drous material. The anhydrous material shows a tendency to be hygroscopic.

Anal. Calcd. for $C_8H_{15}N_3O_6S_2$: C, 32.3; H, 5.1; N, 14.2; S, 21.6. Found: C, 32.4; H, 5.1; N, 14.1; S, 21.3.

Disulfide Interchange.—A solution of 600 mg. of glycyl-L-cystine in water is adjusted to *p*H 7.5 with sodium carbonate and the total volume brought to 20 ml. After storage at 25° for 7 days, the *p*H is adjusted to 6, the reaction mixture concentrated *in vacuo*, and the precipitated cystine collected; yield 50 mg. This corresponds to a disulfide interchange of the order of 20%.

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[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Purine N-Oxides. V. Oxides of Adenine Nucleotides[1]

BY MARCUS A. STEVENS, HERMAN W. SMITH AND GEORGE BOSWORTH BROWN

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The 1-N-oxides of adenosine 2′-, 3′- and 5′-phosphates and of adenosine 5′-diphosphate have been prepared from the parent nucleotides by oxidation with hydrogen peroxide–acetic acid mixtures. Oxidation with hydrogen peroxide alone has been used to prepare chromatographic quantities of the 1-N-oxides of adenosine 5′-monophosphate and deoxyadenylic acid. These nucleotide oxides have been characterized by hydrogenation to the parent nucleotide and by acid hydrolysis to 4-aminoimidazole-5-carboxamidoxime. Alkaline hydrolyses of adenosine 1-N-oxide and of the adenosine 2′-, 3′- and 5′-phosphate 1-N-oxides give the 1-ribosyl and 1-phosphoribosyl derivatives of 5-aminoimidazole-4-carboxamidoxime, respectively. The nucleotide oxides in the solid state and in aqueous solution exhibit an instability which is not observed with adenine 1-N-oxide or adenosine 1-N-oxide.

The preparation of mono-N-oxides of adenine and adenosine and the characterization of these oxides as 1-oxides has been reported.[2,3] In the hope of preparing a material which may be an effective antimetabolite for normal nucleotides, or have significance in biological oxidation–reduction systems, the series has been extended to the adenine nucleotide 1-N-oxides.[4] It has been found possible to prepare such oxides in ways similar to those used for the oxidation of adenine and adenosine. Oxidation of AMP-5′ for 16 days with a mixture of acetic acid and hydrogen peroxide gives the 1-N-oxide in a yield of 62%. Under certain conditions, AMP-5′ 1-N-oxide precipitates directly from the oxidizing solution, but the yield is variable. More reproducible yields are obtained when the AMP-5′ 1-N-oxide is separated from the unoxidized AMP-5′ by chromatography on Dowex-1-formate with 0.1 *M* formic acid.[5]

Either AMP-2′ or AMP-3′ gives a mixture of the 1-N-oxides of AMP-2′ and AMP-3′ upon oxidation with acetic acid and hydrogen peroxide. The mixture obtained by oxidizing AMP-3′ for a certain period of time appears to be identical in composition with that obtained from AMP-2′ by oxidation for the same period of time. Equilibration of the 2′-phosphate and 3′-phosphate forms must be occurring in the acetic acid medium used, though it may be that the AMP-2′ and -3′ 1-N-oxides also equilibrate. This type of equilibration was observed by Brown and Todd,[6] with the 2′,3′-phosphodiester proposed as the intermediate. When attempts were made to isolate the separate nucleotides from the mixture of nucleotides produced by the oxidation of either AMP-2′ or AMP-3′ by gradient elution with formic acid from a Dowex-1-formate column, it was found possible to separate the AMP-2′ and AMP-2′ 1-N-oxide from each other, but complete separation of the AMP-3′ from the AMP-3′ 1-N-oxide was not found possible. To circumvent this problem a sample of AMP-3′ (or AMP-2′) was oxidized until it had been largely converted to a mixture of oxides; then separation on a column was carried out by direct elution with 0.1 *N* formic acid. This yielded a little AMP-2′ (1st peak, small amount), AMP-2′ 1-N-oxide (2nd peak, large amount) and AMP-3′ 1-N-oxide (3rd peak, large amount + small amount of AMP-3′). An essentially pure sample of AMP-3′ 1-N-oxide was obtained from this last peak by taking the solution representing the first two thirds of the peak, evaporating to dryness and recrystallizing from acetone–water. After oxidation with an acetic acid–hydrogen peroxide mixture at 25° for 9 days, AMP-2′ 1-N-oxide was obtained in 17.4% yield, and AMP-3′ 1-N-oxide (partially contaminated with AMP-3′) was obtained in 25.4% yield.

