Synthesis of 3-β-D-Ribofuranosyladenine and (3-β-D-Ribofuranosyladenine)-5'-phosphate*

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ABSTRACT: 3-β-D-Ribofuranosyladenine (3-isoadenosine) has been synthesized by alkylation of adenine with bromotribenzoylribofuranose followed by debenzoylation with methanolic ammonia. A comparable amount of the naturally occurring isomer, adenosine, was also formed in this sequence of reactions. The instability of 3-isoadenosine in acid precluded the use of the isopropylidene group for blocking the 2'- and 3'-hydroxyl groups during phosphorylation, and therefore phosphorylation with cyanoethyl phosphate was carried out on the unprotected nucleoside or the 2',3'-ethoxymethylene derivative. Removal of the ethoxymethylene group with acetic acid and the cyanoethyl group with ammonium hydroxide yielded (3-β-D-ribofuranosyladenine)-5'-phosphate and smaller amounts of the 2'(3') isomer.

I

Of the anomalous naturally occurring purines and pyrimidines the 3-substituted purines are of unusual interest. Forrest *et al.* (1961) have isolated 3-ribosyluric acid and the corresponding nucleotide (Hatfield and Forrest, 1962) from beef erythrocytes. In addition, these authors have found that beef blood hemolysates catalyze not only the synthesis of 3-ribosyluric acid phosphate from uric acid and phosphoribosyl pyrophosphate (Hatfield *et al.*, 1963b) but apparently also 3-ribosylxanthine phosphate from xanthine and phosphoribosyl pyrophosphate (Hatfield *et al.*, 1963a; Hatfield and Wyngaarden, 1964). Laster and Blair (1963) have found an enzyme which catalyzes the phosphorolysis of 3-ribosyluric acid to uric acid and ribose-1-phosphate. Unique among the substituted purines is the alkaloid, triacanthine, which has the structure 6-amino-3-(γ,γ-dimethylallyl)purine, or 3-(γ,γ-dimethylallyl)adenine (Leonard and Deyrup, 1962; Denayer, 1962; Denayer *et al.*, 1961; Cavé *et al.*, 1962).

These findings, together with the recently discovered preferential alkylation of adenine on the 3 position (Leonard and Deyrup, 1962; Jones and Robins, 1962; Pal, 1962; Leonard and Fujii, 1963), stimulated us to synthesize 3-β-D-ribofuranosyladenine (I) (3-isoadenosine),[1] the isomer of adenosine in which ribose is attached to N-3 of adenine, in order to compare its chemical and biological properties with those of adenosine.
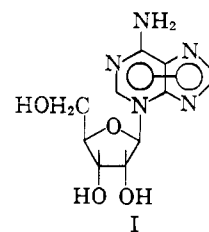
Of the four possible N isomers of adenosine, 3-isoadenosine most resembles adenosine in terms of the relative positions of the ribose and purine rings and of the amino group. Because of these similarities, it seemed not unreasonable that 3-isoadenosine might be able to substitute for adenosine in certain enzymatic reactions in which adenosine derivatives are substrates or cofactors. As a consequence of this, 3-isoadenosine might prove interesting as a possible chemotherapeutic agent or as an analog in studies of the binding sites of enzymes which require adenosine-containing substrates or cofactors. The synthesis and properties of coenzyme analogs derived from 3-isoadenosine are described in the companion article (Leonard and Laursen, 1965).

Synthesis of 3-β-D-Ribofuranosyladenine. Attachment of ribose to N-3 of adenine was accomplished by direct alkylation of adenine (free base) in dimethylformamide or acetonitrile with a benzoylated or acetylated haloribose. Bromotribenzoylribose (compound IV) was the reagent of choice in this reaction because of the ready availability of its precursor, 1-acetyl-2,3,5-tribenzoylribose (Recondo and Rinderknecht, 1959). Alkylation did not occur exclusively at N-3, as exemplified by the formation of comparable amounts of the 3 isomer (compound VI, 25%) and the 9 isomer (compound VIII, 18%), which were readily separable by virtue of their solubility properties. Chromatographic analysis of the mixture resulting from the reaction of chlorotriacetylribose (compound III) with adenine showed the presence of 24% triacetyl-3-isoadenosine (compound V), 22% of triacetyladenosine (compound VII), and
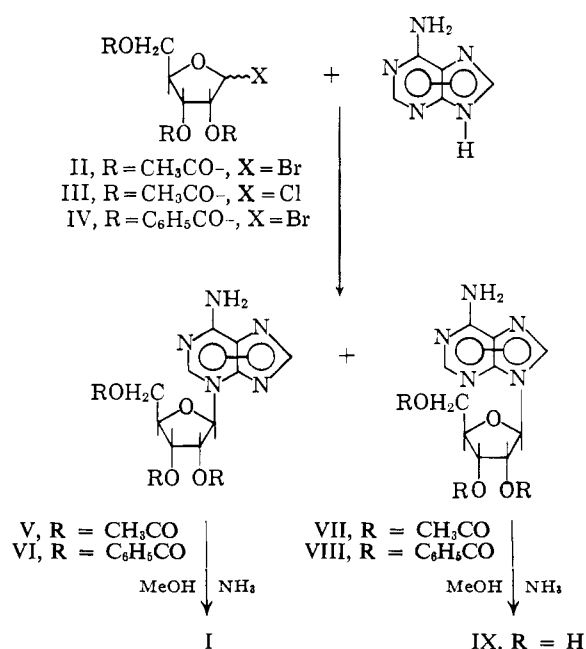
[1] Abbreviations used in this work: 3-isoadenosine, 3-β-D-ribofuranosyladenine; 3-iso-AMP, (3-isoadenosine)-5'-phosphate; DCC, dicyclohexylcarbodiimide.

