Langridge, R., and Rich, A. (1962). In press.
Marsh, R. E., Bierstedt, R., and Eichhorn, E. L. (1962), *Acta Cryst. 15*, 310.
Nirenberg, H. W., and Matthaei, J. H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1588.
Rich, A., Davis, D. R., Crick, F. H. C., and Watson, J. D. (1961), *J. Mol. Biol. 3*, 71.
Speyer, J. F., Lengyel, P., Basilio, C., and Ochoa, S. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 63.
Steiner, R. F., and Beers, R. F., Jr. (1957), *Biochim. Biophys. Acta 26*, 336.
Steiner, R. F., and Beers, R. F., Jr. (1961), chap. 7 and 8 *in* Polynucleotides, Amsterdam, Holland, Elsevier Publishing Co.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962a), *Biochim. Biophys. Acta 55*, 584.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962b), *Proc. Nat. Acad. Sci. U. S. 48*, 686.

The Reaction of Methylamine with Periodate-Oxidized Adenosine 5'-Phosphate

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The periodate oxidation product of adenosine 5'-phosphate reacts with methylamine to form a cyclic, hemialdal type of structure (I) that releases inorganic phosphate in neutral or acid solution. Reduction of the cyclic compound with sodium borohydride gives rise principally to 6-adenyl-5-hydroxy-4-methyl-2-hydroxymethylphosphate morpholine (IV), in which the methylamine residue is firmly incorporated and which is hydrolyzed by acid to *N,N,N*-(ethanolalmethyl-2-hydroxypropyl-3-phosphate) tertiary amine (V). A small amount of a trialcohol monophosphate (III), free of added amine, is also formed.

The release of inorganic phosphate from periodate-oxidized nucleotides was first studied by Whitfeld (1954) and also by Brown *et al.* (1953, 1955). Several independent investigations (Hakamori, 1959; Ogur and Small, 1960; Yu and Zamecnik, 1960) have shown that the release of phosphate occurs by a more complex mechanism than the earlier reports indicated. Many amino compounds react with periodate-oxidized 5'-nucleotides to form addition complexes whose stability is dependent upon *p*H (Khym and Cohn, 1961). Inorganic phosphate is released slowly at *p*H > 8 but is released rapidly and quantitatively at *p*H < 8, the rate depending upon the amino compound used. A typical example of these interactions is given in Reaction 1 for oxidized AMP and methylamine (partial structures are used for the nucleotide derivatives). The addition compounds formed by primary amines above *p*H 8 can be stabilized with respect to phosphate released by reduction with sodium borohydride.

This paper describes the compounds formed when methylamine-treated periodate-oxidized adenosine 5'-phosphate is reduced with sodium borohydride. This reduction step sheds considerable light on the over-all reaction of periodate-oxidized nucleotides with amines, and, in particular, clarifies the relation of *p*H to stability of the amine complexes formed. The reduction, as demonstrated in Reaction 2, yields two compounds, both of which have been isolated in crystalline form. One is a morpholine derivative (IV), isolated in 77% yield, containing one nitrogen atom more than the five initially present in the adenine moiety. The other compound (III), isolated in 15% yield, contains no additional nitrogen and was found to be identical with the trialcohol monophosphate that results when oxidized adenosine 5'-phosphate is reduced with sodium borohydride in the absence of methylamine.

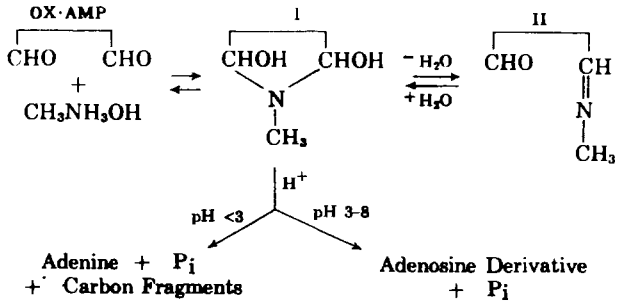
The compounds were identified from the fragments produced by acid hydrolysis. The trialcohol derivative (III) yields adenine, glycolaldehyde, and α-glycerophosphate. The morpholine derivative (IV) yields adenine and a compound which does not absorb ultraviolet light and which contains N and P in a 1:1 ratio. This latter compound, to be designated here as compound V, has been identified as a six-carbon amine having either structure Va or structure Vb of Reaction 3, which shows the products obtained when V is oxidized with periodate.

* Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

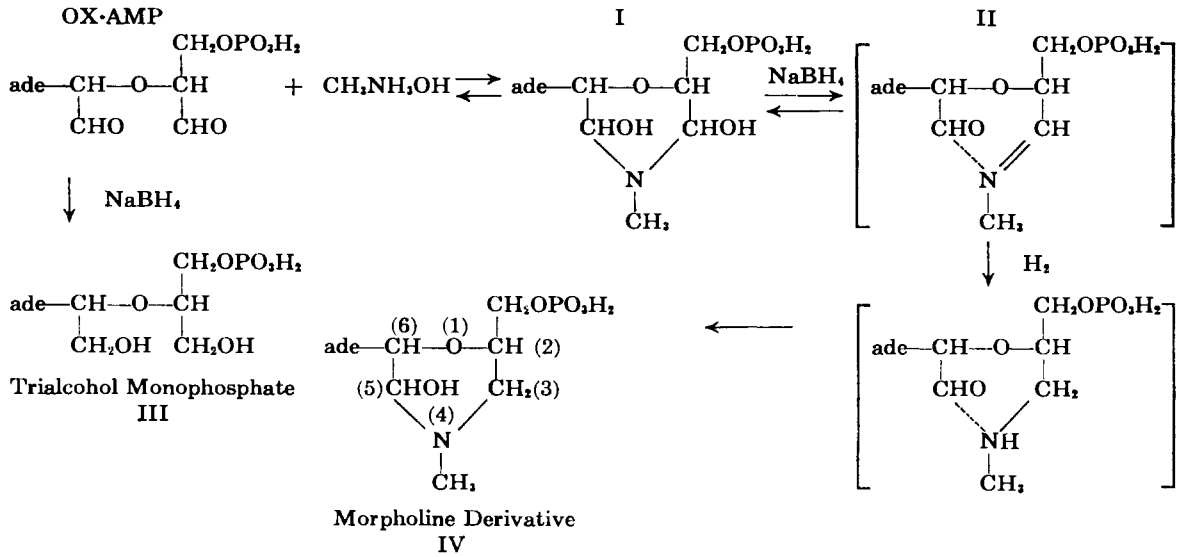
Experimental

Methylamine, Formic Acid, and Phosphorus Measurements.—The nitrogen still described by Steyermark (1951) was used to determine both *N*-methylformamide (as methylamine) and formic acid. Authentic *N*-methylformamide hydrolyzes readily to yield formic acid and methylamine under the conditions used here to determine either of these compounds. Methylamine was detected by paper chromatography (Block *et al.*, 1955) only after steam distillation of *N*-methylformamide solutions from concentrated alkali into dilute boric acid solutions. The amine was determined quantitatively by titrations of the distillates (Jones, 1944). Methylamine was determined similarly from periodate-oxidized V by the addition of excess ethylene glycol before transfer of the sample to the nitrogen still. If a volume reduction was necessary, excess acid was added after the ethylene glycol and the sample was concentrated by vacuum distillation before transfer of the sample to the nitrogen apparatus. In some cases, methylamine was determined in the effluent from a Dowex-1 column that had removed IO_3^-, IO_4^-, and phosphate derivatives and that contained only methylamine and formaldehyde. The average yield of methylamine found in oxidation mixtures was about 65%, and this value did not change significantly when samples were withdrawn for analysis any time from 6 to 72 hours.

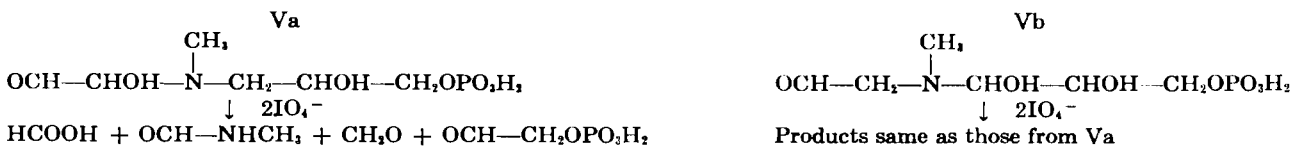
Formic acid was determined in the same steam still used for the methylamine determinations by a method similar to that described by Bell *et al.* (1944). The oxidized solution was vacuum distilled almost to dryness



Reaction 1



Reaction 2



Reaction 3

after addition of 1 ml of 1 M ethylene glycol and then transferred to the steam still with an additional 2 ml of ethylene glycol. One-half ml of 10 N H_2SO_4 was added, and the mixture was steam distilled until at least 110 ml of distillate was collected. Titration with 0.01 N NaOH of distillates from known samples containing about 50 μmoles of HCOOH showed that 90 to 96% of the acid could be recovered. In the oxidation of V, two moles of HCOOH were always found if the oxidation had proceeded for at least 48 hours.

Total and inorganic phosphate were determined by either the Fiske and Subbarow (1925) or the Griswold (Griswold *et al.*, 1951) method.

Preparation of Glycolaldehyde Phosphate.—Authentic glycolaldehyde phosphate was prepared by oxidizing α-glycerophosphate (~ 100 μmoles) with periodate. The oxidation was carried out in 0.25 M $NaHCO_3$, and after 2 hours the sample was diluted to 200 ml and percolated through 2 ml of Dowex-1 (acetate) resin. The column was washed with water, and a quantitative amount of CH_2O was found in the effluent by the chromotropic acid test (Khym and Cohn, 1960), and an 80% yield of CH_2O as its dimedon derivative, m.p. 190–191°, was obtained by the method of Bell (1948) after the effluent was neutralized with acetic acid and the *p*H adjusted to 4.5 by the addition of acetate buffer. It was not necessary to reduce the volume of the effluent or treat the solution for removal of IO_3^- and IO_4^- (these ions are retained by the resin) before the addition of the dimedon.

Glycolaldehyde phosphate was eluted from the resin column with 50 ml of 0.1 M NaCl, and 80 mg of $NaBH_4$ was added to obtain ethylene glycol phosphate. Excess $NaBH_4$ was removed by the method described previously (Khym and Cohn, 1960). Prostatic phosphatase quantitatively removed the phosphate group from the glycol phosphate to yield ethylene glycol, which was identified by a procedure given in detail elsewhere (Khym and Cohn, 1960).