ADP is also oxidized by hydrogen peroxide–acetic acid to a 1-N-oxide, but chromatography was not a satisfactory method of isolating the product. Fractional precipitation of the barium salt was used to give virtually pure ADP 1-N-oxide (as barium salt). ATP and deoxyAMP decompose to adenine when attempts are made to oxidize them with mixtures of acetic acid–hydrogen peroxide at room temperature.

The structures of the products obtained by the oxidation of AMP-2′, 3′ and -5′, and of ADP have been determined from elemental analysis, by reduction to the unoxidized nucleotide with hydrogen and Raney nickel catalyst and by hydrolysis to adenine 1-N-oxide (II) or to 4-aminoimidazole-5-carboxamidoxime (III). They are all 1-N-oxides. The reactions used for characterization are il-

(1) This investigation was supported in part by funds from the American Cancer Society (Grant #MET-27), National Cancer Institute, National Institutes of Health, Public Health Service (Grant #CY-3190) and from the Atomic Energy Commission (Contract #AT930-1-910).

(2) M. A. Stevens, D I. Magrath, H. W. Smith and G. B. Brown, THIS JOURNAL, **80**, 2755 (1958).

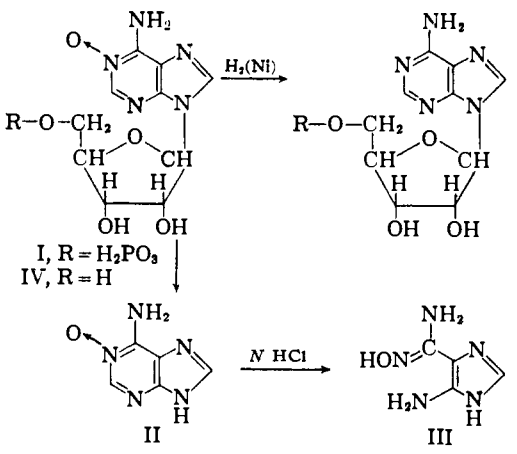
(3) M. A. Stevens and G. B. Brown, *ibid.*, **80**, 2759 (1958).

(4) The following abbreviations are used: AMP-2′, -3′ and -5′ for the 2′-, 3′- and 5′-phosphates of adenosine, respectively, ADP and ATP for adenosine 5′-diphosphate and 5′-triphosphate, and deoxyAMP for 2′-deoxyadenosine 5′-phosphate.

(5) W. E. Cohn, THIS JOURNAL, **72**, 1171 (1950).

(6) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952).

SCHEME I



lustrated below (for the case of AMP-5′ 1-N-oxide (I)).

A complication has been encountered in attempts to get chromatographically pure samples of the nucleotide N-oxides. This difficulty appears to result from an internal oxidation–reduction reaction that occurs in adenine nucleotide 1-N-oxides in the crystalline state or in concentrated solution.

Earlier it had been noted[2] with adenine 1-N-oxide that in water there was no tendency to lose oxygen, but, in acetic acid, there was a tendency to convert to a substance resembling adenine in R_f and ultraviolet spectrum. It is postulated that adenine 1-N-oxide slowly re-establishes the equilibrium with acetic acid.

$$\text{Adenine 1-N-oxide} + CH_3CO_2H \rightleftharpoons CH_3CO_3H + \text{Adenine}$$

At equilibrium about 0.5% of the adenine N-oxide appears to be converted to a substance resembling adenine in spectrum and chromatographic properties.

With AMP-5′ 1-N-oxide[7] a similar type of instability is observed without acetic acid. The slow decomposition of AMP-5′ 1-N-oxide can be demonstrated by two-dimensional paper chromatography with the same solvent (solvent B, an alkaline phosphate system) for development in each direction. Were AMP-5′ 1-N-oxide stable, the AMP-5′ 1-N-oxide spot from the first development would migrate as a single spot during the second development, in fact it gives a further spot in the second development with an R_f identical to AMP-5′. This indicates that decomposition occurs when the chromatogram is dried between the two developments, and since no smearing of the spots is observable after either development, it appears that little decomposition occurs during the actual development of the chromatograms. If the chromatogram is kept for 12 hr. between developments, the amount of nucleotide decomposition product which builds up amounts to 6%[8] of the AMP-5′ 1-N-oxide present. Adenosine N-oxide under the same conditions suffers no decomposition even when phosphate is present in the solvent used.

(7) The remarks that follow about the instability of AMP-5′ 1-N-oxide apply also to the 1-N-oxide of the other nucleotides.

(8) Using relative a_m values of adenosine and adenosine 1-N-oxide at 260 mμ.