354

52% of adenine. Although the highest yields were obtained when alkylation was carried out in acetonitrile, the yields appeared to be relatively independent of solvent (dimethylformamide or acetonitrile). The slight differences observed may have been owing to the type of alkylating agent used or to other factors. As a solvent for adenine, dimethylformamide is far superior to acetonitrile, the former dissolving *ca.* 4 g/100 ml and the latter 0.003 g/100 ml at room temperature. Acetonitrile was used in most of the reactions mainly because of its ease of purification.

The relatively large amount of substitution at N-9 was at first surprising in view of the previously observed (Leonard and Deyrup, 1962; Leonard and Fujii, 1963; Jones and Robins, 1962; Pal, 1962) preference for N-3. One logical explanation is that the haloribose derivatives, being α-haloethers, are so reactive (Hine, 1956) that the directive forces in adenine are no longer as important. We have also observed this lack of specificity with the α-haloethers, methoxymethyl chloride, and 1-bromo-2,3-dibenzoyl-5-diphenylphosphoryl-ribofuranose (Ukita and Hayatsu, 1962). It was hoped that the latter reagent would prove valuable as an intermediate in the synthesis of 3-isoadenosine monophosphate, but the low yields obtained in the alkylation reaction discouraged its further use. R. Ning (unpublished results), using reactive aziridinium salts as alkylating agents, has noted the formation of comparable amounts of 3- and 9-substituted adenines.

II, R = CH₃CO-, X = Br
III, R = CH₃CO-, X = Cl
IV, R = C₆H₅CO-, X = Br

V, R = CH₃CO
VI, R = C₆H₅CO

VII, R = CH₃CO
VIII, R = C₆H₅CO

MeOH | NH₃

MeOH | NH₃

I

IX, R = H

3-Isoadenosine itself was obtained by removal of the protecting groups from compound V or VI with methanolic ammonia. Some decomposition seemed to occur during this step, since the product, when attempts were made to crystallize it from water, usually precipitated as a gel. Some of the impurities were removed by passing an aqueous solution of crude 3-isoadenosine through a Dowex 1 column. One of the impurities was

not adsorbed by the resin. Although initially colorless, it was slowly converted to an insoluble purple substance (λ_{max} 540 mμ) on standing in solution. The purple substance, however, was adsorbed on Dowex 1. No further attempt was made to identify the impurities. It is interesting that a purple compound, pterorhodin, having spectral properties similar to the substance just described, is formed as a degradation product of uric acid in water at high temperatures (Pfleiderer, 1957). Removal of the protecting groups from compound VIII yielded adenosine (compound IX), as determined from ultraviolet spectral data and a mixture melting point with an authentic sample of adenosine.

Structure Proof of 3-Isoadenosine. The position of substitution of ribose in 3-isoadenosine was established as N-3 by comparison of its ultraviolet spectra (Table I) and pK_a' value of the conjugate acid (Table II) with those of substituted adenines having known structures (Leonard and Deyrup, 1962; Denayer, 1962; Denayer *et al.*, 1961). The nucleoside consumed 1 mole of periodate, thus establishing the furanose structure.

In a preliminary communication of this work (Leonard and Laursen, 1963), we discussed the assignment of the β configuration at C-1' of 3-isoadenosine by use of NMR spectroscopy and the equations of Karplus (1959). If the coupling constant, $J_{H_1'H_2'}$, of the proton at C-1' of a nucleoside or derivative is *low enough*, assignment of β configuration is possible (Lemieux and Lown, 1963; Goldman *et al.*, 1963; Karplus, 1963). In our case, $J_{H_1'H_2'}$ of 2',3'-isopropylidene-(3-isoadenosine) was 2.2 cps, which predicts, without allowance for perturbations, a dihedral angle between the intersecting planes defined by H₁'—C—C and H₂'—C—C of 120°, corresponding to that observed in a molecular model of 2',3'-isopropylidene-(3-isoadenosine) having the β configuration. The dihedral angle predicted for an α configuration could not be accommodated in a model without gross distortion.

Clark *et al.* (1951) demonstrated the β configuration for adenosine by making the cyclonucleoside derivative from the 5'-*p*-toluenesulfonate derivative. In an analogous reaction (compound X → XI) with 3-isoadenosine derivatives, a small amount (20%) of material having the expected spectral properties of the cyclonucleoside (compound XI) was obtained, but the 2',3'-isopropyl-

X

XI
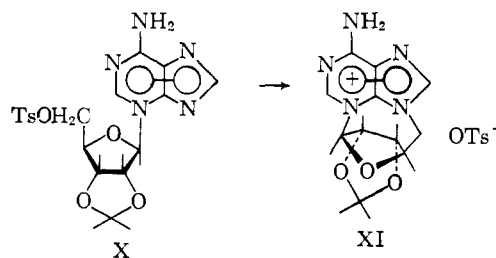
idene-9,5'-cyclo-3-isoadenosine *p*-toluenesulfonate was not well characterized.

