosphate Elimination. Base-catalyzed elimination reactions may be considerably enhanced upon transfer from an aqueous solution into a dipolar aprotic solvent such as dimethyl sulfoxide, dimethylformamide, or acetonitrile (Parker, 1962; Cockerill, 1967). Since previous evidence was consistent with a scheme (Figure 6) whereby phosphate was liberated from periodate-oxidized 5′-AMP *via* a β-elimination reaction in which methylamine acted as a base, it became of interest to investigate the effect of the above three dipolar aprotic solvents on P_i release. A systematic study at pH 6.0 of aqueous mixtures in which the organic solvent content was increased at 10% intervals from 0 to 90% (v/v) revealed that the aqueous mixtures of all three solvents behaved similarly and followed an optimum curve with respect to solvent composition. In terms of the corresponding mole fractions, the maxima occurred at $\bar{X}_{solvent}$ = 0.20, 0.26, and 0.30 (50, 60, and 60%, v/v) in the mixtures of dimethyl sulfoxide, dimethylformamide, and acetonitrile, respectively, in the presence of 125 mM methylamine (Figure 4a). The maximum

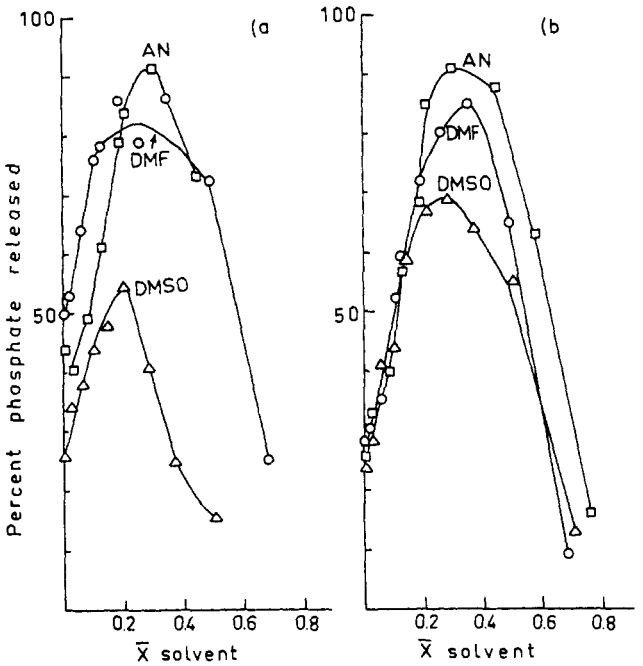

FIGURE 4: Effect of dimethyl sulfoxide, dimethylformamide, and acetonitrile on P_i liberation. (a) Reaction mixtures (0.9 mM in periodate-oxidized 5′-AMP) containing sodium cacodylate and methylamine (125 mM) both at pH 6.0 were kept for 20 min at 25° in the presence of 0–90% v/v (increasing at 10% intervals) dimethyl sulfoxide (△), dimethylformamide (○), or acetonitrile (□), as described in Materials and Methods. No buffer was present in the sample containing 90% solvent. Mole fractions were calculated neglecting possible change in the volume of the binary mixture. Reaction mixtures were assayed in duplicate. Acetonitrile, average of two experiments. In a similar experiment but for 30 min the position of the maximum in dimethyl sulfoxide and acetonitrile was reproduced followed by gradual decrease in P_i down to 3 and 7% in 90% (v/v) dimethyl sulfoxide and acetonitrile, respectively. (b) As in part a but using 10 mM methylamine, 90 min. Reaction mixtures were assayed in duplicate.

shifted to $\bar{X}_{solvent}$ = 0.28, 0.35, and 0.30 (60, 70, and 60%, v/v) in the presence of dimethyl sulfoxide, dimethylformamide, and acetonitrile, respectively, when methylamine was 10 mM only (Figure 4b). That only a small amount of phosphate was released at high organic solvent concentration did not result from concomittant destruction of aldehyde groups, since periodate-oxidized 5′-AMP (0.9 mM) treated at "pH 6.0" with 125 mM methylamine for 105 min at 25° in 90% dimethyl sulfoxide, dimethylformamide, or acetonitrile still remained intact as judged from the electrophoretic mobility in the presence of bisulfite (Theander, 1957).

