Periodate Oxidations of Enamines. I. Oxidation of Adenosine 5′-Monophosphate in the Presence of Methylamine*

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ABSTRACT: A novel mechanism useful in studies concerned with RNA sequence analyses is proposed for the rapid phosphate and adenine elimination from periodate-oxidized methylamine-treated adenosine 5′-monophosphate (AMP). Periodate oxidation of AMP in the presence of methylamine uses 4 equiv of periodate instead of 1. The isolated products of this reaction are adenine, inorganic phosphate, formic acid, and carbon dioxide. Inorganic phosphate formation from AMP was shown to be dependent on 1 equiv of periodate and methylamine and to be too rapid to be accounted for by elimination from an aldimine. Adenine formation was dependent not only on methylamine but also on an additional 3 equiv of periodate. A mechanism which requires the formation of a cyclic enamine is used to explain both phosphate and adenine formation, the former by rearrangement of the enamine to a conjugated system and the latter by periodate oxidation of the enamine. This oxidative mechanism has value in biochemistry because it predicts that certain functional group arrangements often encountered in natural products such as amino sugars will require more periodate than expected on glycol content. One such substance is 5′-amino-5′-deoxyuridine which has a single glycol group but uses 4 equiv of periodate for complete oxidation.

In recent years attention has been focused upon the ribonucleotide sequence in biologically active RNA. Among the different procedures which have been used in RNA sequence analysis there exists only one chemical method for stepwise sequencing (see Gilham, 1970, for general references). In this procedure, the RNA molecule must be terminated by a nucleoside because the initial step in the method is oxidation of its *cis*-glycol group with periodate. This reaction is shown in Figure 1 (**1**) for an RNA molecule terminated with adenosine.

In order for this oxidation to be useful in sequence analysis procedures, the oxidized nucleoside must be cleaved quantitatively from the RNA polymer and must be obtained in a readily identifiable form. Brown and his collaborators (1953) and Whitfeld and Markham (1953) suggested a method for the cleaving reaction. It consisted of treating the oxidized nucleoside at pH 10 for prolonged periods and was rationalized as occurring *via* a base-catalyzed β elimination as shown in Figure 1 (**2**). However, this method proved to be less than satisfactory because elimination of the phosphate ester was not quantitative (Brown *et al.*, 1955). Hakamori (1959) showed that quantitative phosphate elimination could be achieved when the oxidized nucleoside was treated with cyclohexylamine. Following reports showed that other primary amines such as lysine (Ogur and Small, 1960), methylamine (Khym and Cohn, 1961), and aniline (Fraenkel-Conrat and Steinschneider, 1968) were also effective in the elimination reaction, and that the use of these amines allowed complete reaction to occur at pH's substantially less than 10 (Khym and Cohn, 1961).

The precise reason for the increase in the rate of phosphate elimination with primary amine was not understood; however, it was suggested (Khym and Cohn, 1960; Steinschneider, 1971) that the primary amine condenses with the aldehydes of the oxidized nucleoside to form an aldimine from which phosphate elimination occurs. Thus, in the absence of amine, the dialdehyde, Figure 1 (**2**), most probably exists as a hydrate **2a,a′** from which base abstraction of the proton at $C_{4'}$ would appear unlikely, particularly at slightly alkaline pH's. On the other hand, the $C_{4'}$ hydrogen of the bisaldimine **2b** was assumed to be sufficiently acidic to allow proton abstraction with concomitant β elimination of the phosphate ester. Aldimine formation is clearly possible and the rates of phosphate elimination for an aldimine should be much faster than from the hydrate but both reactions would be fast only at high pH's. The problem is to reconcile the expected slow rate of β elimination from an aldimine system (Tener, 1961) at pH's around 7 with the very rapid rates actually found (Khym and Cohn, 1961).

Another problem associated with this chemical approach is the identification of the nucleoside obtained after oxidation. From Figure 1, it can be seen that several intermediates in addition to adenine can be obtained from the elimination reaction. Thus, an analysis of the reaction solution immediately after phosphate elimination could yield confusing results not only as to the nature of the terminal purine or pyrimidine base but also as to its amounts. In order to circumvent this difficulty, the purine or pyrimidine can be obtained free by an additional step, namely, subjecting the reaction solution to either base (Whitfeld, 1954) or acid (Khym and Cohn, 1961) hydrolysis. In another study, it was found that this acid or base step could be eliminated and the purine or pyrimidine obtained free if the oxidation solution contained not only primary amine but also excess periodate (Neu and Heppel, 1964).

The nature of this second reaction requiring excess periodate is not known; however, it is clear that excess periodate is required for complete elimination of the purine or pyrimidine base at pH's between 6 and 9.