Acid and Base Hydrolysis of 3-Isoadenosine. Some observations made in the early experiments with 3-

355

TABLE I: Ultraviolet Spectra of 3-β-D-Ribofuranosyladenine Derivatives.[a,b]

Compound (Adenine Derivative)	Solvent	λ_{max} (mμ)	ϵ_{max}	λ_{min} (mμ)	ϵ_{min}
3-β-D-Ribofuranosyl- (I)	H₂O, 0.1 N HCl	275	18,200	237	3100
		220	s[c]		
	H₂O, pH 7	277	12,900	245	2800
		215	16,700		
	H₂O, 0.1 N NaOH	278	13,100	247	3800
3-β-(2',3'-Isopropylidene-D-ribofuranosyl)-	H₂O, 0.1 N HCl	275	20,300	237	3500
		220	s		
	H₂O, pH 7	277	14,100	245	3100
		213	19,000		
	H₂O, 0.1 N NaOH	277	13,900	245	3450
3-β-(2',3',5'-Triacetyl-D-ribofuranosyl)- (V)	H₂O, 0.1 N HCl	276	17,800	237	3100
		220	s		
	H₂O, pH 7	278	11,900	246	2950
		213	18,000		
	H₂O, 0.1 N NaOH	278	12,900	247	3750
3-β-(2',3',5'-Tribenzoyl-D-ribofuranosyl)- (VI)	EtOH, 0.1 N HCl	277	22,900	252[d]	9750
	EtOH	282	14,600	253[d]	6150
		275	14,400		
	EtOH, 0.1 N NaOH	280	14,800	251[d]	6150
		274	14,800		
3-β-(2',3'-Dibenzoyl-5'-diphenylphosphoryl-D-ribofuranosyl)-	EtOH, 0.1 N HCl	278	20,500	252[d]	9250
	EtOH	283	13,300	252[d]	6350
		276	13,200		
	EtOH, 0.1 N NaOH	280	13,200	252[d]	6500
		274	13,300		
3-β-(5'-Phosphoryl-D-ribofuranosyl)- (XII)	H₂O, 0.1 N HCl	275	16,600	237	3100
		220	s		
	H₂O, pH 7	274	11,800	245	3250
		212	16,400		
	H₂O, 0.1 N NaOH	275	11,900	246	3400

[a] Determined on a Cary Model 15 spectrophotometer. [b] Supersedes values (ϵ) quoted in Leonard and Laursen (1963). [c] s = shoulder. [d] Minimum position dependent upon tailing of benzoyl absorption (not recorded).

TABLE II: Physical Constants of 3-β-D-Ribofuranosyladenine Derivatives.

Compound (Adenine Derivative)	Melting Point (°C)	pK_a'[b] 50% DMF[a]	pK_a'[b] H₂O	$[\alpha]^{26}D$
3-β-D-Ribofuranosyl- (I)	210–211 (decomp)	4.7	5.5	−35° (c 0.606 in 0.05 N HCl)
3-β-(2',3'-Isopropylidene-D-ribofuranosyl)-	250–251			−79° (c 0.538 in 0.05 N HCl)
3-β-(2',3',5'-Triacetyl-D-ribofuranosyl)- (V)	224–225 (darkens at 220)	4.8	5.6	−11° (c 0.430 in DMF)
3-β-(2',3',5'-Tribenzoyl-D-ribofuranosyl)- (VI)	246–247	4.9		−69° (c 0.89 in DMF)
3-β-(5'-Phosphoryl-D-ribofuranosyl)- (XII)	170–210 (darkens and decomp)			−6.6° (c 0.91 in 0.1 N NaOH)

[a] DMF = dimethylformamide. [b] Determined by Dr. H. Boaz, Eli Lilly and Co., Indianapolis, Indiana.
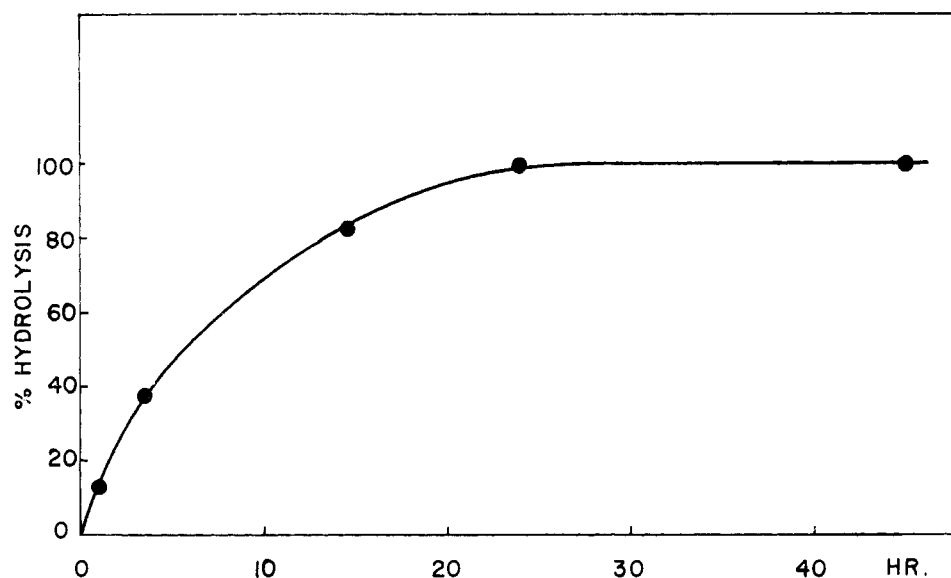
FIGURE 1: Hydrolysis of 3-isoadenosine in 0.3 N NaOH at 37°.

isoadenosine suggested that it might be unstable in acidic and basic solutions. Since 3-isoadenosine was to be subjected to these conditions in later experiments, e.g., for the removal of protecting groups, we felt it necessary to study these properties in some detail. Moreover, it seemed that a knowledge of these properties would be useful if 3-isoadenosine were considered a compound potentially isolable from natural sources.