Since an acid-catalyzed step was presumably included in the overall reaction, a comparison was conducted over the range of pH 4–10 of the effectiveness in promoting phosphate release of the "protophobic" solvents dimethyl sulfoxide (Me_2SO) and dimethylformamide (DMF) and the "protophilic" acetonitrile (AN) (Kolthoff *et al.*, 1968). This was possible when the organic solvent content was 50% (v/v) corresponding to $\bar{X}_{Me_2SO}$ = 0.20, $\bar{X}_{DMF}$ = 0.19, and $\bar{X}_{AN}$ = 0.21. As shown in Figure 5 essentially similar behavior was observed in all three solvents all of which enhanced phosphate liberation on the acid side of the pH optimum. While little effect was observed here on the alkaline side of the curve, the expected inhibition of this reaction was observed in a similar experiment but in the presence of 70% dimethyl sulfoxide. The position of the maximum in Figure 5, which shifted by about 1 pH unit toward the acid in 10 mM methylamine, was again dependent on the nature of the solvent and the concentration of methylamine. The organic solvent was capable also of enhancing catalysis of phosphate elimination by bases other than methylamine as for instance in a 50% (v/v) dimethyl sulfoxide "pH 10" control in which 57% of the phosphate of 1.8 mM periodate-oxidized 5′-AMP was liberated after 90 min at 25° with only 8% in the absence of dimethyl sulfoxide.

That the observed enhancement of phosphate liberation was due primarily to a direct effect on the β-elimination step rather than on the formation of the initial carbinolamine adduct (Figure 6, III) was inferred from: (1) the failure to observe breaks in the kinetic curves in a variety of binary mixtures, expected if the organic solvent affected the initial and rapid formation of the carbinolamine only; (2) an approximate calculation showing that in the absence of the attendant inhibition, phosphate elimination would have been enhanced by 15–50-fold in pure organic solvent as expected for the type of interaction studied; (3) considerations (see Discussion) indicating that under the conditions of this study it was the β-elimination step which was rate limiting in aqueous solution.

Discussion

Addition of methylamine to periodate-oxidized 5′-AMP (Figure 6, II) will result initially in the formation of a carbinolamine adduct (Khym and Cohn, 1961) (III), a reaction which does not *per se* lead to the release of P_i as these authors have already noticed. The results of the present study indicate that methylamine may engage in two additional activities. First and foremost, it may function as a base catalyst in the β elimination of the phosphoryl residue. This is inferred from: (1) the enhancement of phosphate release upon increasing the concentration of methylamine well beyond that required for the formation of the initial carbinolamine complex; (2) the formation in conjunction with the release of the phosphoryl residue of a chromophore likely to represent a double bond between C-4′ and C-5′ in conjugation with a C-3′=N imino group. This point of view is also compatible with the enhancement of the methylamine-catalyzed liberation of phosphate from periodate-oxidized 5′-AMP observed in aqueous mixtures of dimethyl sulfoxide, dimethylformamide, and acetonitrile, dipolar aprotic solvents known to enhance base-catalyzed reactions (Parker, 1962). Judging from the relationship between phosphate elimination and methylamine concentration it appears that rather than the formation of the carbinolamine adduct (III) it is the elimination reaction itself which is rate limiting on the acid side of the pH optimum curve. The absence of breaks in the kinetics of phosphate liberation in mixed solvents is in accord with this idea. Secondly, methylamine may act to inhibit phosphate release, an effect which became apparent when the reagent was present in a molar excess over periodate-oxidized 5′-AMP higher than 100-fold. The inhibitory effect of excess methylamine is considered equivalent to that of increasing the pH on the alkaline side of the pH optimum curve and is probably due to competition for the protons in the medium with an acid-catalyzed step, to be discussed next.

Previous observations (Khym and Cohn, 1961) confirmed in this study indicate that at alkaline pH an acid-catalyzed reaction becomes rate limiting. Since the elimination of phosphate appears to be directly catalyzed by methylamine acting as a base, the acid-catalyzed step will probably occur some time earlier. A likely candidate for this reaction is the hydrogen