In another experiment, 97 μmoles of α-glycerol phosphate was oxidized as before. After 3 hours, 17 ml of 1 M formic acid was added to the solution, and, after dilution to 150 ml, the mixture was sorbed on a 9 cm × 1 cm^2 column of Dowex-1 (formate) resin. The glycolaldehyde phosphate was eluted with 0.6 M HCOOH as shown in Figure 1A. Authentic *N*-

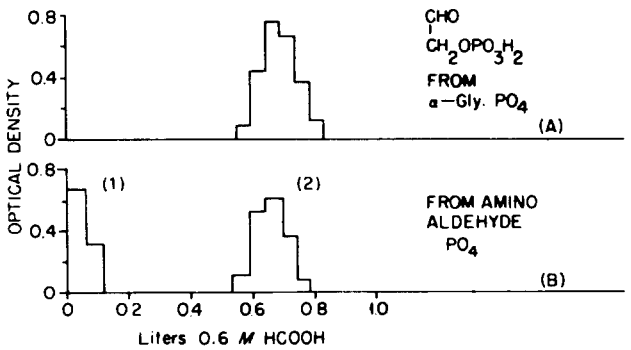


FIG. 1.—Ion-exchange analyses of periodate oxidation mixtures. *A*, quantitative recovery of glycolaldehyde phosphate from periodate-oxidized α-glycerophosphate. *B*, 65% recovery of glycolaldehyde phosphate (peak 2) and 35% recovery of an *N*-methylamine derivative of glycolaldehyde phosphate (peak 1) after amino aldehyde phosphate (V) was treated with IO_4^- for at least 16 hours. Abscissa includes volume of initial sample; ordinate *A*, phosphate color assay for recovery of 100 μmoles total P by Fiske-Subbarow method; *B*, recovery of 24 μmoles total P by Griswold method. Column, 9 cm × 1.0 cm², Dowex-1 (formate). Flow rates about 2 ml per minute.

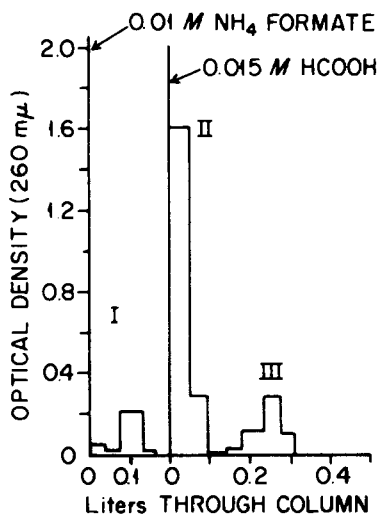


FIG. 2.—Ion-exchange separation of products after methylamine-treated oxidized AMP was reduced with $NaBH_4$. Peak I adenine, 7%; II morpholine derivative, 77%; III trialcohol phosphate, 15%. Column, 9 cm × 1.0 cm², Dowex-1 (formate). Flow rate about 2 ml per minute. Eluting reagents as indicated. Assay of fractions at 260 mμ. Ten μmoles total calculated as AMP used for this analysis.

methylformamide (Eastman Organic Chemicals) was added in varying molar proportions with the periodate in some of the oxidations of α-glycerol phosphate; the presence of the amide did not change the yield of glycolaldehyde phosphate. The glycolaldehyde phosphate fractions were vacuum distilled to a 10-ml volume. The grass-green color characteristic for glycolaldehyde was obtained when the Dische diphenylamine test (Morrison *et al.*, 1955) was applied to aliquots from the concentrated glycolaldehyde phosphate fractions. Phenylhydrazine reacted with the isolated glycolaldehyde phsophate to give the bisphenylhydrazone of glyoxal (Khym and Cohn, 1960). The column-isolated material was also reduced by $NaBH_4$ and analyzed for ethylene glycol phosphate by the procedures given previously (Khym and Cohn, 1960).

Formation of Addition Complex, Reduction with Sodium Borohydride, and Isolation of Products.—One g of adenosine 5′-phosphate was dissolved in 50 ml of 0.1 M $NaIO_4$. After 20 minutes, 3 ml of 1 M ethylene glycol was added, and the mixture was poured with stirring into 280 ml of 0.05 M methylamine. After about 45 minutes for the formation of the addition complex, 1.5 g of $NaBH_4$ dissolved in 100 ml of 0.001 M methylamine was added in small portions, with stirring, over a period of 30 minutes. The mixture was set aside for 16 hours, and then 50 ml of 1 M HCOOH was added to destroy excess $NaBH_4$; after 1 hour 50 ml of 1 M NH_4OH was added and the solution diluted to 1 liter. Isolation of the products was accomplished by ion-exchange chromatography on a Dowex-1 (formate) column 9 cm × 4 cm². This amount of resin conveniently handled 250 ml of the stock preparation at one time. After sorption of the sample, about 150 ml of 0.01 M ammonium formate removed a small amount of adenine (7%); then, after a water wash of 200 ml, 0.015 M HCOOH was started through the column. This last reagent eluted the two major components of the reduction. The morpholine derivative (IV) was eluted rapidly and was almost completely separated from the alcohol phosphate (III), which was collected after a few fractions containing small amounts of both components were discarded. Both fractions were concentrated by vacuum distillation and crystallized by addition of alcohol. In Figure 2 the separation is demonstrated for smaller amounts of material.

The morpholine derivative (IV) was dissolved in a minimum of hot water and crystallized by the addition of 5 to 7 volumes of ethyl alcohol. After cooling the crystals were filtered, washed with alcohol, and dried *in vacuo* over P_2O_5. They gave a m.p. of 222–224° with decomposition.

Anal. Calcd. for 6-adenyl-5-hydroxy-4-methyl-2-hydroxymethylphosphate morpholine (IV), $C_{11}H_{17}N_6O_6P$ (360.28): C, 36.67; H, 4.76; N, 23.33; P, 8.60. Found: C, 36.93; H, 5.23; N, 23.80; P, 8.80.