A number of unusual nucleosides have been isolated from s-RNA by hydrolysis in 0.3 N sodium hydroxide at 37° for 18 hours (Dunn et al., 1960; Cantoni et al., 1962). Under these conditions 3-isoadenosine is almost completely degraded (Figure 1) to an as yet unidentified product, having the ultraviolet spectrum shown in Figure 2. 3-Isoadenosine was also found to be quite unstable in acid, being hydrolyzed to adenine and ribose about fifty times faster than adenosine (Figure 3). This instability prevented the use of the isopropylidene group for protecting the 2'- and 3'-hydroxyl groups in later experiments, since it was found that the glycosidic linkage was hydrolyzed at about the same rate as the isopropylidene group.

Phosphorylation of 3-Isoadenosine. Because of the lability of 3-isoadenosine, it was necessary to carry out phosphorylation under as mild conditions as possible. The method of Tener (1961), who employed β-cyanoethyl phosphate and dicyclohexylcarbodiimide (DCC), seemed ideally suited for our purposes, since the cyanoethyl group can be removed by mildly alkaline hydrolysis.

Rather than experiment with protecting groups for the 2'- and 3'-hydroxyls other than isopropylidene, we decided first to phosphorylate the unprotected nucleoside (Tener, 1961), using an excess of nucleoside to avoid diphosphate formation and trusting that the primary 5'-hydroxyl would be more reactive than the secondary 2'- and 3'-hydroxyls.

Phosphorylation of 3-isoadenosine (compound I)

with β-cyanoethyl phosphate, followed by removal of the cyanoethyl group in 9 N ammonium hydroxide (Tener, 1961), gave, 3-isoadenosine-5'-phosphate (3-iso-AMP, compound XII), in about 20% yield, together
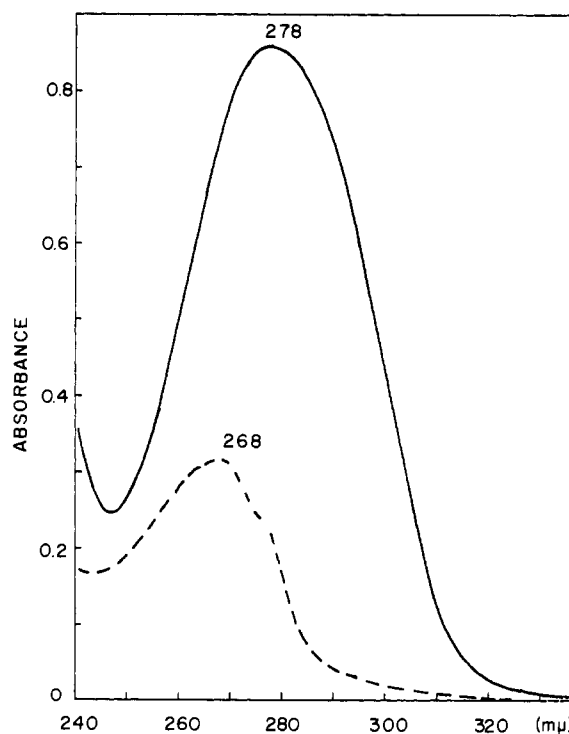

FIGURE 2: Alkaline degradation of 3-isoadenosine. Ultraviolet spectra in (————) 0.3 N NaOH and in (– – – –) 0.3 N NaOH after 40 hours at 37°.

357

with smaller amounts of the 2'(3')-phosphate (compound XIII) and diphosphates (compound XIV).

The monophosphates were separated from 3-isoadenosine and diphosphates by ion-exchange chromatography, and the 5'-phosphate was separated from compound XIII by fractional crystallization. The product obtained in this manner consumed 1.03 moles of periodate and was completely cleaved to inorganic phosphate and 3-isoadenosine by snake venom 5'-nucleotidase, thus confirming that the phosphate was on the 5' position (compound XII).

3-Isoadenosine-2'(3')-phosphate was isolated from a mixture of isomeric monophosphates by treating the mixture with crude snake venom 5'-nucleotidase which selectively hydrolyzed the 5' isomer. Treatment of compound XIII (which may have been a mixture of the 2'- and 3'-phosphates, although it ran as a single spot on chromatography) with dicyclohexylcarbodiimide according to Dekker and Khorana (1954) gave 3-isoadenosine-2',3'-cyclicphosphate (compound XV).