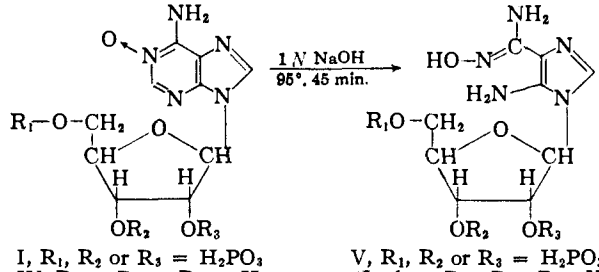
Hydrolysis of a sample of AMP-5′ 1-N-oxide containing this nucleotide decomposition product gives mainly 4-aminoimidazole-5-carboxamidoxime, but also trace quantities of adenine and a substance which gives a purple color with Pauly's imidazole reagent. The formation of this last indicates that AMP-5′ is only part of the decomposition product. The observation that there is a 328 mμ peak as well as a 260 mμ peak in the spectrum of the decomposition product at *p*H 8 also supports the belief that this decomposition product contains some substance other than AMP-5′. The nature of the complex decomposition process that AMP-5′ 1-N-oxide undergoes is not fully known, but it occurs with AMP-5′ 1-N-oxide and not with adenosine 1-N-oxide, with or without phosphate. Decomposition of AMP-5′ 1-N-oxide occurs slowly in dilute solution (as shown by the clean-cut separation of AMP-5′ 1-N-oxide from AMP-5′ on paper or on ion exchange resin) and more rapidly in concentrated solution or the crystalline state.

If it is the esterified phosphate moiety which is involved in the decomposition of AMP-5′ 1-N-oxide to AMP, then it should be possible to oxidize AMP-5′ to its oxide, but not adenosine to its oxide, with hydrogen peroxide alone rather than with mixtures of hydrogen peroxide and acetic acid. This proves to be true, though conversions and rates of reaction at room temperature are low. AMP-5′ with 6% hydrogen peroxide for 21 days gives a mixture of AMP-5′ and AMP-5′ 1-N-oxide in the ratio of 86:14. During the oxidation process, about 63% of the ultraviolet absorbing material is broken down to non-ultraviolet absorbing material. The yield of oxide from this process is about 7.5%.[8] Oxidation with hydrogen peroxide provides a method of making, in chromatographic quantities, the 1-N-oxides of those nucleotides and deoxynucleotides which are unstable in acetic acid–hydrogen peroxide mixtures. DeoxyAMP, for example, upon oxidation with 6% hydrogen peroxide for one week, gives a mixture of deoxyAMP, deoxyAMP 1-N-oxide, adenine, plus an additional substance. The deoxyAMP 1-N-oxide has been identified by its characteristic N-oxide spectrum. Its high R_f in solvent B suggests the oxidation product has not lost its deoxyribose phosphate moiety. The approximate yields of products from the hydrogen peroxide oxidation of deoxyAMP have been determined by chromatography of the crude oxidation product, elution of the spots after development, and comparison of the ultraviolet absorbencies of the solutions produced. Analysis of the oxidation mixture, after one week, shows it to contain deoxyAMP, deoxyAMP 1-N-oxide, adenine and unknown substance in the ratios 30:7:6:6. Since during the oxidation five-sixths of the material is decomposed to non-ultraviolet-absorbing material, the yield of the oxide is 2.5%.[8] Purines such as adenine, though hydrolyzed by acid, are very stable in alkali. Conversion of adenine to its N-oxide results in a labilization of the pyrimidine moiety of the adenine molecule and an increased sensitivity of the molecule to cleavage by acid hydrolysis.[3] Decomposition of the adenine 1-N-oxide in hot concentrated alkali is not observed, however.

The derivatives of adenine 1-N-oxide with a 9-ribosyl group (IV)[2] or a 9-phosphoribosyl group (I) are more labile to acid hydrolysis (scheme I) than adenine 1-N-oxide. In the ribosyl and phosphoribosyl derivatives cleavage of the molecule by dilute acid is somewhat more facile than that of adenosine or AMP. Concomitantly, an instability in alkali[9] is observed. Heating adenosine 1-N-oxide (IV), or AMP-2′, 3′ or 5′ 1-N-oxide (I) with 1 *N* sodium hydroxide at 95° for 45 minutes causes almost complete hydrolysis of the starting materials. One main hydrolysis product is formed in each case. Isolation of these hydrolysis products in quantities needed for analysis has not been found possible, since the products are not stable enough to be separated over ion-exchange resins. By paper chromatographic techniques it has been found possible to separate the hydrolysis products from by-product materials and unhydrolyzed starting materials.

Spray tests are used to identify the products on chromatogram papers. Presence of imidazole, oximino and ribose moieties in the hydrolysis products is indicated by the Pauly test,[10] the ferric chloride test[11] and the *cis*-glycol test,[12] respectively. The presence of phosphate in the hydrolysis products of the nucleotide oxides is confirmed by the usual phosphate test.[13] A positive Bratton–Marshall test,[14] a test for diazotizable aromatic amines, indicates that an amino rather than a formamido imidazole is formed, and thus the products are 1-phosphoribosyl (V) and 1-ribosyl (VI) derivatives of 5-aminoimidazole-4-carboxamidoxime (Scheme II).