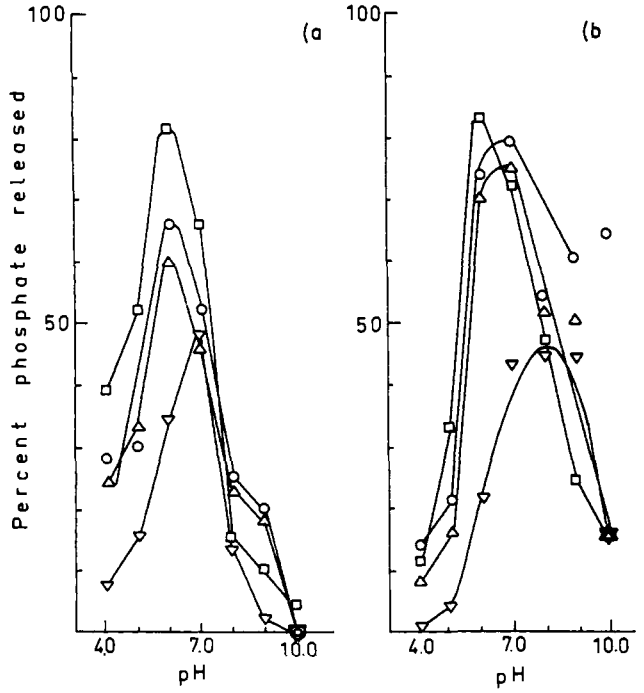

FIGURE 5: Effect of pH on phosphate liberation in presence or absence of 50% organic solvent. (a) Reaction mixtures containing 0.9 mM periodate-oxidized 5′-AMP were prepared as described in Materials and Methods and allowed to react for 30 min at 25°. Methylamine was 250 mM and organic solvent when present was at 50% (v/v). Buffers used were: pH 4.0, 5.0, sodium acetate; pH 6.0, 7.0, sodium cacodylate; pH 8.0, 9.0, sodium barbital (freshly prepared, 0.2 ml of 50 mM); pH 9.0, 10.0, sodium bicarbonate. Values for pH 9.0 represent the average of both buffers. (b) Same as in part a but using 10 mM methylamine. Samples withdrawn after 90 min at 25°. The amount of P_i liberated in the absence of methylamine under the conditions of both experiments has been separately determined and duly subtracted. All values represent the average of two experiments, each assayed in duplicate. Solvents used were: dimethyl sulfoxide (△), dimethylformamide, (○), acetonitrile (□), and H_2O (▽).

ion catalyzed elimination of a water molecule from the carbinolamine (III) to form the corresponding Schiff base (IV), a well-established feature of various Schiff base forming reactions (Cordes and Jencks, 1962; Jencks, 1959). Chemical considerations suggest that the carbinolamine addition complex may not render C-4′ sufficiently acidic to permit subsequent abstraction of the attached proton by the attacking base in the initial step of the elimination reaction. Sufficient electron-withdrawing power may, however, be generated with the formation of the corresponding Schiff base and especially so if the latter were to exist in the protonated form. The acid-catalyzed removal of water may therefore represent a prerequisite for β elimination to subsequently proceed.

In Figure 6 an attempt is made to satisfactorily account for those features of the reaction considered by this author relevant to its mechanism. The sequence of reactions proposed here is at variance with previous suggestions (Khym and Cohn, 1961; Cohn and Khym, 1962; Khym, 1963) which maintained that initially a cyclic morpholine-like adduct formed between periodate-oxidized 5′-AMP and one methylamine molecule, this compound then liberating P_i upon further acidification. While such a compound may indeed exist in solution, its identification rested on the proposed structure of a borohydride reduction product (Khym, 1963; Brown and Read, 1965) obtained when only 8% of the total

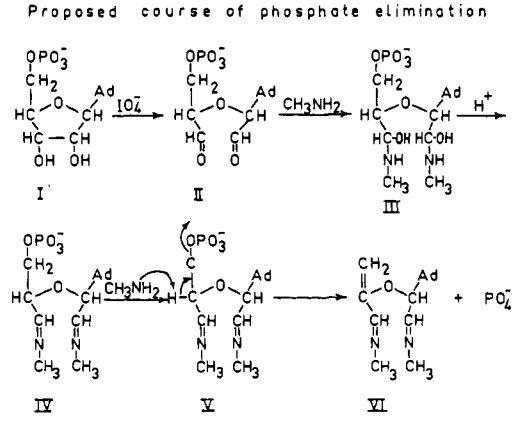

FIGURE 6

5′-AMP had been liberated in the presence of a fivefold molar excess of methylamine (Khym, 1963). Since the treatment with borohydride may have also shifted the equilibrium in solution toward this particular compound simultaneously reducing double bonds if present, the evidence appears insufficient to establish the morpholine derivative as an intermediate in phosphate release. While there is no direct evidence with regard to the exact number of methylamine residues bound, it appears likely that under the conditions used here both aldehyde groups condensed with one methylamine molecule each as shown in Figure 6. Possibly a sequence of reactions similar to that proposed here may take place also during internal nucleic acid degradations such as those of DNA (Burton and Petersen, 1960), apurinic acid (Livingston, 1964), or tRNA (Phillipsen *et al.*, 1968), in which amines catalyze the breakdown of a polynucleotide bearing free aldehyde groups in position β to carbon atoms linked to phosphoryl residues of the sugar phosphate backbone.