In subsequent phosphorylation reactions the alkoxymethylene group described by F. Cramer (personal communication) and Žemlička (1964) was used to block the 2'- and 3'-hydroxyls. The group can be removed in 80% acetic acid at room temperature, under which conditions 3-isoadenosine is relatively stable.
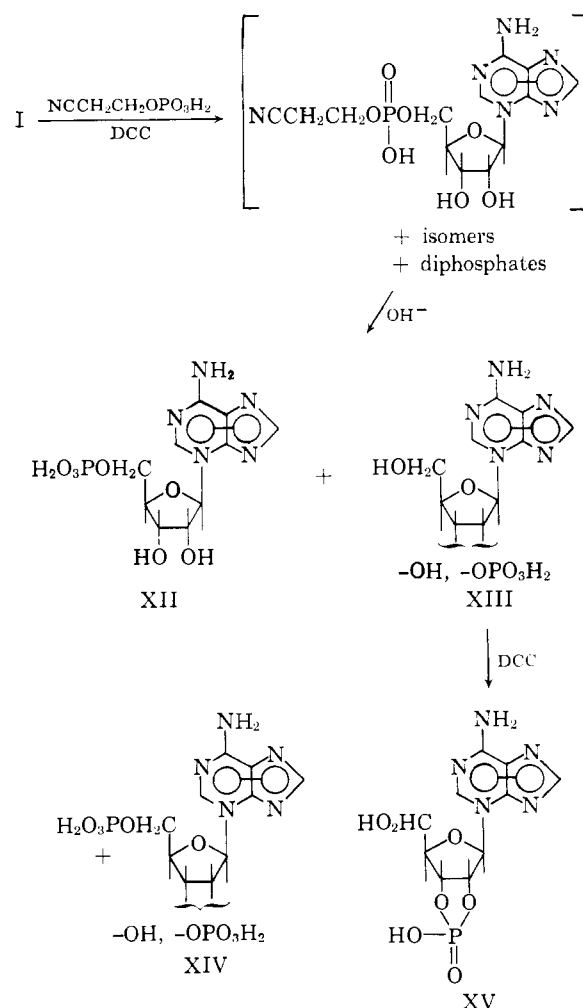
Di-(p-nitrophenyl) phosphate (Hampton ,1961) was used to catalyze the transesterification between methyl or ethyl orthoformate and the nucleoside. Attempts to isolate the intermediate compound XVI in pure form failed, although thin-layer chromatography showed only a single spot. In general, the over-all yield by this route was not much better than when direct phosphorylation was employed.
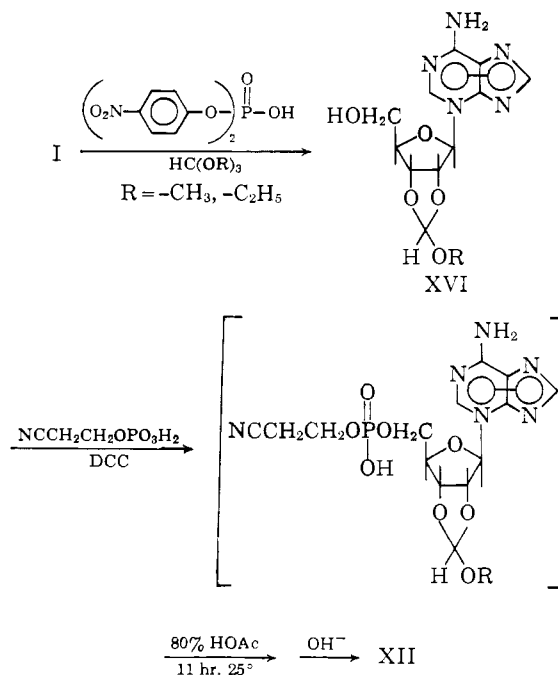
Biological Activity of 3-Isoadenosine. The ability of 3-isoadenosine to support the growth of an adenine-requiring *Escherichia coli* mutant and to inhibit the growth of certain mammalian cell cultures (Leonard and Laursen, 1963; Gerzon *et al.*, 1964) will be discussed in detail in a forthcoming publication. Adenine and adenosine do not reverse the growth inhibition by 3-isoadenosine in the systems investigated to date.

Experimental

Melting points are corrected. Ultraviolet spectra were obtained on a Cary Model 15 recording spectrophotometer. Spectra were obtained as follows: To 3.0 ml of solution was added 0.05 ml of 6.1 N HCl, giving a solution 0.1 N in HCl; 0.05 ml of 6.1 N NaOH, giving a solution 0.1 N in NaOH; or 0.05 ml of 0.61 M sodium phosphate buffer (pH 7.0), giving a solution 0.01 M in sodium phosphate (pH 7.0). Solvent base lines were run in all cases, and corrections for dilution were made. Optical rotation measurements were made on a Bendix Ericsson ETL-NPL automatic polarimeter, Type 143A, in a 1.0-cm path length cell. Solvents were removed using a Büchi "Rotavapor" thin-film rotary evaporator and a vacuum pump. The bath temperature was kept as low as was practical to prevent decomposition.

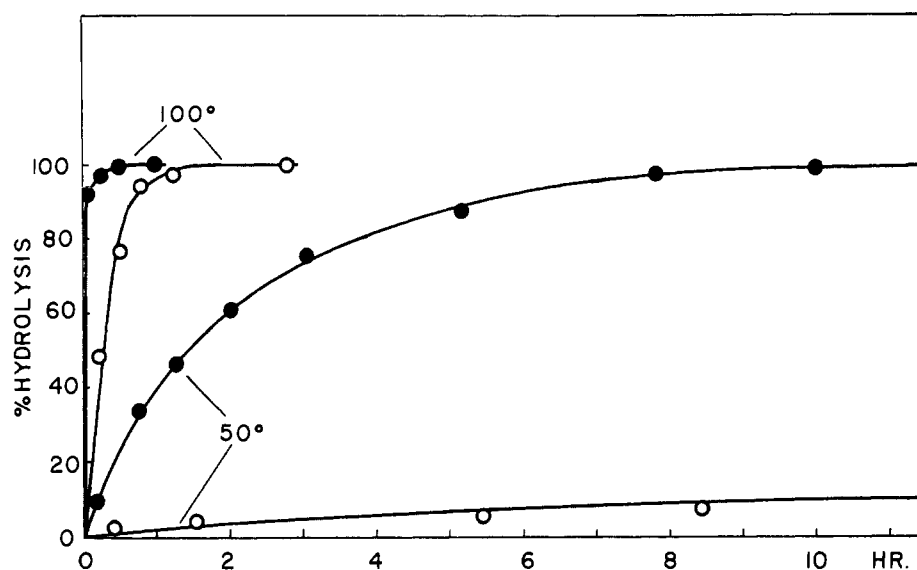*Purification and Manipulation of Solvents.* Pyridine

358

FIGURE 3: Hydrolysis of adenosine (O—O) and 3-isoadenosine (●—●) in 0.1 N HCl.

was distilled from calcium hydride and was stored over calcium hydride or Linde molecular sieves (Type 4A). Acetonitrile and dimethylformamide were distilled from phosphorus pentoxide and were stored over molecular sieves.