The alcohol phosphate (III) was recrystallized and dried in a similar manner and was identical with the product obtained when oxidized AMP is directly reduced with sodium borohydride. To obtain this material, 250 mg of AMP was oxidized with 10 ml of 0.1 M $NaIO_4$ and after about 20 minutes excess 1 M ethylene glycol was added. The solution was diluted to 40 ml, and 250 mg $NaBH_4$ was added and the mixture was set aside for 16 hours. Excess $NaBH_4$ was destroyed as before, and the solution was diluted to 200 ml (conc. of ammonium formate ~ 0.05 M) and adsorbed on 15 cm³ of Dowex-1 (formate) in a 4-cm² column. After a water wash, 0.01 M ammonium formate was put through the column until the effluent was neutral and essentially optically clear at 260 mμ. The alcohol phosphate (III) was eluted with 300 ml of 0.1 M HCOOH and then concentrated by vacuum distillation. The compound was crystallized and dried in the same manner as the morpholine derivative. The yield of product was 87%, with m.p. 201–203°.

Anal. Calcd. for adenyl-hydroxymethylphosphate diethylene glycol (III); $C_{10}H_{16}N_5O_7P$ (349.24): C, 34.39; H, 4.62; N, 20.05; P, 8.87. Found: C, 34.65; H, 4.83; N, 19.77; P, 8.87.

Hydrolysis of the Trialcohol Monophosphate.—This compound (III), prepared by the two methods given, hydrolyzes in 0.25 N HCl at 100° in 15 minutes to yield quantitatively adenine, glycolic aldehyde, and glycerophosphate. A hydrolysate that contained initially 50 μmoles of the trialcohol phosphate in 4 ml of 0.25 N HCl was diluted to 100 ml and passed through 2 ml of Dowex-1 (acetate) resin, which retains the glycerophosphate and allows adenine and glycolic aldehyde to be collected in the effluent. A portion of this solution

(~ 15 ml) was made alkaline and sorbed on 9 cm × 1 cm² of Dowex-1 (formate) and the presence of adenine quantitatively demonstrated by elution with 0.01 M ammonium formate. The remaining portion of the effluent was concentrated by vacuum distillation to ~ 1 ml, and glycolic aldehyde was determined as its bisphenylhydrazone (Khym and Cohn, 1960).

Glycerophosphate was eluted from the acetate resin with 20 ml of 1 N HCl and the solution vacuum distilled to remove the HCl. The glycerophosphate was dissolved in 5 ml of 0.2 M acetate buffer, *p*H 5.5, and incubated at 37° in the presence of prostatic phosphatase. After inorganic phosphate had been released, the mixture was concentrated and the presence of glycerol detected qualitatively by paper chromatography and determined quantitatively by oxidation with periodate (Khym and Cohn, 1960).

Results

Hydrolysis of the Morpholine Derivative.—This compound (IV) did not hydrolyze in 0.25 N HCl but was completely degraded after about 30 minutes in 6 N HCl at 100° to adenine and a fragment, compound V, containing nitrogen, phosphorus, and carbon in the ratio of 1:1:6. This degradation product responded to the arsenomolybdate test (Nelson, 1944) for aldehydes but gave a negative reaction (dithiocarbamate test) for primary and secondary amine residues (Umbreit, 1961). Preparation of suitable quantities of compound V for its identification was carried out as follows: 100 mg of the morpholine derivative (IV) was dissolved in 2 ml of 6 N HCl and heated for 30 minutes; after cooling, the solution was vacuum distilled to remove HCl. The residue was taken up in 5 ml of 0.05 M acetic acid and washed through 4 cm × 1 cm² of Dowex-50 (hydrogen) resin with two more portions of dilute acetic acid. Compound V was removed from the column with 50 ml of water, and the adenine was eluted with 25 ml of 5 N NH_4OH and identified as before after removal of most of the NH_4OH.

The water solution of compound V, after concentration by vacuum distillation, was used in the periodate oxidation studies.

Periodate Oxidations.—Several 30-μmole samples of compound V were oxidized in 10 ml of 0.02 M $NaIO_4$ that was 0.15 M with respect to $NaHCO_3$. After definite time intervals at room temperature, either 5 or 10 ml aliquots were passed through 3 cm × 1 cm² of Dowex-1 (acetate). The columns were washed with water until 30 ml of effluent was collected, and these fractions were analyzed for formaldehyde (Khym and Cohn, 1960). One hundred μmole samples diluted to 200 ml before passing through the same size column gave formaldehyde as its dimedon derivative (Bell, 1948) at 90% yield, m.p. 190–191°. Iodate was eluted from the columns with 40 ml of 0.1 M NH_4Cl, and the amount present was determined spectrophotometrically at 232 mμ (Khym and Cohn, 1960).

Periodate oxidations were also carried out after dephosphorylation of compound V with prostatic phosphatase. Several 30-μmole samples were incubated with enzyme in *p*H 5.5 acetate buffer for 16 to 24 hours. After the incubation, the solutions were adjusted to the appropriate concentration of $NaIO_4$ and $NaHCO_3$, after a small amount of base was added to destroy the buffering capacity of the solution, and then the oxidations were followed by the procedures given.