From the foregoing observations, it appears that more than one type of reaction is involved in the phosphate ester elimination and purine or pyrimidine base formation from the oxidized nucleoside. Although several mechanisms for these reactions have been proposed (Khym and Cohn, 1961; Steinschneider, 1971), they cannot be generalized to include all the

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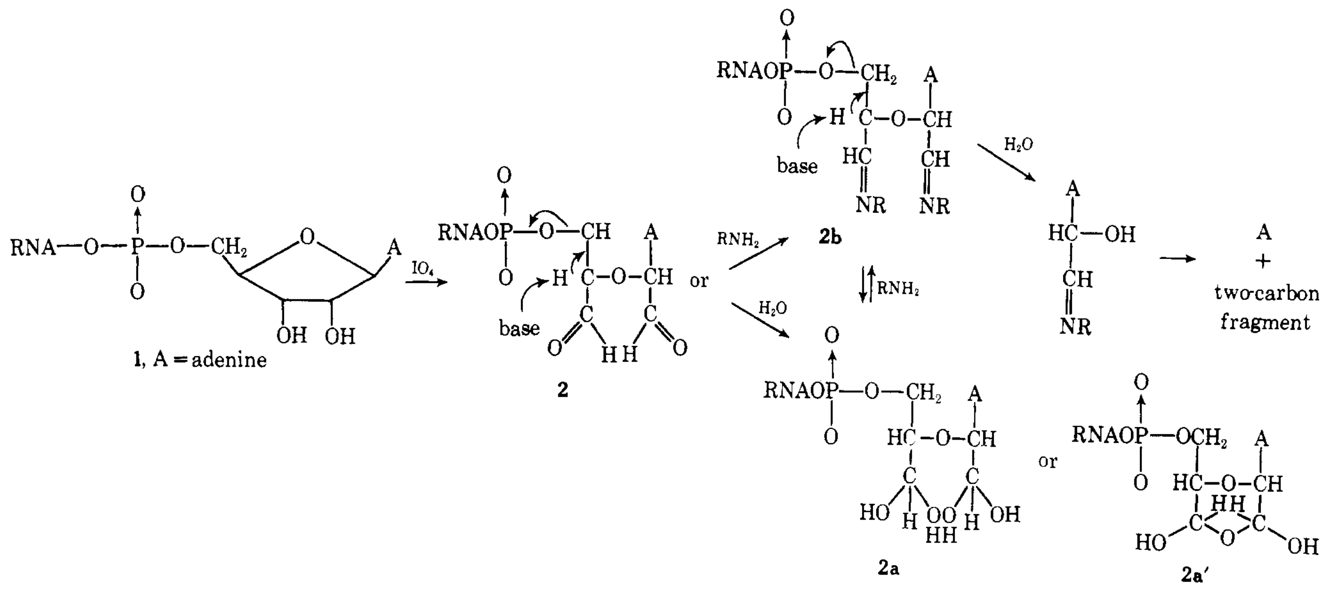

FIGURE 1: Base-catalyzed phosphate elimination.

observations noted (see Schmidt, 1968, for general references).

The purpose of this report and its companion paper (L. Werthemann and D. H. Rammler, 1971)[1] is to document studies which explain the involvement of primary amine and periodate in these elimination reactions at neutral pH and to establish the conditions required for these reactions in sequence studies on RNA.

Materials and Methods

All chemicals were the highest grade commercially available. The pH of the periodate oxidations was kept constant using a Radiometer pH-Stat equipped with a jacketed titration cell kept at 37°. Periodate was measured using the arsenite–iodine method described by Guthrie (1962). Paper chromatography was performed using the descending technique on double-acid-washed Whatman No. 44 paper. A neutral solvent system, 1-butanol–water (86:14, v/v), was used to separate adenine from the other components in the oxidation mixture. Adenine was eluted from paper with HCl (0.01 M) for 18 hr and its concentration was determined spectrophotometrically. Its authenticity was determined by comparing its chromatographic and spectral properties with a known sample. Formic acid was measured enzymatically using the method described by Rammler and Rabinowitz (1962).

Results and Discussion

As noted earlier, the mechanism by which the phosphate is released from the oxidized nucleotide is still obscure. It has been suggested that this reaction occurs *via* a β-elimination reaction (Steinschneider, 1971) but its rates around neutrality appear to be too rapid to justify this conclusion. Further, β-cyanoethyl phosphate, a substance known to eliminate its phosphate *via* a base-catalyzed elimination (Tener, 1961), is stable under conditions which totally release phosphate from periodate-oxidized adenosine 5′-monophosphate (AMP). It has also been proposed that the elimination occurs *via* an aldimine formed by the addition of methylamine to carbon $C_{4'}$ of oxidized AMP (**3**, Figure 2). If this were the primary mechanism, the rate of phosphate released from **3** should approximate that of its structural analog glyceraldehyde 3′-phosphate (**4**). Shown in Figure 2 is a comparison of the two rates and it is apparent from these data that the rates are substantially different. Thus, elimination from **3**, curve 1 (▲), is more than 90% complete in the time for 35% from **4**, curve 2 (●). These results indicate that the notion that phosphate is released from an aldimine of periodate oxidized AMP is probably not correct and that the driving force for the rapid elimination from **3** is uniquely associated with its structure.

