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A Chemical Preparation of 5-Amino-4-imidazolecarboxamide Ribotide

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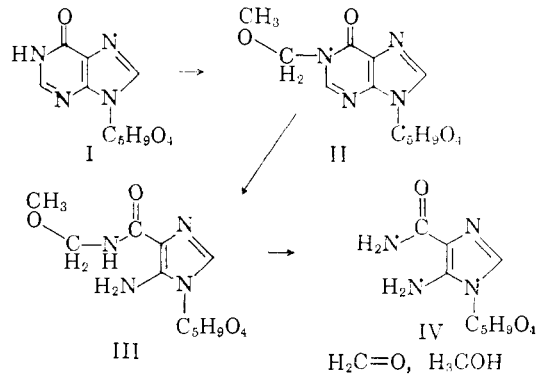
The N-methoxymethyl group was shown to be stable to alkali when attached to N-1 of inosine but to be hydrolytically removable from an amide nitrogen. Consequently, it was useful in directing the ring-opening of inosine and an inosinic acid derivative to 5-amino-4-imidazolecarboxamide riboside and ribotide.

Earlier work has shown that 5-amino-4-imidazolecarboxamide riboside is readily obtainable from inosine (hypoxanthine riboside) by ring-opening reactions which permit retention of the ribose.[1] Since the actual intermediate in purine biosynthesis is the 5′-phosphate of this imidazole riboside,[2] the preparation of the latter was undertaken. Conceivably the nucleotide could be prepared from the nucleoside by some of the phosphorylating agents that have been developed. Alternately, a ring-opening reaction could be applied to an isosinic acid derivative. The latter approach is described in this paper.

The substituents used earlier to labilize the pyrimidine ring in inosine were not suitable for use with inosinic acid for various reasons and a new grouping was sought which could be introduced into the 1-position of an inosinic acid derivative and, after ring-opening, be removed without loss of the 5′-phosphate. The methoxymethyl group, it seemed, might satisfy these requirements since its introduction by way of the reactive methyl chloromethyl ether suggested no difficulty, while its ultimate removal by a mild acid hydrolysis appeared a possibility. The selective acidic removal of a 2′,3′-isopropylidene group from purine ribosides without cleavage of the acid-sensitive ribosidic linkage is a common procedure and, in 5-amino-4-imidazolecarboxamide riboside, the ribosidic linkage is less sensitive to acid hydrolysis than in purine nucleosides.[3]

Before attempting the use of methyl chloromethyl ether on a nucleotide, its behavior with inosine triacetate was investigated to determine the ease of its removal. The alkylation was carried out with sodium hydride in dimethylformamide or dioxane. Initially, 1-methoxymethylinosine (II, or triacetate) was not characterized but the alkylation mixture, after removal of solvent, was directly subjected to alkaline hydrolysis for ring-opening. It was an unexpected finding that an amino-imidazole, presumably 5-amino-4-imidazole-(N-methoxymethyl)-carboxamide riboside (III), appeared only transiently in the hydrolysis mixture, as detected by paper chromatography, hydrolyzing further to amino-imidazolecarboxamide riboside (IV) itself.

The ability to promote ring-opening and to depart under the same alkaline reaction conditions were promising properties of the methoxymethyl group.



Inosine was present among the reaction products, a finding which could be explained either by incomplete alkylation initially or by the sensitivity of the N-methoxymethyl group in the inosine derivative (II) to hydrolysis back to inosine (II → I) as a competing reaction with the ring opening. Conceivably, the methoxymethyl group in II is relatively stable to cleavage whereas, after ring opening, the imidazole derivative III forms an anion, $H_3C{-}O{-}CH_2{-}N{-}\overset{-}{\underset{\|}{C}}{-}R$ (R = 1-ribosyl-5-amino-4-imidazolyl), which promotes methoxide elimination followed by hydrolysis to the amide and formaldehyde. In this case, maximal yield of the imidazole derivative would depend on the efficiency of the alkylation step. Subsequent work supports this interpretation since when great care was taken to dry all reagents for that step, spectroscopic examination of the reaction mixture after the alkaline ring opening sometimes gave optical density ratios of 270/250 mμ as high as 1.3; the theoretical value is 1.5,[4] whereas for inosine the ratio is 0.34.[5] Consequently, a quantitative conversion was being approached. Subsequently, the formation of amino-imidazolecarboxamide riboside was confirmed by isolation of the picrate.