The results of these oxidations are shown in Figure 3. It is seen from this figure that when the phosphate group is present only one mole of formaldehyde per mole of phosphorus is released during the oxidation

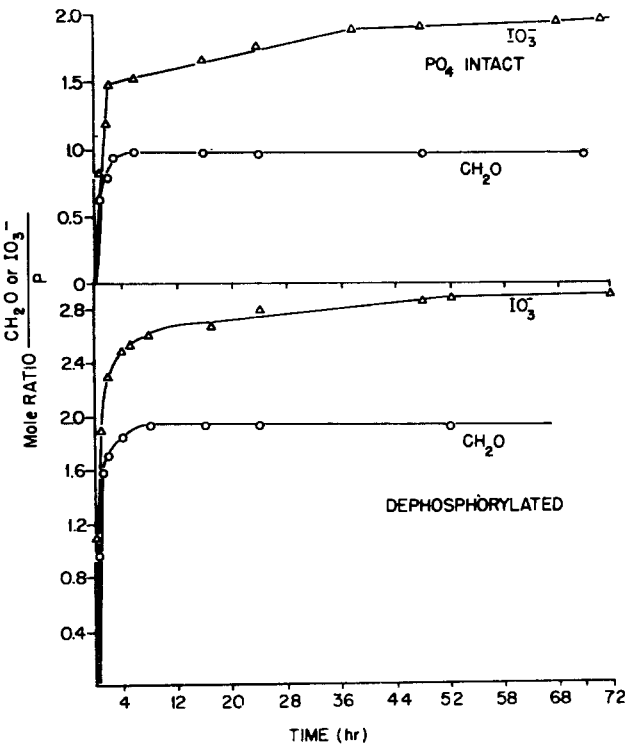


FIG. 3.—Periodate oxidation of tertiary amino aldehyde phosphate (V). Top curves, production of CH_2O (O) and IO_3^- (△) per mole of phosphorus *vs.* time with phosphate intact. Bottom curves, same only compound dephosphorylated with enzyme before additions of periodate.

but that two moles of iodate are produced over a period of 72 hours and that the final uptake of periodate is very slow. However, when compound V is dephosphorylated, two moles of formaldehyde are rapidly liberated during the oxidation and the consumption of periodate approaches a final value of three moles per mole of phosphorus. The oxidation of the amino phosphate with periodate is sensitive to *p*H, and the oxidation is extremely slow at *p*H values below 6.0.

Identification of Glycolaldehyde Phosphate as an Oxidation Product.—One hundred μmoles of compound V were treated with 10 ml of 0.5 M $NaHCO_3$ and 7 ml of 0.1 M $NaIO_4$, the volume was adjusted to about 25 ml, and the mixture was set aside for at least 16 hours. Next, 17 ml of 1 M HCOOH was added and the sample diluted to 150 ml and placed on a 9 cm × 1 cm² column of Dowex-1 (formate). Elution was carried out with 0.6 M acetic acid and gave rise to two peaks containing bound phosphorus as shown in Figure 1B. For identification purposes, the elution was carried out in a slightly different manner than shown in Figure 1B. Before addition of samples to columns, bicarbonate was removed by adjusting the *p*H to 3.5. Alkali was added to readjust the *p*H to about 8.5 and the samples were sorbed on the columns followed by a wash with alkaline 0.02 M sodium formate. This procedure retains both phosphate compounds but allows free *N*-methylformamide and formaldehyde to pass through the column. The material represented by peak 1 of Figure 1B was then eluted with 0.05 M HCOOH and that of peak 2 with 0.6 M HCOOH. The phosphate compound contained in peak 1 gave a nitrogen-to-phosphorus ratio of 1:1 and reacted with phenylhydrazine to give glyoxal bisphenylhydrazine. After enzymic removal of phosphate, it was oxidized with periodate to yield formaldehyde.

The second peak from the column run of Figure 1B

matched in position that of glycolaldehyde phosphate and, after concentration, gave the same test and reactions as did authentic glycolaldehyde phosphate.

Discussion

The reaction of oxidized AMP with methylamine was initially thought to involve two methylamine residues (Khym and Cohn, 1961). However, the findings here, the isolation of a reduced crystalline product containing only one methylamine moiety, and titration data (Cohn and Khym, 1962) showing the release of one mole of acid when methylamine is added to the oxidized nucleotide before reduction with sodium borohydride indicate that only one mole of methylamine is involved in the reaction with oxidized AMP, in accordance with the equations of Reaction 1. In alkaline solution one methylamine residue appears to form an addition compound with oxidized AMP to give the cyclic hemialdal I, a type of structure proposed by Barry and Mitchell (1953b) for the addition of an amine to a dialdehyde. Apparently whether one or two amines add to a dialdehyde depends on the particular dialdehyde itself or the type of amine used or both. The isolation of both mono- and diamino derivatives of dialdehyde have been reported in the literature (Khym and Cohn, 1960; Barry and Mitchell, 1953a; Guthrie *et al.*, 1959).

It has been shown that amines react characteristically with aldehydes to produce *N*-alkyl aldimines commonly called Schiff's bases (Sprung, 1940; Gilman, 1953; Royals, 1954), and that the mechanism of this condensation appears to be simple addition followed by the elimination of water from the addition product. Both steps are reversible (Gilman, 1953; Royals, 1954), and thus an equilibrium exists between the complexes formed and the original products. The extent of complex formation (determined by phosphate elimination) when oxidized AMP reacts with amines depends upon the particular amine used (Khym and Cohn, 1961). The presence of unreacted oxidized AMP (shown by its isolation as a reduced alcohol phosphate) supports the concept of an equilibrium between oxidized AMP and the methylamine addition products.

The complexes formed by the interaction of amines and aldehydes are stable in dilute alkaline solution but are decomposed with great ease by acids to give the initial products (Gilman, 1953; Royals, 1954). In fact, the ease of acid hydrolysis is the basis of a quantitative method for the determination of isolated *N*-substituted aldimines by the release of aldehyde when aldimines are made acid to methyl red indicator (Hillenbrand and Pentz, 1956). It is during this reversion (Reaction 1) that phosphate is released from amine-treated oxidized AMP, by a mechanism as yet undetermined, when the *p*H of a reaction mixture is lowered to neutrality. The release of phosphate prevents formation of the original products, but apparently the C2′ and C3′ carbons of oxidized AMP are still involved in some addition product of methyl amine even after phosphate elimination, since an "adenosine-like" fragment appears in the hydrolysate and readily yields adenine at *p*H values lower than 3, while oxidized AMP untreated with amine is stable in 1 N HCl for several days (Khym and Cohn, 1961).