In the prior experiments, the reactions contained only methylamine and the oxidized nucleotide. The possibility that periodate can be involved in the phosphate elimination reaction with methylamine has been suggested (Neu and Heppel, 1964). If periodate is involved, it can only be by the further oxidation of **3** which would yield in methylamine the aldimine of glycol aldehyde phosphate **5**. The elimination rate of phosphate from **5** in the presence of periodate and methylamine is given by curve III (□). Curve IV (○) shows the rate of phosphate released from **3**, under identical conditions, and it is considerably different from that found for **5**. Thus, it appears that **5** is not an intermediate in this elimination reaction.

Adenine Elimination Reaction. In the complete oxidation reaction, namely, nucleotide, excess periodate, and methylamine, adenine is rapidly released from the nucleotide. This reaction was next investigated because of the possibility that the reactions leading to phosphate elimination were similar to those which released the adenine. The initial experiments were concerned with the amount of methylamine which condensed with the oxidized product. If methylamine condensed with each of the aldehydes of the oxidized nucleotide (Steinschneider, 1971), the resulting bisaldimine would be expected to eliminate its phosphate at a rate similar to that found for the aldimine of glyceraldehyde 3-phosphate. This was shown not to be the case suggesting that either 1 or more than 2 equiv of methylamine react with the oxidized nucleotide. A titrimetric method was used to estimate this quantity of methylamine; however, because the reaction with the nucleotide re-

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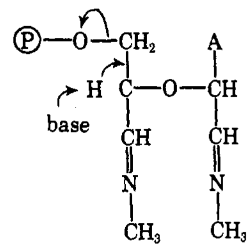
[1] To be published.



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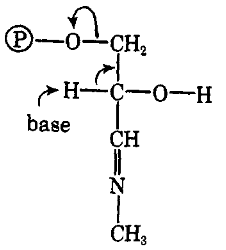

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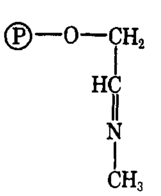

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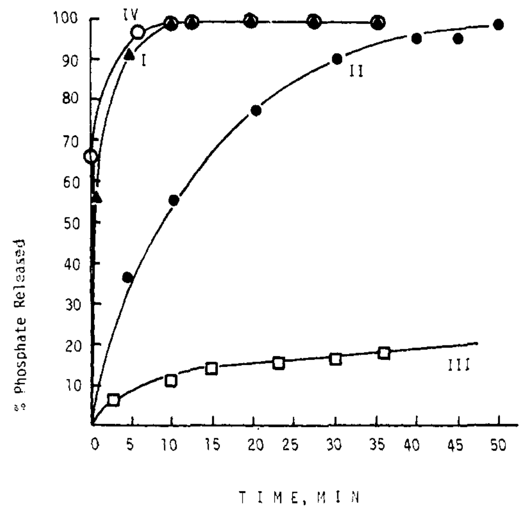
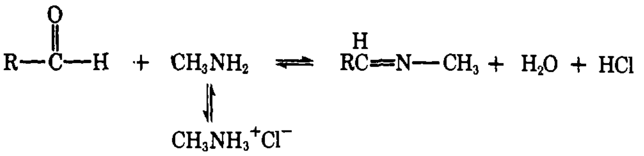


FIGURE 2: Kinetics of phosphate elimination at 37°. Curve I (▲) is the rate of phosphate elimination from methylamine-treated periodate-oxidized AMP; curve II (■) from methylamine-treated D,L-glyceraldehyde 3′-phosphate; curve III (□) from methylamine-treated glycolaldehyde phosphate; curve IV (○) from methylamine-treated periodate-oxidized AMP in the presence of tenfold excess of periodate. The reaction solutions contained the phosphate esters (30 μmoles); methylamine·HCl (2.0 M brought to pH 7.5 with NaOH and buffered with triethanolamine 0.3 M; pH 7.5) in a final volume of 4 ml. Oxidized AMP was prepared by oxidizing with 1.1 equiv of periodate for 30 min prior to the addition of methylamine. Glycolaldehyde phosphate was prepared *in situ* from α-glycerol phosphate and 1 equiv of periodate. Phosphate analysis was performed using the method described by Ames and Dubin (1960). The percentages plotted are the ratios of phosphate found to AMP carried through the same procedure, but without periodate, and determined at 260 nmoles (ϵ 15.4 × 10^3). These values differed from the absolute AMP concentration (stock solution) by between 3 and 10%. Ⓟ = $(HO)_2P(O)$–.

leases acid, that is, inorganic phosphate, these measurements were made on periodate-oxidized adenosine. The reaction which forms the basis of this measurement is shown in Figure 3. When methylamine hydrochloride at pH 7.5 is added to oxidized adenosine at pH 7.5, acid is formed, as shown, and its quantity is proportional to the amount of aldimine formed. The HCl released can be readily measured by a pH-Stat and Figure 3 shows the pH-Stat tracing which indicates that about 1 equiv of acid is released in 5 min, the time required for essentially complete phosphate elimination. This measurement and the oxidation of 5′-aminouridine, which is discussed later, indicate that the oxidized nucleotide condenses with about one methylamine, and that the second aldehydic group is either unreactive or is blocked by a second rapid reaction with the aldimine.