SCHEME II



I, R_1, R_2 or R_3 = H_2PO_3
IV, R_1 = R_2 = R_3 = H

V, R_1, R_2 or R_3 = H_2PO_3
VI where R_1 = R_2 = R_3 = H

The above cleavage of the pyrimidine ring and the loss of the formyl group is to be contrasted with the behavior[15] of 9-phosphoribosylpurine (VII) (Scheme III). In that case it is the imidazole moiety of the purine which is alkali labile, and the first product of the hydrolysis is the formamido derivative, 5-formamido-4-phosphoribosylaminopyrimidine (VIII).

ADDED IN PROOF: In a note F. Cramer and K. Randnath (*Angew. Chem.*, **70**, 571 (1958)) have reported obtaining oxides from adenine nucleotides.

(9) Alkaline decomposition of 1-benzylinosine to 5-amino-1-ribosyl-4-imidazole-N-benzylcarboxamide has been reported recently by E. Shaw, THIS JOURNAL, **80**, 3899 (1958).

(10) H. Pauly, *Z. physiol. Chem.*, **42**, 508 (1904).

(11) A. Hantzsch and C. H. Besch, *Ann.*, **323**, 23 (1902).

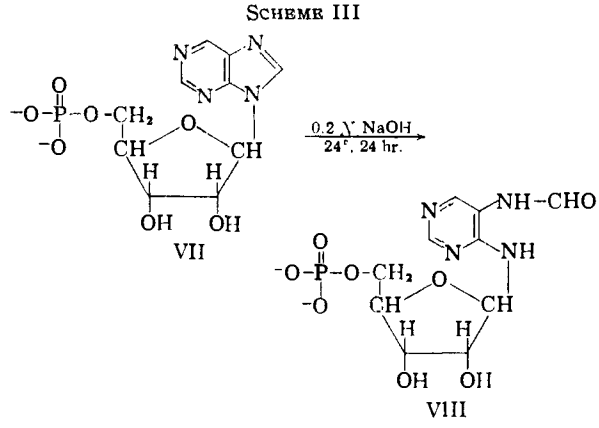
(12) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

(13) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(14) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

(15) D. I. Magrath and G. B. Brown, THIS JOURNAL, **79**, 3253 (1957).

SCHEME III



VII

VIII

Experimental

Except where noted, all chromatographic analyses were performed ascending on Whatman No. 1 paper at 25° with solvents A (1% ammonium sulfate–isopropyl alcohol = 1:2 vol./vol.),[16] B (5% disodium phosphate–isoamyl alcohol = 3:2 vol./vol.),[17] or C (saturated ammonium sulfate solution–water–isopropyl alcohol = 79:12:2 vol./vol.).[18] (See Table I for R_f values.) Electrophoresis was carried out on Whatman 3MM paper in 0.05 *M* ammonium formate/0.05 *M* formic acid buffer at *p*H 3.5 and at 25° on an apparatus manufactured by the E-C Apparatus Co. of Swarthmore, Pennsylvania. Measurements of ultraviolet absorption were carried out on a Beckman Spectrophotometer, Model DK-2.

TABLE I

Compound	R_f A[a]	R_f B	R_f C	Electrophoretic movement, cm./2 hr. at 25 v./cm.
Adenosine-2′-phosphate	0.48	0.70	0.33	6.6
Adenosine-3′-phosphate	.47	.63	.24	6.6
Adenosine-5′-phosphate	.34	.71	.38	6.6
Adenosine-5′-diphosphate	.21[b]	.76	.39	..
Adenosine-5′-triphosphate	.12[c]	.80	.56	..
Deoxyadenylic acid	.41	.71	.30	..
Adenosine-2′-phosphate 1-N-oxide	.40	.81	.46	14.8
Adenosine-3′-phosphate 1-N-oxide	.40	.78	.40	14.8
Adenosine-5′-phosphate 1-N-oxide	.28	.83	.56	14.8
Adenosine-5′-diphosphate 1-N-oxide	.15[b]	.86	.54	..
Deoxyadenylic acid 1-N-oxide	.32	.81	..	..

[a] Due to weak buffering power of 1% $(NH_4)_2SO_4$, R_f's are variable. [b] Ba salt. [c] Na salt.