It is known that Schiff's bases are easily reduced by the addition of hydrogen across the NC double bond of the compounds (Gilman, 1953; Royals, 1954; Billman and Diesing, 1957). However, since a primary alcohol group is not found in the compound (IV) that firmly bonds only one methylamine residue, it appears that very little Schiff base is formed and therefore the majority of the oxidized AMP compound is in the hemialdal form (I of Reaction I). It is assumed that the addition of sodium borohydride displaces the equilibrium toward the true Schiff's structure (II) and allows reduction to occur. The Schiff base structure shown in Reaction 2 is rapidly reduced and the secondary amine structure that is formed reacts immediately with the 2′ aldehyde group to yield the stable compound IV.

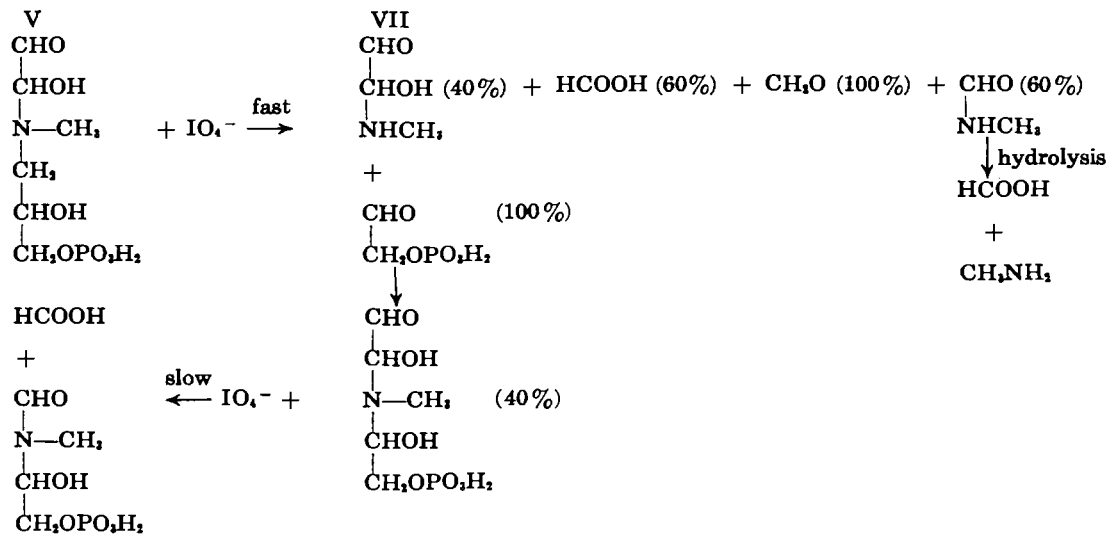
The trialcohol monophosphate III hydrolyzes readily in dilute acid at 100° to give the expected degradation products, and these products are consistent with the properties of its dephosphorylated derivative, adenine-hydroxymethyl diethylene glycol (Khym and Cohn, 1960). The reduced product (IV), containing methylamine, is resistant to hydrolysis in dilute acid and gives products, when hydrolyzed in concentrated acid, that are not consistent with an ethylene glycol derivative. This fact and the observation that it does not form a dithiocarbamate indicate that no free secondary amine structure is present in this reduced compound. Therefore the cyclic structure IV, a morpholine derivative, is proposed for the reduced compound.

The most direct evidence for the assignment of a cyclic structure to the reduced compound IV comes from the properties of the six-carbon fragment V that results from the degradation of this compound in concentrated acid. The fact that the degradation product is an intact fragment comes from the observation of its amphoteric nature as demonstrated by its behavior on hydrogen-form resin (any noncationic phosphate compound would not absorb on the resin column), its response to reducing tests, and the finding of bound phosphorus and nitrogen in the compound in the ratio of 1:1. These facts are consistent with the open-chain structures Va or Vb of Reaction 3 for the six-carbon fragment. Structure Vb is the alternate structure for the reduction of a Schiff base involving the 2′ carbon of oxidized AMP.

The decision between Va and Vb for the structure of the six-carbon fragment is made on the basis of its reactivity toward periodate. Dyer (1956) has reviewed the action of periodate on hydroxyalkylamines, and it has been demonstrated that α-amino alcohols and α-amino aldehydes respond to periodate best in a slightly alkaline medium. Many tertiary amines do not respond to the action of periodate (Dyer, 1956), although in the determination of the structure of mycaminose (Hochstein and Regna, 1955) and of desosamine (Flynn *et al.*, 1954) it was shown that periodate readily cleaves a $HOC–CN(CH_3)_2$ bond. In accordance with these facts and the type of products that were isolated, the expected action of periodate on structures Va and Vb is that given in Reaction 3.

As seen in Figure 3, one mole of formaldehyde per mole of phosphorus is liberated well before the final uptake of two moles of periodate is realized. This would appear to rule out structure Vb and cyclic hemiacetal structures for both Va and Vb, since an α-glycol group would be expected to be more readily oxidized than an α-amino alcohol or α-amino aldehyde group (Dyer, 1956; Bobbitt, 1956). Therefore, the six-carbon fragment is assigned the open-chain structure Va, *N,N,N*-(ethanolal-methyl-2-hydroxypropyl 3-phosphate) tertiary amine.