Periodate Oxidation of AMP in the Presence of Methylamine. The rate of oxidation of AMP and UMP at pH 7.5[2] is shown in Figure 4. Curve I is the rate and periodate consumption for the oxidation of AMP and UMP, respectively, in solution not containing methylamine, and as shown, only the expected 1 equiv of periodate is used. However, when methylamine is added to the oxidation, an additional 3 equiv are used (curve II).[8] This is true whether all the reactants are together initially or the methylamine is added later. The fact that the reaction is similar for both types of nucleotides and the overoxidation requires 3 equiv suggests that it is not a random overoxidation but rather is specific and dependent on the product of the methylamine reaction with the oxidized nucleotide.



$$\mathrm{R{-}\overset{\overset{O}{\|}}{C}{-}H + \underset{\underset{CH_3NH_3^{+}Cl^{-}}{\updownarrow}}{CH_3NH_2} \rightleftharpoons R\overset{H}{C}{=}N{-}CH_3 + H_2O + HCl}$$

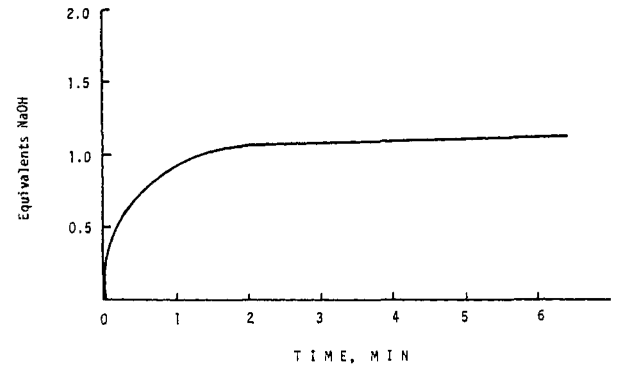


FIGURE 3: Periodate-oxidized adenosine and methylamine. The solution at 37° contained adenosine (0.60 μmole) in 1.3 ml of aqueous sodium periodate (0.60 μmole). The pH of this solution was brought to 7.5 after 15 min. To this was added methylamine.HCl (2.7 ml; pH 7.5, 37°) and sodium hydroxide was added to maintain the pH at 7.5. Figure 3 is a tracing of the amount of alkali added by the titrimeter to a nitrogen gas shielded oxidation solution for 6 min.

Analysis of the products of these reactions by paper chromatography after 10-min oxidation revealed very little adenine or uracil in the reactions containing no methylamine or methylamine and 1 equiv of periodate, while a considerable amount was found in reaction with excess periodate and methylamine. These results suggest that the rapid release of the heterocyclic base from the oxidized nucleotide depends on both excess periodate and methylamine, unlike the phosphate elimination reaction. This dependency is more clearly demonstrated in the following experiments.

Adenine Formation and Its Dependency on Periodate. In Table I are noted the amounts of adenine formed after 5- and 30-min oxidation as a function of periodate. When 1.25 equiv of periodate is used only 17% of the adenine is released from the oxidized nucleotide in 5 min while 2.75 equiv yields 45% in a similar period. Increasing the periodate concentration to 6.4 equiv, that is, an excess over the 4 equiv required for complete oxidation, provides essentially quantitative yields of adenine in 30 min while a large excess, 13 equiv, substantially increases the rate and gives complete yields in 5 min.

These data demonstrate that overoxidation is required for rapid adenine formation in the presence of methylamine and that this reaction utilizes 3 equiv of periodate.

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[2] pH 7.5 was chosen for the oxidations because RNA is maximally stable at this pH.

[8] The rate of phosphate elimination in the presence of dimethylamine is considerably slower than that for methylamine.