Inosine, without protecting groups, can be benzylated in the 1-position in good yield in dimethylformamide in the presence of sodium bicarbonate.[1a] The greater alkylating ability of methyl chloromethyl ether is seen in its formation of a 1,5′-dimethoxymethylinosine at room temperature in dimethylformamide with sodium bicarbonate. After purification of this derivative by paper chromatography, the liberation of formaldehyde during the ring-opening reaction was demonstrated.

(1) (a) E. Shaw, *J. Am. Chem. Soc.*, **80**, 3899 (1958); (b) **81**, 6021 (1959).

(2) J. M. Buchanan and S. C. Hartman, *Advances in Enzymology*, **21**, 199 (1959).

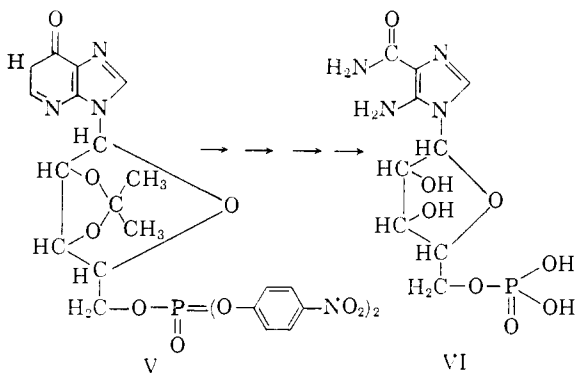
(3) J. G. Flaks, M. J. Erwin and J. M. Buchanan, *J. Biol. Chem.*, **228**, 201 (1957).

(4) G. R. Greenberg and E. L. Spilman, *ibid.*, **219**, 411 (1956).

(5) G. H. Beaven, E. R. Holiday and E. A. Johnson, Chapter 14 in "The Nucleic Acids," Vol. I, ed. E. Chargaff and J. N. Davidson, Academic Press, Inc., New York, N. Y., 1955.

For the synthesis of 5-amino-4-imidazolecarboxamide ribotide by this method a protected form of inosinic acid was desirable. 2′,3′-O-Isopropylideneinosine was phosphorylated by means of tetra-(*p*-nitrophenyl) pyrophosphate, as described by Chambers, Moffat and Khorana,[6] which provided the desired 5′-di(*p*-nitrophenyl)-phosphate of isopropylideneinosine in crystalline form in good yield. This nucleotide (V) was then alkylated as the sodium salt in dioxane by means of chloromethyl methyl ether. Following alkaline hydrolysis, an estimate of ring opening was made either by quantitative Bratton–Marshall analysis[3] for the diazotizable amino group or by measurement of the optical density ratio, 270/250 mμ. Evidence for good yields of amino-imidazolecarboxamide formation was regularly obtainable when the procedure given in the Experimental section was followed. (When such evidence was not obtained, completion of a given preparation was not worthwhile.) Removal of the remaining protective groups was carried out by venom diesterase and mild acid hydrolysis as described in the literature.[6] The product was purified on an anion exchange column and was obtained in an over-all yield of 11–16% from isopropylideneinosine-5′-di-(*p*-nitrophenyl) phosphate, a satisfactory yield considering the number of steps involved (alkylation, ring-opening, dealkylation, removal of protecting groups).