Solvents were stored in bottles equipped with a gas-inlet tube and an outlet tube extending to the bottom of the bottle. Solvents were forced out with dry nitrogen.

3-β-(2′,3′,5′-Triacetyl-D-ribofuranosyl)adenine (V). (A) FROM COMPOUND II. Anhydrous hydrogen bromide (10 ml) was condensed in a Carius tube containing 5.0 g (0.0158 mole) of 1,2,3,5-tetraacetylribofuranose in a Dry Ice–isopropyl alcohol bath. The tube was sealed and warmed to room temperature; after 25 minutes it was cooled again and opened. The tube was removed from the cooling bath and hydrogen bromide was allowed to evaporate. Traces of hydrogen bromide were removed under high vacuum overnight (Howard *et al.*, 1947). The residue was dissolved in 10 ml of dry dimethylformamide, and the solution was added to a suspension of 2.2 g (0.0162 mole) of adenine in 35 ml of dry dimethylformamide. The mixture was stirred for 1 hour at 90°, and the solvent was removed under vacuum. The dark residue was dissolved in 150 ml of water, and the solution was adjusted to pH 3 with phosphoric acid. The solution was extracted with three 50-ml portions of chloroform to remove unreacted sugar, and was then basified to pH 8.5 with ammonium hydroxide and extracted with four 50-ml portions of chloroform. (In some cases a solid precipitated during extraction, and more chloroform had to be used to dissolve it.) The chloroform extracts were combined, dried over sodium sulfate, and evaporated to dryness. Recrystallization of the residue from ethanol yielded 1.22 g (20%) of very pale yellow needles, mp 224–225° (darkens at 220°).

Anal. Calcd for $C_{16}H_{19}N_5O_7$: C, 48.85; H, 4.89; N, 17.90. Found: C, 48.83; H, 5.11; N, 17.67.

Optical rotation, $[\alpha]_D^{26}$ −11° (c 0.430, dimethylformamide). Plain negative optical rotatory dispersion curve. Dissociation constant, $pK_a′$, of the conjugate acid (in all cases) 5.6 (water), 4.8 (water-dimethylformamide, 50:50).

(B) FROM COMPOUND III. A mixture of 2.14 g (0.0159 mole) of adenine and 1-chloro-2,3,5-triacetylribofuranose (compound III, prepared from 5.0 g [0.0158 mole] of 1,2,3,5-tetraacetylribofuranose according to Davoll *et al.*, 1948) in 25 ml of dry dimethylformamide was stirred at room temperature for 4.5 days. The mixture was then diluted to 250 ml with ethanol (heating was necessary to dissolve all the solid) and a 2-ml aliquot was removed for analysis (*vide infra*). The solvent was removed under vacuum, and the residue was worked up as in the previous experiment; yield, 1.105 g (18%).

Analysis of Products from Reaction of Compound III with Adenine. Aliquots of 50-μl (3.12 μmoles, based on the initial amount of adenine) of the ethanolic solution obtained from the reaction of 1-chloro-2,3,5-triacetyl-ribofuranose (compound III) with adenine (*vide supra*) were spotted along a 6-cm line on Whatman No. 1 filter paper. The chromatogram was developed in 1-butanol–acetic acid–water (75:5:20). Spots were located under ultraviolet light and were excised and extracted with 25 ml of 0.1 N HCl (Table III).

Alkylation of Adenine with 1-Bromo-2,3,5-tribenzoyl-ribose (IV). (A) ISOLATION OF 3-β-(2′,3′,5′-TRIBENZOYL-D-RIBOFURANOSYL)ADENINE (VI). To a solution of 50.4 g (0.10 mole) of 1-acetyl-2,3,5-tribenzoylribofuranose (Recondo and Rinderknecht, 1959) in 100 ml of dry methylene chloride was added 300 ml of 30–32% hydrogen bromide in acetic acid. The solution was allowed to stand for 75 minutes, and acetic acid and hydrogen bromide were removed by evaporation under vacuum (bath temperature less than 35°), and by codistillation under vacuum with five 50-ml portions of dry toluene. The reddish oil was dissolved in 650 ml of dry acetoni-

359

TABLE III: Analysis of Alkylation Products.

Spot	R_F	λ_{max} (mμ)	ϵ_{max}	A_{max}	μmole	%
Adenine	0.53	262	13,100	0.841	1.61	52
Triacetyl-3-isoadenosine (V)	0.72	276	17,800	0.523	0.74	24
Triacetyladenosine (VIII)	0.87	257	14,600	0.400	0.68	22
Total					3.03	98%

trile, and 13.5 g (0.10 mole) of dry adenine was added to the solution. The mixture was stirred for 36 hours at 50°, and 15 ml of concentrated ammonium hydroxide was added. After the solvent had been removed under vacuum, the viscous residue was triturated with 500 ml of ether. The ether was decanted, 500 ml more of ether was added, and the solid was removed by filtration and was washed with ether. (The ether extracts, containing tribenzoyladenosine and other tribenzoylribose derivatives, were saved for recovery of tribenzoyladenosine.) The solid (31 g, consisting of adenine, tribenzoyl-3-isoadenosine, and ammonium bromide) was dissolved by heating on the steam bath in a mixture of 400 ml of water, 25 ml of concentrated ammonium hydroxide, and 1500 ml of ethyl acetate. (Ammonium hydroxide was added to promote solution of the adenine. It was found in later experiments that ammonium hydroxide was unnecessary and the adenine would dissolve by the addition of sufficient water, thus minimizing the danger of debenzoylation in the basic solution.) The layers were separated hot, and the ethyl acetate layer was decolorized with charcoal and was evaporated to about 300 ml. After the mixture had been cooled, the crystals were removed by filtration and were washed with ethyl acetate, methanol, and ether, 14.9 g (26%), mp 244–246°. A single recrystallization from ethanol provided an analytical sample, mp 246–247°.