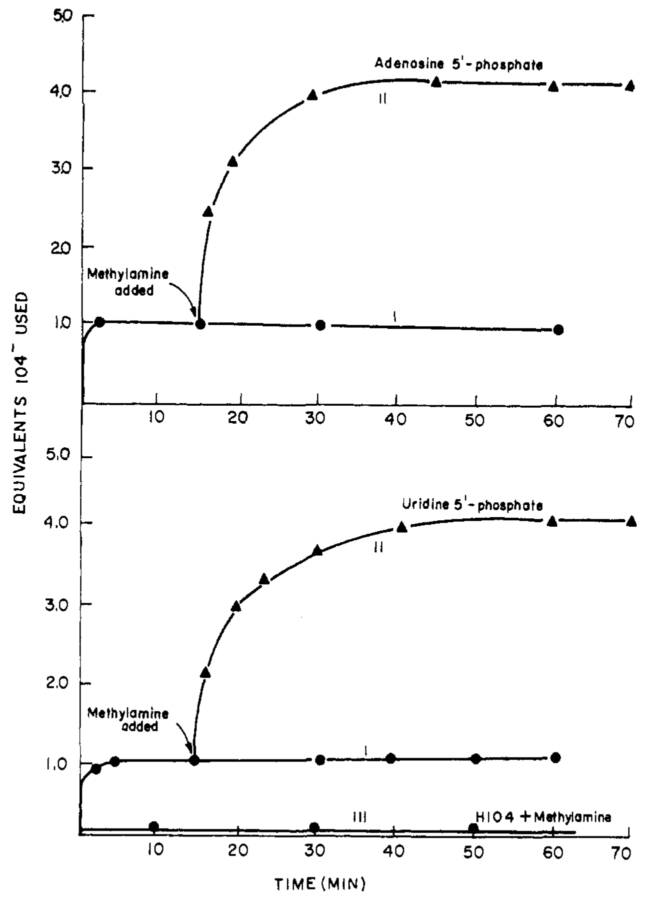

FIGURE 4: Periodate oxidation of nucleotides in methylamine solution. The oxidations were carried out as follows. Curve I (●): to a solution of nucleotide (0.12 ml, 0.25 M) at 37° was added sodium periodate (0.8 ml, 0.5 M). Water was added to give a final volume of 2.0 ml. The pH was maintained at 7.5 and samples were removed to determine periodate (Guthrie, 1962). Curve II (▲): after 15 min methylamine·HCl (2.0 ml, 4.0 M, pH 7.5, at 37°) was added. The pH was maintained by the addition of sodium hydroxide. Samples were removed at the periods indicated and analyzed for periodate. No periodate was used in a solution not containing the nucleotide, curve III.

TABLE I: Adenine Formation and Its Dependence on Periodate.[a]

IO_4:AMP	Oxidation Time	Adenine Found (%)
1.25	5	17
1.25	30	22
2.75	5	45
2.75	30	52
6.4	5	83
6.4	30	97
13.0	5	96
13.0	30	97

[a] This reaction was carried out in the following manner. To a solution of AMP (0.12 ml; 0.25 M) at 37° was added sodium periodate. The amount of periodate was varied to yield the ratios given above. The pH of this aqueous solution was around 4.5. After 15 min, the reaction was quickly brought to pH 7.5 and methylamine·HCl (2.7 ml; 4.0 M; pH 7.5 at 37°) was added. The pH was kept at 7.5 by the addition of sodium hydroxide in the pH-Stat. Where necessary, water was added to bring the final volume to 4 ml. Aliquots were removed periodically for periodate, phosphate, formic acid, and adenine analysis. Adenine was determined after chromatography in butanol–water (86:14, v/v). It was eluted in HCl (0.01 M) and its concentration determined spectrophotometrically.

TABLE II: Formic Acid.[a]

Expt	Periodate to AMP	Oxidation Time After Addn of Methylamine	Formic Acid: AMP
1	1	30	0.01
2	9	5	1.2
2	9	30	1.4
3	13	5	1.55
3	13	30	1.64

[a] The oxidation solutions were prepared as described in Table I. Formic acid analysis was performed directly on aliquots from the oxidation solution in expt 1 and 2. In expt 3, the oxidation solution was brought to pH 11 with potassium hydroxide. After 40 min at this pH at 50°, the solution was neutralized and assayed for formic acid. Control reactions with no adenosine 5′-phosphate yielded small amounts (less than 5%) of formic acid.

It is interesting to note that the recoveries of adenine are always less than theoretical. When adenine is added to the oxidation solution in place of AMP and carried through the same experimental procedure, it can be recovered with less than 1% loss. These results suggest that the oxidation conditions do not break down this purine. In other experiments, not reported, similar results were found for UMP and uracil recoveries. The reason for our inability to isolate all of the adenine from the oxidized AMP is not understood; however, the difference, 3%, if real, will not affect the mechanistic conclusions derived from this study.

Nonultraviolet-Absorbing Products. The products of the oxidation reaction were next investigated in an attempt to determine the nature of the overoxidation reaction. In the oxidation of adenosine it was observed that addition of excess periodate caused a decrease in the pH of the oxidation solution, suggesting that acid was being formed. Examination of the acid products from the oxidation reaction showed that one of the acids was formic acid. This substance was detected and characterized using an enzymatic method specific for formic acid (Rammler and Rabinowitz, 1962). The results of this analysis are shown in Table II. A comparison of expt 1 with 2 and 3 indicates that formate formation is dependent on excess periodate, that is, it appears to be a product of the overoxidation reaction. The amount of formate released does not increase significantly after treatment with strong base, indicating the absence of formate esters, and these data show that each equivalent of AMP yields more than 1 equiv of formate as a result of the overoxidation reaction.

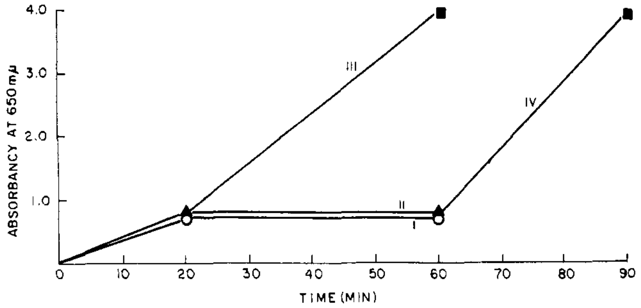

FIGURE 5: Carbon dioxide formation. The composition of the oxidation solution was similar to that described in Table I except that the oxidation represented by curve I contained only 1 equiv of periodate and that by curve II contained no methylamine. The oxidations were carried out at pH 6.5 with carbon dioxide free nitrogen gas bubbling through the reaction solution and into a column of barium hydroxide. After the periods indicated, the opacity of the barium hydroxide was measured at 650 nm.