Amino-imidazolecarboxamide ribotide (VI) has previously been obtainable only by enzymatic procedures.[3,7] The chemical method described in this paper makes this nucleotide readily accessible. It was converted to the N-succino derivative by means of the yeast enzyme, adenylosuccinase, in the presence of excess fumarate as described by Miller, Lukens and Buchanan.[8]



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Experimental[9]

5-Amino-4-imidazolecarboxamide Riboside from Inosine Triacetate.—Inosine triacetate (2.0 g., dried at 110° *in vacuo*) was dissolved in anhydrous dioxane (50 ml.), treated with sodium hydride (290 mg. of a 50% mineral oil emulsion), and left 1 hour to permit salt formation. Freshly distilled methyl chloromethyl ether (0.36 ml.) was added and the mixture was left overnight at room temperature. After removal of the solvent under reduced pressure, the residue was taken up in water (10 ml.) and filtered through wet filter paper to remove mineral oil. For ring opening the solution was refluxed with 95% ethanol (80 ml.) and 5 *N* sodium hydroxide (10 ml.) for one-half hour.[10] The optical density ratio, 270/250 mμ, was 1.1. A quantitative Bratton–Marshall analysis using crystalline aminoimidazolecarboxamide riboside as a standard indicated a yield of 60%.

This is an excellent way of forming this nucleoside; however, an efficient recovery of it from the reaction mixture free of inosine and other products was not developed. For confirmation of the identity of the product, a sample was brought to about *p*H 2 and treated with 20% ethanolic picric acid. The crystalline picrate which formed at 4° was identical with authentic material[1] in infrared absorption spectrum.

Reaction of Inosine with Methyl Chloromethyl Ether.—Inosine (1.0 g.) was dissolved in dimethylformamide (25 ml.) and left overnight with sodium bicarbonate (1.0 g.) and methyl chloromethyl ether (0.7 ml.). The solvent was removed at 40° under reduced pressure. The residue was extracted with absolute alcohol until no further ultraviolet-absorbing materials were obtained. The extract was concentrated and an aliquot applied to a sheet of Whatman #3 filter paper for chromatography in 1-propanol and water (4:1). Two major products were found with R_f 0.70 and 0.50. Little inosine remained.

The faster moving zone was eluted from the paper by means of methanol and found to have a maximum absorption in the ultraviolet at 250 mμ. Assuming the product to have the same molar extinction coefficient as inosine, the yield was calculated to be about 40%. Apparently, this product was a dimethoxymethylinosine (presumably 1,5′). Substitution in the 1-position was indicated by ring opening in alkali with shift of maximum to 265 mμ and by failure of the compound to migrate when examined electrophoretically in 0.05 *M* tris buffer, *p*H 8.8, at 350 volts for 1.5 hours in contrast to inosine. The product from prolonged alkaline hydrolysis was not amino-imidazolecarboxamide riboside itself, but a faster moving material found at R_f 0.52 (instead of 0.31 for the riboside) and was probably the 5′-methoxymethyl derivative. The formaldehyde determination of Tanenbaum and Bricker[11] showed that the material in the 0.7 zone gave no evidence for free formaldehyde, even when boiled with water. Acid hydrolysis gave twice the yield of formaldehyde that was obtained on alkaline hydrolysis, consistent with the interpretation that a second methoxymethyl group was present attached to an oxygen atom, stable to alkali, unstable to acid.

2′,3′-O-Isopropylideneinosine.—The reaction of dry inosine with acetone was carried out as described for guanosine by Chambers, *et al.*[6] The yield of recrystallized material was 65% m.p. 271–276° (lit.[12] m.p. 267°).

2′,3′-Isopropylidene-inosine-5′-di-(*p*-nitrophenyl) Phosphate (V).—2′,3′-Isopropylideneinosine (10.3 g.) and di-(*p*-nitrophenyl) phosphate (27.3 g.), both of which had been previously dried at 110° *in vacuo*, were dissolved with warming in anhydrous dioxane (150 ml.). At room temperature, di-(*p*-tolyl)-carbodiimide (8.6 g.) was added and the reaction mixture was left overnight, protected from moisture. The precipitated di-*p*-tolylurea was removed by filtration and washed thoroughly with dioxane. The filtrate and washings were combined, and, after removal of the dioxane under reduced pressure, the residue was taken up in methylene dichloride (400 ml.). This solution was stirred with water (100 ml.) with addition of aqueous potassium bicarbonate until the aqueous layer remained at *p*H 7–7.5. The organic layer was then washed with water, dried with anhydrous magnesium sulfate, and taken to dryness under reduced pressure. The residue usually crystallized. It

(6) R. W. Chambers, J. G Moffat and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3750 (1957).