Anal. Calcd for $C_{31}H_{25}N_5O_7$: C, 64.24; H, 4.35; N, 12.09. Found: C, 64.58; H, 4.56; N, 12.24.

Optical rotation $[\alpha]_D^{26}$ −69° (c 0.89 in dimethylformamide). Dissociation constant, pK_a', 4.9 (water-dimethylformamide, 50 : 50).

Tribenzoyl-3-isoadenosine could be recrystallized conveniently as follows: 17.8 g of tribenzoyl-3-isoadenosine was dissolved by heating in 200 ml of dimethylformamide on the steam bath. Heating was continued and 1.7 liters of methanol was added. Crystallization began almost immediately. The solution was cooled and allowed to stand, and the crystals (fine needles) were removed by filtration and were washed with methanol and ether, 14.6 g, mp 246–247°.

(B) 2',3',5'-TRIBENZOYLADENOSINE HYDROCHLORIDE. The ether extracts from the previous experiment (part A) were shaken with 200 ml of 1 N hydrochloric acid, and a solid, insoluble in both phases, precipitated. The solid was removed by filtration, was washed with ether, and was recrystallized from 500 ml of isopropyl alcohol. The yield was 11.0 g (18%), mp 194–196°.

(C) 2',3',5'-TRIBENZOYLADENOSINE (VIII). 2',3',5'-Tribenzoyladenosine hydrochloride (56.5 g, 0.092 mole) was added to a mixture of 100 ml of water, 25 ml of concentrated ammonium hydroxide, 700 ml of ether, and 200 ml of ethyl acetate. The mixture was shaken until all of the solid had dissolved, and the layers were separated. The organic phase was washed with two 100-ml portions of water, dried over sodium sulfate, and evaporated. The product was recrystallized by dissolving the glassy residue in 500 ml of ethanol-isopropyl alcohol (1 : 1), cooling the solution to incipient cloudiness, and adding 200 ml of ether. The yield was 37 g (70%) of light-tan prisms. Recrystallization of a sample from methanol gave colorless crystals melting at 100–103°.

9-β-D-*Ribofuranosyladenine* (*Adenosine*) (IX). 2',3',5'-Tribenzoyladenosine (compound VIII, 950 mg) was dissolved in 150 ml of dry methanol, and ammonia was bubbled into the solution for a few minutes. The solution was kept at room temperature for 24 hours and the solvent was evaporated. The solid was triturated in ether to remove methyl benzoate and was removed by filtration. The yield of crude material was 447 mg (100%). A sample recrystallized from water melted at 238–240°. The melting point was not depressed on admixture with authentic adenosine.

3-β-D-*Ribofuranosyladenine* (*3-Isoadenosine*) (I). Tribenzoyl-3-isoadenosine (compound VI), 28.3 g, 0.102 mole) was dissolved in 900 ml of dimethylformamide by heating on the steam bath. Methanol (2.8 liters, commercial anhydrous) was added, and heating was continued until solution was complete. Methanolic ammonia (300 ml, saturated at 40°) was added, and the solution was allowed to cool to room temperature and to stand for 12 hours. The solvent was removed under vacuum, and the residue was triturated in three 100-ml portions of ether to remove methyl benzoate. The solid was dissolved with heating in about 800 ml of water, and the solution was basified (pH 9.5–10) with ammonium hydroxide. The solution was passed through a heated column (3.6 × 20 cm; at 60–70°, to prevent crystallization of 3-isoadenosine) of Dowex 1 (chloride, 200–400 mesh). The column was washed with water, and 100-ml fractions were collected every 6 minutes; elution was followed by measuring the absorbance at 275 mμ. Fractions 3–14 were cooled in the refrigerator overnight and the crystals (short white needles) which formed were removed by filtration, 9.86 g (76%), mp

210–211° (decomp). Additional material could be obtained by concentration of the mother liquors.

Anal. Calcd for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.20. Found: C, 44.91; H, 4.82; N, 26.48.

Optical rotation, $[\alpha]_D^{26}$ −35° (*c* 0.606 in 0.05 N HCl). Plain negative optical rotatory dispersion curve. Dissociation constant, pK_a', 5.5 (water), 4.7 (water–dimethylformamide, 50:50).

Acidic Hydrolysis of 3-Isoadenosine and Adenosine. Acidic hydrolysis of 3-isoadenosine and adenosine in 0.1 N HCl was followed by measuring the liberation of reducing sugar by Nelson's method (Gunsalus, 1959). Alternatively, the hydrolysis of 3-isoadenosine could be followed by measuring the decrease in absorption at 275 mμ.

Alkaline Hydrolysis of 3-Isoadenosine. Hydrolysis of 3-isoadenosine in 0.3 N NaOH was followed by measuring the decrease in absorption at 275 mμ.