On acidification, the overoxidation reaction solution appeared to release a gas. The nature of this gas was investigated in the following manner. Carbon dioxide free nitrogen gas was passed through an oxidation solution containing 1 equiv of periodate, AMP, and methylamine and thence into a tower containing a clear solution of barium hydroxide. At different time intervals, aliquots of the barium hydroxide solution were removed and their opacities measured spectrophotometrically at 650 nm. The results of this study are shown in Figure 5 (curve 1). As shown, the opacity of the solution did not change significantly after 60-min gassing with nitrogen (curve 1). Similar results were obtained when nitrogen was passed through an oxidation solution containing AMP, 10 equiv of periodate, but no methylamine (curve II, to 60 min). However, when methylamine was added after 60 min of nitrogen gassing, curve IV, a threefold increase in opacity was obtained. This amount corresponded to that found with the complete oxidation solution, as shown by curve III. A solution of periodate and methylamine gave results similar to those shown in curve I.

These data indicate that a product of the overoxidation reaction is a gas and it is reasonable to assume from its reaction with barium hydroxide that the gas is carbon dioxide.

A Summary of Results and a Mechanistic Rationalization. The preceding results can be summarized as follows: 1 equiv of periodate is used to oxidized AMP in the absence of methylamine and 4 in its presence. The oxidized nucleotide reacts with about 1 equiv of methylamine. The oxidized methylamine-treated nucleotide rapidly eliminates inorganic phosphate, and this reaction does not depend on periodate. The rapid release of adenine from the oxidized methylamine-treated nucleotide depends on the consumption on an additional 3 equiv of periodate and the isolated products of the reaction, in addition to adenine, are formic acid and carbon dioxide.

A novel oxidation mechanism which will accommodate all of these observations is shown in Figure 6. The initial oxidation of **6** yields a dialdehyde which condenses with methylamine to form the monoaldimine **7** which undergoes intramolecular condensation yielding the carbinolamine **8** (Khym, 1963). This substance dehydrates to form the enamine **9** which rearranges **10** to form the conjugated ammonium ion **11** with elimination of phosphate.

This series of reactions explains why only 1 equiv of both methylamine and periodate is required for the elimination of

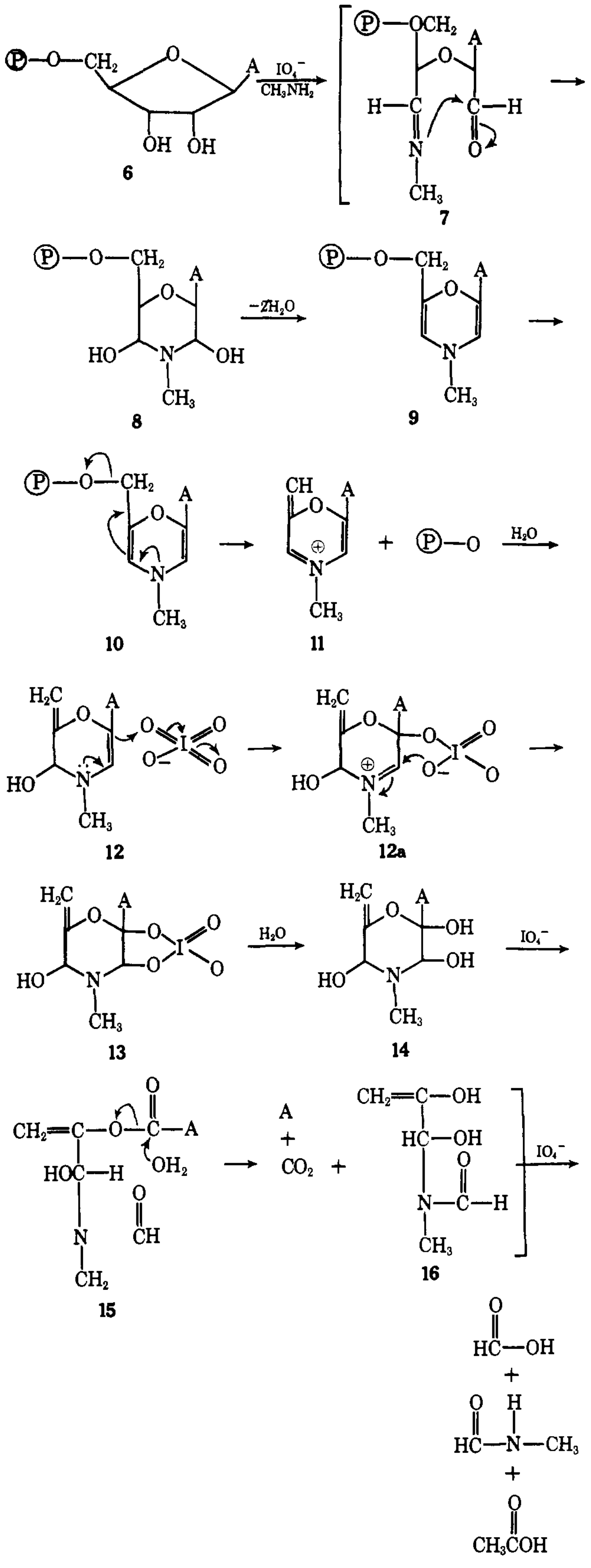

FIGURE 6: Enamine formation and the oxidation of AMP. A = adenine; Ⓟ = $(HO)_2P(O)$–.