Preparation of the 1-N-Oxides of Adenosine-2′- and -3′-Phosphates.—AMP-3′ (500 mg.) was dissolved in a mixture of acetic acid (25 ml.) and 30% hydrogen peroxide (5 ml.) and allowed to stand for 9 days. At the end of this time chromatograms developed from this solution showed that the product from the reaction were numerous, due to an equilibration in the oxidizing solution between the various 2′- and 3′-nucleotides. The oxidized solution was treated with 10% palladium on charcoal for 2 days to destroy the excess hydrogen peroxide, then was evaporated to dryness under vacuum at room temperature. The residue was dissolved in water (6 liters) and fed to a 15 cm.-high (250 ml.) Dowex-1-formate ion-exchange column. Of the 9,300

(16) N. Anand, V. M. Clark, R. H. Hall and R. Todd, *J. Chem. Soc.*, 3665 (1952).

(17) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(18) J. D. Smith and R. Markham, *Biochem. J.*, **46**, 509 (1950).

O.D. units (at 260 mμ) fed, 8,610 O.D. units were absorbed. The column was eluted slowly with 0.1 *M* formic acid. Fractions of the eluate of 15-ml. volume were collected and their optical density at 253 mμ was measured. A plot of the O.D. against the fraction (Fig. 1) clearly shows the presence of three fractions of ultraviolet absorbing material in the eluate. Peak A represents a total of 1012 O.D. units and peaks B_1 and B_2 represent 3150 units each. Thus, of the 8,600 O.D. units absorbed on the column, 7,300 O.D. units appear in these three major peaks. These peaks were chromatographed in solvent A and compared with AMP-2′ and -3′. Oxides of both AMP-2′ and -3′ were expected, since these 2′- and 3′-phosphates interconvert in the oxidizing medium used. The following is a description of the chromatograms obtained from the materials in the three peaks.

Peak A.—Mainly a spot R_f 0.69 corresponding to the AMP-2′, plus an unidentified spot of R_f 0.74. Elution of the main spot and demonstration that this material has an absorption maximum at 260 mμ (only) is additional evidence that this peak is the unoxidized AMP-2′.

Peak B_1.—The main spot is at R_f 0.81 with trace contamination (after evaporation) with AMP-2′. This peak consists of AMP-2′ 1-N-oxide.

Peak B_2.—The main spot is at R_f 0.78 with contamination with AMP-3′. This peak consists mainly of AMP-3′ 1-N-oxide.

The materials in peaks B_1 and B_2, AMP-2′ and -3′ 1-N-oxides were regained by evaporating each peak separately *in vacuo* at room temperature. Since peaks B_1 and B_2 were not entirely separate, a little contamination of the AMP-2′ 1-N-oxide with the AMP-3′ 1-N-oxide was expected, but this could barely be discerned on the chromatogram. The yield of the AMP-2′ 1-N-oxide was 91 mg. (17.4%) and of the AMP-3′ 1-N-oxide was 133 mg. (25.4%). Both the AMP-2′ 1-N-oxide and the AMP-3′ 1-N-oxide were recrystallized from acetone–water. Crystallization could not be used to reduce the contaminant quantity below 4%. Better separation of AMP-2′ 1-N-oxide and AMP-3′ 1-N-oxide could be achieved by decreasing the original loading on the ion-exchange column or by elution with formic acid of gradually increasing strength up to 0.1 *N* instead of elution with 0.1 *N* formic acid alone. Neither of these changes brings about a complete separation of the AMP-3′ 1-N-oxide from the AMP-3′.

After drying *in vacuo* over phosphorus pentoxide for one day, AMP-2′(3′) 1-N-oxide was found to be a tetrahydrate.

Anal. (AMP-2′ 1-N-oxide) Calcd. for $C_{10}H_{14}N_5O_8P \cdot 4H_2O$: C, 27.53; H, 5.16; N, 16.09. Found: C, 27.24; H, 4.82; N, 16.16.

Preparation of Adenosine 5′-Phosphate 1-N-Oxide. A. With Peroxyacetic Acid.—AMP-5′ (dihydrate) (333 mg.) was suspended in a mixture of 30% hydrogen peroxide (2.6 ml.), water (0.7 ml.) and acetic acid (3.3 ml.) and then allowed to stand at room temperature. After standing for 2 days, most of the solid dissolved. After standing 16 days, chromatographic analysis of the mixture showed that it was about half converted to the oxide. At this point the solution was evaporated to dryness under vacuum at room temperature. During the final stages of the evaporation the solution was handled with care, because of the possible presence of peroxides. As quickly as possible after the evaporation to dryness, the residue was dissolved in 2 liters of distilled water. This solution (8,600 O.D. units) was fed to a column of Dowex 1-formate (200–400 mesh) (15 cm. by 4 cm. diam.; 300 ml.), the effluent having an O.D. of 0.075 (150 O.D. units). The ultraviolet absorption spectrum and the chromatographic behavior of the material in the effluent suggested it to be adenine. The column was eluted with 0.1 *M* formic acid at the rate of about 100 ml./min., with excellent separation of the peaks. The first peak (from 500 to 700 ml.) representing 59 O.D. units was a material of unknown composition with absorption maxima at 320, 260 and 235 mμ. The second peak (from 2600 to 4200 ml.) representing 4,380 O.D. units was shown by ultraviolet absorption and R_f in solvents A and B to be AMP-5′. On evaporation of the solution representing this peak, AMP-5′ (120 mg.) was regained.