The behavior of phosphate elimination in the presence of either dimethyl sulfoxide, dimethylformamide, or acetonitrile appeared similar and was consistent with a multistep mechanism including partial reactions which differ with respect to acid and base catalysis. Notwithstanding small differences in promoting phosphate release, which appeared inversely related to the solvating power toward cations (Parker, 1962) and the affinity for the proton (Kolthoff *et al.*, 1968) of these solvents, this behavior can be qualitatively accounted for in terms of the hypothesis (Parker, 1962) that the organic solvent was increasing the effective concentration of the basic species in solution. The ability to affect the nature of the rate-limiting step is thus considered analogous to that of the pH and the concentration of methylamine. It is unlikely that the optimum curve resulted from maxima in the degree of structure of the solvent since no such effect was observed when an isolated β-elimination reaction was studied (Cockerill, 1967), the positions of the maxima observed here varied with the concentration of methylamine in an accountable manner, and did not usually coincide with those observed in the physical properties of the aqueous mixtures of dimethyl sulfoxide (Cowie and Toporowski, 1961) or dimethylformamide (Fratiello, 1964).

Recent findings in this laboratory indicated that on the one hand 5′-AMP may be oxidized in dimethyl sulfoxide and dimethylformamide according to Yu and Bishop (1967) and on the other that these solvents inhibited bovine pancreatic RNase and RNase T1 (A. Steinschneider and K. Druck, to be published). Together with the present findings,

it appears that procedures may be developed for the stepwise degradation of RNA in the presence of dipolar aprotic solvents which could improve the conditions with respect to nonspecific internal degradation of the polynucleotide chain.

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Charge Effect on the Interaction of Free Radicals from Phenazine Methosulfate with Deoxyribonucleic Acid*

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ABSTRACT: The complexes of the neutral and of the protonated forms of the 5-methylphenazyl-2-nitrile free radical with deoxyribonucleic acid have been studied with ultraviolet–visible and electron spin resonance spectroscopy. For the uncharged free radical at pH 10 a binding constant $K_I = 4 \times 10^4$ M^{-1} was determined. The binding constant for the cation radical at pH 4.7 was found to be $K_I = 3 \times 10^6$ M^{-1}. Both these measurements were made in acetone–water mixed solvent (1:9, v/v) with Na^+ concentration 0.001 M. These quantities can be combined to obtain an estimate of the contribution of the charge to the binding free energy of 2.5 kcal mole^{-1}. This result is compared to previous estimates obtained for complexes of deoxyribonucleic acid with aminoacridine cations.

Binding of planar aromatic molecules to nucleic acids is a subject of considerable interest and the determination of the structure of resulting complexes has been the aim of many investigations in recent years. Much of this work has involved dye molecules of the aminoacridine class and a useful review of this subject is available (Blake and Peacocke, 1968). At neutral pH, most of the biologically active aminoacridines exist in the cationic form and bind to DNA in two clearly distinguishable ways. The type I complex is formed at low concentrations of the dye relative to nucleic acid phosphate and bears most resemblance to the proposed intercalated structure which provides an appealing model for the frame-shift mutational activity of these dyes (Lerman, 1961; Crick *et al.*, 1961). For high dye concentration in which the number of bound dyes is approximately equal to the number of polymer phosphates, the type II complex predominates and is characterized by binding on the outside of the double helix. Most of the investigations have been focused on the type I complex because of its obvious biological relevance.

It is a well established fact that an increase in ionic strength leads to a decrease in the extent of binding of both types of complexes (Drummond *et al.*, 1965). On the other hand, it also is clear that uncharged aromatic hydrocarbons and heterocyclic molecules likewise bind to nucleic acids and are thereby rendered soluble in aqueous solution (Boyland and Green, 1964; Nagata *et al.*, 1966). These observations prompted our interest in the contribution of the charge of the dye to the binding free energy of type I complexes. Previous estimates of the effect have been made by comparing the

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