When V is dephosphorylated with enzyme before the addition of periodate, an additional mole of periodate is reduced, liberating a total of two moles of formaldehyde per mole of phosphorus. This observation demonstrates that the initial furanoid carbons 4 and 5 have remained intact through all the treatment given oxidized AMP.



Reaction 4

Not readily explained is the incomplete yield of glycolaldehyde phosphate and methylamine, which were isolated in equivalent yields of about 65%. The low yields are considered to arise from a recombination of glycolaldehyde phosphate with the ethanolal *N*-methylamine fragment VII of Reaction 4 in accordance with the reactions already discussed here, rather than a partial migration of the phosphate group from carbon 5 to 4 in V during the acid hydrolysis, which would have given an incomplete yield of formaldehyde.

This recombination is illustrated in Reaction 4 and is consistent with the rate of oxidation of V as shown in Figure 3 in that, after the oxidation is about 70% complete, the final rate of the oxidation, approaching the end-point of two moles of periodate, is extremely slow. Also, this viewpoint is strengthened by the ion-exchange analysis shown in Figure 1B, in which one of the fragments isolated contained nitrogen and phosphorus in a ratio of 1:1 and was shown to contain an aldehydo-carbon as demonstrated by its reaction with phenylhydrazine. After dephosphorylation of the compound with enzyme, an α-glycol group was shown to be present by the action of periodate on this fragment to produce formaldehyde.

It was shown that authentic *N*-methylformamide did not combine with glycolaldehyde phosphate; however, the amide readily hydrolyzes in acid or base to yield formic acid and methylamine.

On the basis of the properties of the reduced product (IV) derived from methylamine-treated oxidized AMP and the reaction of periodate with the six-carbon fragment V arising from its degradation with acid, the structure assigned to this product is 6-adenyl-5-hydroxy - 4 - methyl - 2 - hydroxymethylphosphate morpholine.

Added in Proof

Dr. D. M. Brown (University of Cambridge), who kindly read a preprint of this paper, has suggested that Structure I of Reaction 2 is reduced directly to give a morpholine derivative in which positions (3) and (5) of structure IV are both methylene groups. Consequently Structure V of Reaction 4 would be a 2-deoxy derivative. This interpretation would better fit the acid stability of V but does not account for the production of two moles of formic acid that are found when V is oxidized with periodate. Further investigations of these types of reactions are being carried out by Dr. Brown.

References

Barry, V. C., and Mitchell, P. W. D. (1953a), *J. Chem. Soc.* 3610.

Barry, V. C., and Mitchell, P. W. D. (1953b), *J. Chem. Soc.* 3631.

Barry, V. C., and Mitchell, P. W. D. (1954), *J. Chem. Soc.* 4020.

Bell, D. J. (1948), *J. Chem. Soc.* 992.

Bell, D. J., Palmer, A., and Jones, A. T. (1944), *J. Chem. Soc.* 1536.

Billman, J. H., and Diesing, A. C. (1957), *J. Org. Chem. 22,* 1068.

Block, R. J., Durrum, E. L., and Zweig, G. L. (1955), A Manual of Paper Chromatography and Paper Electrophoresis, New York, Academic Press, Inc., p. 163.

Bobbitt, J. M. (1956), *Adv. Carbohydrate Chem. 11,* 1.

Brown, D. M., Fried, M., and Todd, A. R. (1953), *Chem. and Ind.* 352.

Brown, D. M., Fried, M., and Todd, A. R. (1955), *J. Chem. Soc.* 2206.

Cohn, W. E., and Khym, J. X. (1962), *in* Acides ribonucléiques et polyphosphates. Structure, synthèse et fonctions, Paris, Editions du Centre national de la recherche scientifique, pp. 217–226.

Colloquium on RNA and Polyphosphates, Strasbourg.

Dyer, J. R. (1956), *in* Methods of Biochemical Analysis, vol. III, Glick, D., editor, New York, Interscience Publishers, Inc., pp. 115–116.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.

Flynn, E. H., Sigal, M. V., Jr., Wiley, P. F., and Gerzon, K. (1954), *J. Am. Chem. Soc. 76,* 3121.

Gilman, H. (1953), Organic Chemistry, An Advanced Treatise, ed. 2, vol. IV, New York, John Wiley and Sons, Inc., pp. 652, 659.

Griswold, B. L., Humoller, F. L., and McIntyre, A. R. (1951), *Anal. Chem. 23,* 192.

Guthrie, R. D., Honeyman, J., and Parsons, M. A. (1959), *J. Chem. Soc.* 2457.

Hakamori, S. (1959), Proceedings of the Eighth Symposium on Nucleic Acids, Kyoto, Japan, p. 16.

Hillenbrand, E. F., Jr., and Pentz, C. A. (1956), *in* Organic Analysis, vol. 3, New York, Interscience Publishers, Inc., p. 194.

Hochstein, F. A., and Regna, P. P. (1955), *J. Am. Chem. Soc. 77,* 3353.

Jones, J. H. (1944), *Assoc. Offic. Agr. Chemists 27,* 462.

Khym, J. X., and Cohn, W. E. (1960), *J. Am. Chem. Soc. 82,* 6380.

Khym, J. X., and Cohn, W. E. (1961), *J. Biol. Chem. 236,* PC9.