The third peak (from 5000 to 7200 ml.), representing 3810 O.D. units was indicated by the 230 mμ absorption to be AMP-5′ 1-N-oxide. The solution representing this peak was evaporated to dryness under vacuum at room temperature to give a white solid (121 mg.; yield on AMP-5′ not regained, 62.5%). This material was purified further by dissolving it in water (20 ml.), filtering and adding acetone (40 ml.) to the filtrate. AMP-5′ 1-N-oxide precipitated slowly in fern-shaped crystals, decomp. point 183–185°.

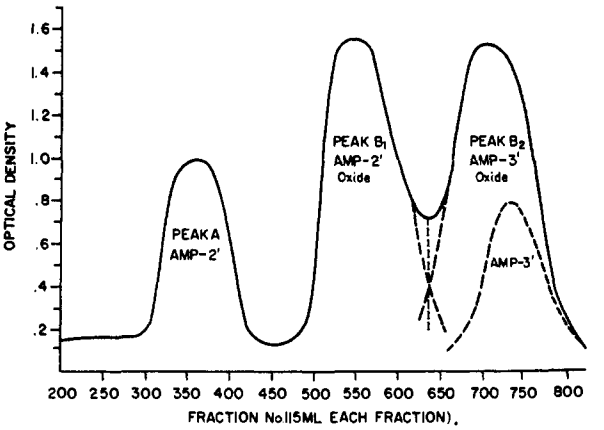


Fig. 1.—Chromatographic separation of AMP-2′, AMP-2′ 1-N-oxide and AMP-3′ 1-N-oxide (AMP-3′) on Dowex-1-formate.

Anal. Calcd. for $C_{10}H_{14}N_5O_8P$: C, 33.07; H, 3.88; N, 19.27. Found: C, 32.81; H, 4.16; N, 18.95.

B. **With Hydrogen Peroxide.**—AMP-5′ dihydrate (10 mg.) was dissolved in a mixture of water (1.5 ml.) and 30% hydrogen peroxide (0.5 ml.) and allowed to stand at room temperature. Eighteen days later, the material in the solution was developed on a chromatogram in solvent B. The two spots formed AMP-5′ (λ_{max} 260 mμ) at R_f 0.71 and AMP-5′ 1-N-oxide (λ_{max} 233 and 260 mμ) at R_f 0.83, were eluted and their optical densities at 260 mμ were compared. The proportion of 1-N-oxide to unoxidized nucleotide was calculated to be 14:86.

Preparation of Adenosine 5′-Diphosphate 1-N-Oxide (Barium Salt).—ADP monobarium salt (25 mg.) was dissolved in a mixture of acetic acid (3 ml.) and 30% hydrogen peroxide (0.5 ml.) and allowed to stand for 8 days, until chromatographic analysis showed that virtually all the starting material had been converted to the N-oxide (R_f solvent B, 0.86). The acetic acid and hydrogen peroxide were carefully removed by evaporation at room temperature *in vacuo*. The product was nearly pure barium salt of ADP 1-N-oxide (23 mg., 90%). To purify the product it was dissolved in water (1.5 ml.), and ethanol (0.3 ml.) was added slowly. Five milligrams of the monobarium salt of ADP 1-N-oxide was obtained in the form of a white microcrystalline powder.

Anal. Calcd. for $C_{10}H_{13}N_5O_{11}P_2Ba$: C, 20.75; H, 2.24. Found: C, 20.76; H, 2.58.

Proof of Structure of Adenine Nucleotide Oxides. Hydrogenation.—The 1-N-oxides of AMP-2′, -3′ and -5′ and ADP were all hydrogenated in high yield to the corresponding unoxidized nucleotide. Roughly, one mole proportion of hydrogen was absorbed in each case. The following example is typical.

AMP-2′ 1-N-oxide (11 mg.) was dissolved in water (3 ml.), and Raney nickel (2 mg.) was added. The resulting mixture was shaken with hydrogen for 3.25 hr. at 25°. After hydrogenation the material was chromatographed in solvent B to give a spot R_f = 0.69 identical with AMP-2′ and different from AMP-2′ 1-N-oxide (R_f = 0.81). The hydrogenation product had a spectrum with an absorption maximum in the ultraviolet at 260 mμ (only), identical with AMP-2′ and differing from AMP-2′ 1-N-oxide which displays two absorption maxima (260 and 230 mμ). Evaporation of the solution after hydrogenation yielded AMP-2′ (8 mg.).