(7) G. R. Greenberg, *J. Biol. Chem.*, **219**, 423 (1956).

(8) R. W. Miller, L. N. Lukens and J. M. Buchanan, *ibid.*, **234**, 1806 (1959).

(9) Melting points were taken on a heated block. Microanalyses were carried out by The Scandinavian Microanalytical Laboratory, Copenhagen, Den.

(10) During the alkaline ring-opening, samples removed for chromatography in 1-propanol and water (4:1) gave evidence of a transient diazotizable amine at R_f 0.56 (presumably III) in addition to the persistant product spot at R_f 0.32).

(11) M. Tanenbaum and C. E. Bricker, *Anal. Chem.*, **23**, 354 (1951).

(12) J. Baddiley, *J. Chem. Soc.*, 1348 (1951).

was thinned with ethanol for filtration and provided 15.0 g. of product, which melted at 147–150° and gave a single spot, R_f 0.90 in propanol–water (4:1). This material was suitable for synthetic use. For analysis, samples were recrystallized from ethyl acetate and hexane or from acetonitrile and benzene. Although the m.p. was sharp, the reproducibility was poor and samples were obtained with a 1–2 degree m.p. range either about 144° or with a similar range about 160°. Possibly two crystalline modifications were at hand, but this was not the only difficulty since successive m.p. determinations of a given sample varied. In any case constant chromatographic and analytical properties were found. *Anal.* Calcd. for $C_{25}H_{23}O_{12}N_6P$: C, 47.63; H, 4.00; N, 13.33. Found: C, 47.80, 47.69; H, 4.18, 3.98: N, 13.20, 13.40 (samples melting about 144° and 160°, respectively).

5-Amino-4-Imidazolecarboxamide Ribotide (VI).—2′,3′-Isopropylideneinosine-5′-di-(*p*-nitrophenyl) phosphate (8.0 g., dried at 100° *in vacuo* prior to use) was added to a suspension of sodium hydride (800 mg. of a 50% mineral oil form) in anhydrous dioxane (320 ml.). Complete solution took place after an hour or so. Chloromethyl methyl ether was distilled under nitrogen and 1.0 ml. was added to the reaction mixture which was left overnight protected from moisture. The dioxane was then removed under reduced pressure and the residue taken up in ethanol (250 ml.). Sodium hydroxide (50 ml. *N*) was added and the solution was refluxed for 1 hour. (Analysis at this point either by a quantitative Bratton–Marshall determination of diazotizable amine or by measuring the optical density ratio of 270/250 mμ of an acidified aliquot was advisable. Successful alkylation and ring opening usually was signalized by a ratio of about 1.3. (In the case of low values, proceeding with the preparation may be inadvisable.)

After removal of the ethanol under reduced pressure, the residue was taken up in water (200 ml.), brought to *p*H 5 with dilute hydrochloric acid, filtered through wet filter paper to remove mineral oil, and extracted repeatedly with ether to remove *p*-nitrophenol. For the enzymatic cleavage of remaining di-ester groups, the aqueous layer, adjusted to *p*H 7, was mixed with 0.1 *M* tris buffer, *p*H 8.8 (200 ml.), $MgCl_2.6\ H_2O$ (1.8 g.) and *Crotalus adamanteus* venom (20 mg., Ross Allen's Reptile Institute, Silver Springs, Fla.) followed by incubation at 37° for 1.5 hours. The liberated nitrophenol was removed by ether extraction at *p*H 5 as before. The isopropylidene group was then hydrolyzed off by heating the solution for 1 hour at *p*H 2.7 in a boiling water-bath. The mixture was brought to *p*H 7 for chromatography.