Morrison, M., Rouser, G., and Stotz, E. (1955), *J. Am. Chem. Soc. 77*, 5156.
Nelson, N. (1944), *J. Biol. Chem. 153*, 375.
Ogur, M., and Small, J. D. (1960), *J. Biol. Chem. 235*, PC60.
Royals, E. E. (1954), Advanced Organic Chemistry, New York, Prentice Hall, Inc., p. 649.
Sprung, M. M. (1940), *Chem. Revs. 26*, 297.
Steyermark, A. (1951), Quantitative Organic Microanalysis, New York, Blakiston Co., p. 148.
Umbreit, G. R. (1961), Abstract of Papers, 139th Meeting of the American Chemical Society, March, 1961.
Whitfield, P. R. (1954), *Biochem. J. 58*, 390.
Yu, C. T., and Zamecnik, P. C. (1960), *Biochim. et Biophys. Acta 45*, 148.

The Effect of Bivalent Metal Ions on the Hydrolysis of Adenosine Di- and Triphosphate*

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Many bivalent metal ions catalyze the hydrolysis of adenosine triphosphate (ATP) at *p*H 5 and 9. In the presence of Cu^{2+} and Zn^{2+} ions, the rate of hydrolysis of ATP passes through an optimum at *p*H 5. These ions increase the rate of hydrolysis of ATP about 60-fold and 12-fold, respectively, at *p*H 5, compared to the rate of hydrolysis in the absence of bivalent metal ions. The rate of hydrolysis of adenosine diphosphate (ADP) is scarcely affected by many bivalent metal ions at *p*H 5 but is accelerated by most bivalent metal ions at *p*H 9. Cu^{2+} and Zn^{2+} ions increase the rate of hydrolysis of ADP about 9-fold and 5-fold, respectively, at *p*H 9. The effect of Mn^{2+} ions on both ATP and ADP hydrolysis is markedly different from that of Cu^{2+} and Zn^{2+} ions. In the hydrolysis of ATP, the amount of inorganic pyrophosphate formed in the presence of bivalent metal ions is negligible at *p*H 5. It is of minor importance at *p*H 9, except in the presence of Ca^{2+} ions, in which case about one third of the orthophosphate plus pyrophosphate formed is pyrophosphate. It is proposed that the metal ion specificities of ADP and ATP hydrolysis are the consequence of different types of chelates which are formed with different bivalent metal ions and of different reaction mechanisms which operate with different bivalent metal ions.

Bivalent metals catalyze the nonenzymatic transfer of phosphate from adenosine triphosphate (ATP) to various acceptors such as orthophosphate, acetate, glycine, and β-alanine (Lowenstein, 1958a,b; Lowenstein and Schatz, 1961). When orthophosphate is the phosphate acceptor, the product of the reaction is pyrophosphate and the most effective bivalent metal ions are Ca, Sr, and Ba, followed in decreasing order of effectiveness by Mn, Cd, and Zn. The *p*H optimum of the reaction is 9.0 (Lowenstein, 1958a, 1960). When acetate is used as acceptor in the presence of hydroxylamine, the product of the reaction is acetohydroxamate and the most effective bivalent metal ions are, in decreasing order of effectiveness, Be, Ni, Co, Zn, and Mn. The *p*H optimum of the reaction is 5.2 (Lowenstein and Schatz, 1961). Similarly, with glycine, the product of the reaction is glycine hydroxamate and the most effective bivalent metal ions are, in decreasing order of effectiveness, Be, Zn, Mn, Ca, and Cd. The *p*H optimum of this reaction is 5.0. Various studies of the effect of Mg, Ca, and Ba ions on the hydrolysis of ATP and adenosine diphosphate (ADP) have been reported (Lohmann, 1932; Spicer, 1952; Hock and Huber, 1956; Nanninga, 1957; Liébecq and Jacquemotte-Louis, 1958a,b; Liébecq, 1959; Blum and Felauer, 1959; Lipkin *et al.*, 1959). The effect of ten different bivalent metal ions at *p*H 8.5 was compared by Liébecq and Jacquemotte-Louis (1958a). Of particular interest was a report which dealt with the complex degradation of ATP in saturated barium hydroxide. It was shown that one of the many products of this degradation is adenosine-3′:5′-cyclic monophosphate (Lipkin *et al.*, 1959). Lastly, the hydrolysis of phosphate esters, including ATP, is also catalyzed by alkaline gels of Ce^{3+} and other trivalent ions (Bamann *et al.*, 1954; Bamann and Trapmann, 1959).

The present paper deals with a study of the bivalent metal ion specificity of the hydrolysis of the phosphoric anhydride linkages of ATP and ADP. The hydrolysis of ATP leads to ADP and AMP, ADP being formed by reaction (1) and AMP by reactions (2) and (3). The hydrolysis of ADP leads to AMP by reaction (3). Detailed analyses of the reaction products are presented, and it is shown that some bivalent metal ions exert an optimum effect at *p*H 5. At this *p*H the hydrolysis of ATP in the presence of copper ions is fifty-seven times faster than the hydrolysis of ATP in the absence of bivalent metal ions.

$$ATP + H_2O \longrightarrow ADP + \text{orthophosphate} \quad (1)$$

$$ATP + H_2O \longrightarrow AMP + \text{pyrophosphate} \quad (2)$$

$$ADP + H_2O \longrightarrow AMP + \text{orthophosphate} \quad (3)$$

METHODS

Measurement of ADP and AMP Formed by Hydrolysis.—The reaction times used were such that the rates of hydrolysis were proportional to time. The reaction was stopped by adding 9 ml of ice-cold water containing 40 μmoles disodium ethylenediamine tetraacetic acid (EDTA) to the reaction mixture. The resulting solution was adjusted to a *p*H of 8–9 by the addition of 2 N NaOH and was then poured on an ion-exchange column which contained the chloride salt of Dowex-1 resin, 200–400 mesh, 8% cross-linked, with a resin bed 1 cm in diameter and 10 cm in height. The adenine nucleo-

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