Acid Hydrolysis.—The 1-N-oxides of AMP-2′, -3′ and -5′ and ADP were hydrolyzed by dilute hydrochloric acid. The formation of adenine 1-N-oxide or of 4-aminoimidazole-5-carboxamidoxime was proof of a 1-N-oxide structure of the nucleotide oxidation products. The following two hydrolyses are typical.

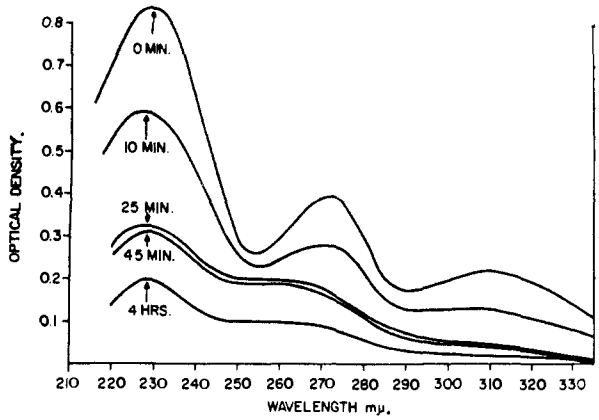


Fig. 2.—Spectral changes accompanying hydrolysis of adenosine 1-N-oxide with 1 *N* sodium hydroxide at 85°.

Hydrolysis of Adenosine 5'-Phosphate 1-N-Oxide.—AMP-5' 1-N-oxide (5 mg.) was dissolved in 1 *N* hydrochloric acid (1 ml.) and the material was brought rapidly to reflux. One minute later a spot of the solution was placed on a chromatogram and developed with solvent B. After development, spots were seen corresponding to adenine 1-N-oxide (R_f 0.42), the starting nucleotide and a trace of 4-aminoimidazole-5-carboxamidoxime. After 5 minutes' refluxing, the main product of the hydrolysis was shown to be the carboxamidoxime.

Hydrolysis of Adenosine 2'-Phosphate 1-N-Oxide.—A sample of AMP-2' 1-N-oxide containing a trace amount of AMP-2' as impurity was purified by loading on 3 MM Whatman paper on a line about 2 cm. long and submitting the material to electrophoresis in 0.05 *M* ammonium formate–0.05 *M* formic acid buffer of *p*H 3.5 for 2 hr. under a potential of 25 volts/cm. At the end of the electrophoresis (see Table I for comparative mobilities) the AMP-2' 1-N-oxide was eluted from the paper with water (3 ml.). Concentrated hydrochloric acid (35%) (1 ml.) was added to the eluate, which was then refluxed for 10 minutes. The hydrolysate was evaporated to dryness and half of the material was developed on chromatograms with solvents A and B. The major spot corresponded in both chromatograms to 4-aminoimidazole-5-carboxamidoxime indicating the 1-N-oxide structure for the starting material. There were three minor spots in the hydrolyzate chromatogram. One was a further hydrolysis product of 4-aminoimidazole-5-carboxamidoxime, namely, 4-aminoimidazole-5-carboxamide. The other two were believed to result from the two nucleotide compounds that form spontaneously in a sample of AMP-2' 1-N-oxide. One is adenine, from the AMP-2' contaminant, and the other an imidazole product. This imidazole product gave a purple color, and the main hydrolysis product (4-aminoimidazole-5-carboxamidoxime) gave an orange-yellow color with Pauly's reagent (alkaline diazotized sulfanilic acid).

Oxidation of Deoxyadenylic Acid.—DeoxyAMP (3 mg.) was dissolved in a mixture of water (0.5 ml.) and 30% hydrogen peroxide (0.1 ml.), and the mixture was allowed to stand one week. At the end of this time the solution was chromatographed on paper using A and B as developing solvents. DeoxyAMP, deoxyAMP 1-N-oxide, adenine and an unknown substance were seen to be present. The nature of the spot at R_f 0.32 in A and 0.81 in B was determined to be the deoxyAMP 1-N-oxide by its spectrum with characteristic 230 mμ N-oxide maximum, its different R_f from adenine 1-N-oxide, and the R_f in solvent B which was in the range where deoxynucleotides or nucleotides are expected. By elution of the adenine, deoxyAMP, deoxyAMP 1-N-oxide and unknown substance from the chromatogram of the product of the oxidation of deoxyAMP and estimation of the O.D.'s of the solutions so formed, it was found that deoxyAMP 1-N-oxide constituted 14% of the ultraviolet absorbing material in the product.