Because of the high ionic content of the reaction mixture at this point due to the sequential operations without purification, demonstration of the presence of aminoimidazolecarboxamide ribotide by paper electrophoresis or chromatography on an ion-exchange column at first did not succeed. A conventional Norite adsorption and elution helped isolation of the nucleotide from inorganic materials prior to column chromatography. The Norite step could be avoided by application to an ion-exchange column in high dilution. For this purpose three-fifths of the reaction mixture was diluted to 1.5 liters and applied to a column of Dowex-1-bromide (200–400 mesh, 2.9 × 32 cm.). The column was washed with water until the eluate was transparent to ultraviolet light, then with 0.008 *N* HBr. The ribotide emerged soon after introduction of the acid. It was identified by its ultraviolet absorption spectrum and diazotizable amino-group; from the extinction coefficient,[3] ϵ 12,600, the yield in the neutralized, pooled eluate was calculated as 11–16% for a number of runs. It was concentrated under reduced pressure. The column purification serves to separate the imidazole ribotide from inosinic acid arising from unreacted starting material as well as from nucleosides arising from hydrolysis of the 5′-phosphate. The identity of the nucleotide was further confirmed by its serving as a substrate for the enzymatic synthesis of the N-succino-derivative on a preparative scale through the use of yeast adenylosuccinase.[8]

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The Base-catalyzed Alcoholysis of Ribonucleic Acids. A Method for the Determination of End Groups[1,2]

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A technique is described for the degradation of ribonucleic acids by methoxide ion-catalyzed methanolysis in methanol-formamide mixtures as the solvent. The products of this reaction were separated, identified and quantitatively determined by means of a combination of anion exchange and paper chromatography, spectrophotometry, and chemical and enzymic degradations. The effects of varying reaction conditions on the course of the methanolysis are discussed. The methanolysis reaction was shown to be applicable to the identification and quantitative determination of 2′(3′)-nucleotide end-groups in two ribonucleic acid samples. Evidence was obtained, furthermore, which rules out the possibility of appreciable numbers of phosphate triester branch points in these samples. The relative amounts of isomeric of 2′- and 3′-nucleotides formed in the alkaline hydrolysis of ribonucleic acids, monomethyl esters of the nucleotides and nucleoside-2′:3′-phosphoric acids were determined. The relative amounts of the monomethyl esters of isomeric 2′- and 3′-nucleotides formed in the methanolysis of the two ribonucleic acid samples also are given.

The various methods which have been devised for the determination of end-groups in polyribonucleotides may be divided into two classes. In the first class are those which permit the quantitative determination, but not identification, of chain ends. The methods in the second class may be used both for the identification and quantitative determination of such ends. The alkalimetric titration of nucleic acids[3] and a study of their dye-binding properties[4] are two methods of the first type. Also, the pyrimidine ends in ribonucleic acids (RNA) have been estimated by comparing the hydroxide ion consumed during the course of an exhaustive ribonuclease (RNAase) digestion with the total number of singly esterified pyrimidine nucleotide phosphate groups present in a total digest.[5] In another procedure which falls in this first category, the polynucleotide is treated with prostate phosphomonoesterase. The amount of

(1) Based on the Ph.D. dissertations of Jonathan S. Dixon, Washington University, 1953, and Preston T. Talbert, Washington University, 1955.

(2) This investigation was supported in part by the United States Atomic Energy Commission. Partial support also was provided by research grant C-3870 from the National Cancer Institute, Public Health Service, and by research grant NSF-G 12451 from the National Science Foundation.

(3) D. O. Jordan in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, Vol. I, pp. 480–482.

(4) L. F. Cavalieri, S. E. Kerr and A. Angelos, *J. Am. Chem. Soc.*, **73**, 2567 (1951); L. F. Cavalieri, A. Angelos and M. E. Balis, *ibid.*, **73**, 4902 (1951).

(5) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).