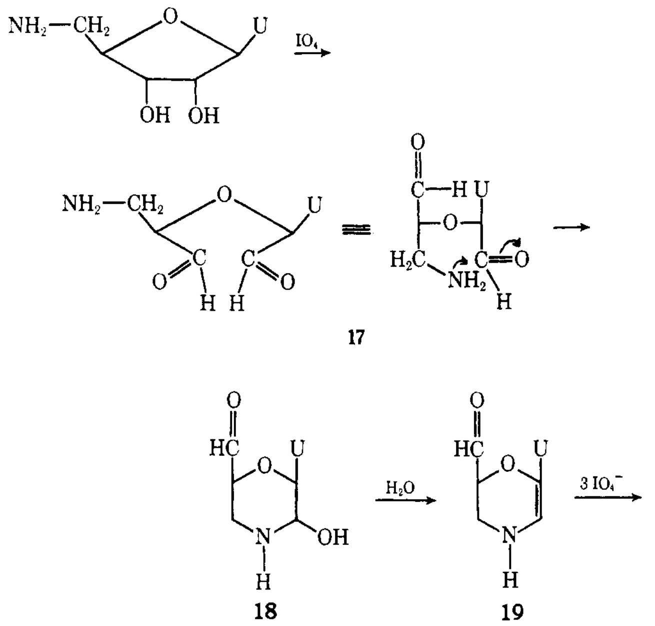

FIGURE 7: Periodate oxidation of 5′-amino-5′-deoxyuridine.

phosphate and provides a step, the formation of the conjugated ammonium ion **11**, with sufficient driving force to explain the rapid phosphate elimination at neutral pH.

The reactions leading to the formation of adenine require the consumption of an additional 3 equiv of periodate. These observations are explained by the following. Hydration of **11** yields the enamine **12** whose oxidation by periodate initiates the overoxidation reactions. There is a nucleophilic attack of C_1 onto an oxygen of periodate, with ring closure to form the cyclic periodate ester **13**. Hydrolysis of this ester provides the diol **14** which utilizes the second equivalent of periodate in the overoxidation reaction. The product of this reaction is the adenine carbamate **15** which can undergo hydrolysis yielding adenine and carbon dioxide. The diol **16** reacts with the third equivalent of periodate yielding *N*-methylformamide (Khym, 1963), formic acid, and presumably acetic acid.

There is precedent for a number of the reactions of this mechanism. Thus, the carbinolamine **8** and its dehydration product **9** can be inferred from the work of Brown and Read (1965) who obtained 2-(9-adenyl)-6-hydroxymethyl-4-methylmorpholine as the principal product after Raney nickel reduction of the condensation product between methylamine and oxidized adenosine. This substance could only have been formed by reduction of a cyclic enamine.

The key step in the overoxidation reaction is the formation of enamine **12** from the aldimine with its subsequent oxidation by periodate. Enamine oxidations are not unusual and have been described (Heyl and Herr, 1950; Dolby and Rodia, 1970; for general references, see Cook, 1969); however, what is unusual is their formation and oxidation in water.

In this mechanism it is proposed that the enamine is in equilibrium with its carbinolamine in water and the reaction is completed because periodate rapidly oxidizes the small amount of enamine present, forcing the reaction away from the more stable carbinolamine. The nucleophilic attack of C_1 onto the oxygen of periodate is similar to a reaction proposed by Bestmann and his collaborators (1969) for the oxidation of a phosphinalkene derivative by periodate.

This mechanism can be used to explain a number of "Abnormal Periodate Overoxidations" of amino sugars reported in the literature (Weiss *et al.*, 1959). These authors found that 5-amino-5-deoxy-β-D-ribofuranosyl-6-dimethylaminopurine used 4 equiv of periodate instead of the expected 1. We have found that a similar amino nucleoside, 5-amino-5-deoxyuridine (Horowitz *et al.*, 1962), also uses 4 equiv of periodate. Both oxidations took place in the absence of added primary amine. These overoxidations are not unusual if they are considered analogous to the oxidation of AMP in the presence of methylamine. This analogy is shown in Figure 7. After the initial periodate cleavage of the *cis*-glycol group, the 3-carbon part containing the primary amino group **17** can rotate and condense with the C_2 aldehyde, forming the carbinolamine **18**. Dehydration of this substance yields the enamine **19** which is subsequently oxidized by 3 equiv of periodate as described in the mechanism shown in Figure 6. The overall result of these reactions is that 5-amino ribonucleosides would be expected to use 4 equiv of periodate instead of 1. These results indicate that care should be exercised in interpreting periodate data obtained from oxidations of amino sugars; of carbohydrates in the presence of primary amine buffers; of sugar phosphates obtained as their alkyammonium salts; and of other substances which can give primary amines.