In order to determine the yield of the deoxyAMP 1-N-oxide from the figures representing the proportion of this material in the final product of the oxidation, it is necessary to determine what proportion of the total units of ultraviolet absorptivity are destroyed during the oxidation. This was done as follows: deoxyAMP (10 mg.) was dissolved in 1 liter of distilled water and the O.D. at 260 mμ was determined. From this the total O.D. (360 units) in the solution could be calculated. In a further batch, deoxyAMP (10 mg.) was dissolved in water (1.5 ml.) and 30% hydrogen peroxide (0.3 ml.). The solution was allowed to stand for 7 days and was then evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in water (1 l.) and the number of O.D. units remaining was determined. All but 57 O.D. units had been destroyed. The yield of deoxyAMP 1-N-oxide can thus be calculated to be approximately 2.5%.

Hydrolysis of Adenosine 1-N-Oxide and AMP 1-N-Oxide.—A solution of adenosine N-oxide in 1 *N* sodium hydroxide with an optical density at 272 mμ of 0.39 was heated to 85°. Aliquots were withdrawn from the solution at 10, 25, 45 and 240 minutes after the start of the hydrolysis, were cooled and their spectra measured (see Figure 2). In the early stages of the hydrolysis, the absorptions at 230, 272 and 310 mμ fell rapidly, without a corresponding fall in absorption at 255 mμ. After about 25 minutes, the decrease of O.D. at 230 and 270 mμ had essentially ceased. To prove that the spectrum at this point was that of the hydrolysis product, 5-amino-1-ribosylimidazole-4-carboxamidoxime, the experiment was repeated with an adenosine 1-N-oxide solution in 1 *N* sodium hydroxide with an O.D. at 230 mμ of 80. After heating for 45 minutes at 85° and diluting 1 to 100, this solution gave a spectrum identical with that of the above more-dilute solution (O.D. = 0.83 at 230 mμ) after the latter had been hydrolyzed for 25 minutes. After hydrolysis for 45 minutes, the more concentrated solution was spotted on paper and developed in solvents A and B. The chief product was seen to be a ultraviolet-absorbing imidazole with R_f's of 0.55 (in A) and 0.64 (in B). Elution with water of the spot which would give the positive Pauly imidazole test gave a solution with a spectrum with maximum at 227 mμ and a shoulder at about 257 mμ at alkaline *p*H. This spectrum is identical with that of the crude 45-minute hydrolysis product of adenosine 1-N-oxide at the same *p*H. If the hydrolysis of adenosine 1-N-oxide is continued past 45 minutes, the first hydrolysis product decomposes further to non-ultraviolet absorbing material. The spectrum after 4 hr. has the same shape as that after 45 minutes, but with reduced O.D. at all wave lengths.

The structure of the hydrolysis product of adenosine 1-N-oxide was determined by using spray tests on chromatograms of the hydrolysis product. The 5-aromatic amino group was detected by a Bratton–Marshall test (diazotization and coupling with N-(1-naphthyl) ethylenediamine hydrochloride to give red color), the oximino group by test with 1% ferric chloride solution (brown color), and the ribosyl moiety by the *cis*-hydroxyl test (sodium metaperiodate and Schiff reagent).

Hydrolysis of AMP 1-N-oxide takes place in a similar manner to the hydroysis of adenosine 1-N-oxide, but the spectral changes in this case are a little less clear cut, partly because of the AMP impurity in the AMP 1-N-oxide. Chromatographic analysis of the hydrolyzate shows it to contain one imidazole product. That this hydrolysis product is a phosphoribosyl derivative rather than a ribosyl derivative or an unribosylated imidazole is evident from its high R_f (0.78) in solvent B, and its positive phosphate reaction. The 5-amino-1-phosphoribosylimidazole-4-carboxamidoximes are spectrally identical to the 5-amino-1-ribosylimidazole-4-carboxamidoxime and give similar Pauly colors.

The hydrolysis products of adenosine 1-N-oxide and of AMP-2', -3' and -5' 1-N-oxides have the following R_f's in solvents A, B and C, respectively:

4-amino-1-ribosylimidazole-5-carboxamidoxime:	0.55, 0.64, 0.48
4-amino-1-(2'-phosphoribosyl)-imidazole-5-carboxamidoxime:	0.14, 0.79, 0.51
4-amino-1-(3'-phosphoribosyl)-imidazole-5-carboxamidoxime:	0.12, 0.75, (−)
4-amino-1-(5'-phosphoribosyl)-imidazole-5-carboxamidoxime:	0.15, 0.78, 0.66

All these hydrolysis products give a pink color on the chromatograms developed in A after spraying with Pauly's reagent and a pink-turning-grey color on the chromatograms developed in B. Spectrally they all show maxima at 210 and 257 mμ at *p*H 3.0 and at 228 and 260 mμ at *p*H 13.0.

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