Most of the intermediates proposed in the mechanism presented in Figure 6 are hypothetical and a number of steps can be described in an alternate manner. Attempts to isolate and define these substances by chemical or physical methods gave inconclusive results. Since the critical mechanistic step is the oxidation of the cyclic enamine **12**, it was therefore important to confirm this step in a simpler system where the intermediates could be isolated. This was carried out and is reported elsewhere (L. Werthemann and D. H. Rammler, 1971).

Acknowledgement

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An Examination of D-Fructose 1,6-Diphosphate and Related Sugar Phosphates by Fourier Transform ^{31}P Nuclear Magnetic Resonance Spectroscopy*

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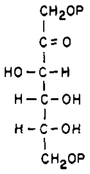
ABSTRACT: D-Fructose 1,6-diphosphate (FDP) and stereochemical analogs of its α- and β-furanose forms have been examined by ^{31}P nuclear magnetic resonance spectroscopy. The 1- and 6-phosphorus resonances of 2,5-anhydro-D-mannitol 1,6-diphosphate and methyl β-D-fructofuranoside 1,6-diphosphate, analogs of the β-furanose form of FDP, have very similar chemical shifts. Conversely, the 1-phosphorus resonances of 2,5-anhydro-D-glucitol 1,6-diphosphate and methyl α-D-fructofuranoside 1,6-diphosphate, analogs of the α-furanose form of FDP, are shifted downfield relative to the 6-phosphorus resonances. The ^{31}P nmr spectrum of FDP contains resonances ascribable to the 1- and 6-phosphate groups of both furanose forms, the β-furanose form being predominant (~90%). The assignment of these phosphorus resonances was confirmed with specifically deuterated reference compounds. In the ^{31}P nmr spectrum of D-fructose-6,6-d_2 1,6-diphosphate, the 1-phosphorus resonances of the α- and β-furanose forms remained as triplets, but the 6-phosphorus resonances collapsed to "singlets" since phosphorus–hydrogen coupling was lost. New evidence is presented confirming earlier work which demonstrated that the acyclic free keto and hydrated keto forms of FDP were not present in solution to an appreciable extent. These studies demonstrate that the four forms of FDP are present in the following percentages: β-furanose (~90), α-furanose (~10), keto (<1.7), and hydrated keto (<0.1).

Since in many reactions in which sugar phosphates participate the free aldehydo or free keto form is the actual substrate (Gracy and Noltmann, 1968; Dyson and Noltmann, 1968; Trentham *et al.*, 1969; Rutter, 1961), it is important to know the exact proportion of the reactive form in the tautomeric equilibrium. Those enzymes with substrates which can exist in multiple tautomeric forms in solution may be able to bind each but may be able to utilize only one in the catalytic reaction. Where only a single form is utilized, the other forms may either be converted to the reactive form by the enzyme (Dyson and Noltmann, 1968) or they may function as inhibitors, especially if the reactive form is present in a very low proportion.

Fructose 1,6-diphosphate aldolase, a good example of this kind of enzyme, catalyzes the conversion of D-fructose 1,6-diphosphate (FDP)[1] to 1,3-dihydroxy-2-propanone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (G-3-P). Each of these substrates may exist in solution in multiple forms. Solutions of G-3-P have been shown (Trentham *et al.*, 1969; Swenson and Barker, 1971) to contain a mixture of the aldehydo form (4%) and the hydrated aldehydo (gem-diol) form (96%); and DHAP exists as a mixture of the free keto (55%, 25°) and hydrated keto (45%) forms (Gray and Barker, 1970; Reynolds *et al.*, 1971). D-Fructose 1,6-diphosphate would be expected to exist in solution as a mixture of the keto **1**, hydrated keto **2**, β-furanose **3**, and α-furanose **4** forms, but a considerable controversy has arisen regarding the relative proportion of each. In an examination of aqueous solutions by ultraviolet spectroscopy, McGilvery (1965) concluded that Na_2FDP existed predominately in form **1**, but Gray and Barker (1970), in a study of FDP and various acyclic 2-ketose phosphates in D_2O by ^{1}H nuclear magnetic resonance (nmr) spectroscopy and infrared spectroscopy, found that the keto **1** and hydrated keto **2** forms of FDP were not present in solution to an appreciable extent (<2%). In another study of 2-ketoses and 2-ketose phosphates by ultraviolet and circular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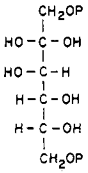
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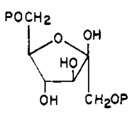
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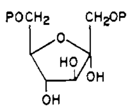
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[1] Abbreviations used are: FDP, D-fructose 1,6-diphosphate; DHAP, 1,3-dihydroxy-2-propanone phosphate; G-3-P, D-glyceraldehyde 3-phosphate.