3-(2′,3′-Dibenzoyl-5′-diphenylphosphoryl-D-ribofuranosyl)adenine. To a solution of 6.78 g (11.2 mmoles) of methyl 2,3-dibenzoyl-5-diphenylphosphoryl-D-ribofuranoside (Ukita and Hayatsu, 1962) in 25 ml of dry methylene chloride was added 1.0 ml of acetic anhydride and 25 ml of 32% hydrogen bromide in acetic acid. The solution was allowed to stand at room temperature for 40 minutes and was evaporated to an oil under vacuum (bath temperature less than 40°). Traces of acetic acid were removed by azeotropic distillation with three 25-ml portions of toluene. Adenine (1.50 g, 11.1 mmoles) was dissolved by heating in 100 ml of dry dimethylformamide. The solution was cooled rapidly to 50°, and before crystallization occurred a solution of 1-bromo-2,3-dibenzoyl-5-diphenylphosphorylribose in 15 ml of dry dimethylformamide was added. The solution was stirred at 30–40° for 2 days. Dimethylformamide was removed under vacuum and the residue was partitioned between 100 ml each of chloroform and water. The aqueous layer was adjusted to *p*H 9.5–10 with ammonium hydroxide, the mixture was shaken, and the layers were separated. The aqueous layer was extracted with three 100-ml portions of chloroform, and the chloroform extracts were combined, dried over sodium sulfate, and evaporated to a dark oil (7.1 g). The oil was dissolved in 20 ml of chloroform and was applied to a column (4 × 60 cm) of Florisil (350 g) packed in benzene. The column was eluted with benzene (2 liters), which removed phenol, and with benzene–ethyl acetate mixtures (98:2, 0.5 liter; 95:5, 0.5 liter; 90:10, 1 liter; 80:20, 4 liters; 40:60, 4 liters), which removed several unidentified noncrystalline compounds, presumably ribose derivatives. Fractions of 100 ml were collected, evaporated and weighed. Ethyl acetate (1.5 liter) removed a compound (390 mg) which crystallized after standing for several days. Recrystallized once from ethanol, the compound melted at 141–146° and had ultraviolet spectra (λ_{max}^{EtOH,H^+} 258 mμ, λ_{min}^{EtOH,H^+} 250; λ_{max}^{EtOH} 259, λ_{min}^{EtOH} 249; $\lambda_{max}^{EtOH,OH^-}$ 260, $\lambda_{min}^{EtOH,OH^-}$ 247 mμ) consistent with the structure 9-(2′,3′-dibenzoyl-5′-diphenylphosphoryl-D-ribofuranosyl)adenine; however, the compound was not further

characterized. Ethyl acetate–methanol (95:5) removed 296 mg of a compound which crystallized almost immediately after removal of the solvent. Two recrystallizations from ethanol yielded 133 mg (1.7%) of tiny white rosettes, mp 171–173°. The compound was identified as 3-(2′,3′-dibenzoyl-5′-diphenylphosphoryl-D-ribofuranosyl)adenine on the basis of the similarity of its ultraviolet spectra with those of tribenzoyl-3-isoadenosine (compound VI) (see Table I).

Anal. Calcd for $C_{36}H_{30}N_5O_9P$: C, 61.11; H, 4.37; N, 9.95. Found: C, 60.94; H, 4.29; N, 9.83.

3-β-(2′,3′-Isopropylidene-D-ribofuranosyl)adenine. Dry 3-isoadenosine (compound I, 134 mg, 0.50 mmole) was suspended by stirring in 5 ml of anhydrous acetone containing 0.42 ml of dimethoxypropane (Hampton, 1961). Di-(*p*-nitrophenyl) phosphate (210 mg, 0.62 mmole) was added, and the solution became yellow. After 45 minutes, all the material had dissolved. Stirring was continued for a total of 5 hours. To the nearly colorless solution was added 13 ml of 0.1 N sodium bicarbonate solution. The solution was evaporated to dryness and the residue was extracted with three 25-ml portions of hot chloroform. (Alternatively, the di-(*p*-nitrophenyl) phosphate could be removed by neutralizing an aqueous solution of the reaction mixture with ammonium hydroxide and passing the solution through a column of Dowex 1-X8.) The solvent was evaporated and the solid was recrystallized from about 10 ml of ethanol. The yield was 132 mg (86%) of colorless prisms, mp 250–251°.

Anal. Calcd for $C_{13}H_{17}N_5O_4$: C, 50.83; H, 5.58; N, 22.79. Found: C, 50.88; H, 5.70; N, 23.00.

Optical rotation, $[\alpha]_D^{26}$ −79° (*c* 0.538 in 0.05 N HCl).

Attempted Preparation of 2′,3′-Isopropylidene-9,5′-(3-β-D-ribofuranosyladenine) Cyclonucleoside Chloride (Clark et al., 1951) (Chloride Derivative of Compound XI). Isopropylidene-3-isoadenosine (150 mg, 0.49 mmole) was suspended in 1.5 ml of dry pyridine. Toluenesulfonyl chloride (100 mg, 0.53 mmole) was added and the mixture was stirred until solution was complete. After standing for 20 hours at room temperature, the initially yellow solution became colorless and deposited a sludge. To the solution was added 1 ml of ice water, followed by 10 ml of ice-cold saturated sodium bicarbonate solution. The mixture was extracted with three 15-ml portions of chloroform. The chloroform extracts were evaporated to dryness, the solid was redissolved in dry chloroform, and the solution was filtered and evaporated to dryness. The residual glass was further dried under high vacuum, 108 mg (48%). The glass was heated for about 4 hours in 10 ml of anhydrous dioxane at reflux. Dioxane was removed under vacuum, and the residue was dissolved in 25 ml of water. The solution was adjusted to *p*H 7 with ammonium hydroxide and was extracted with four 10-ml portions of chloroform to remove uncyclized material. An ultraviolet spectrum of the aqueous solution showed $\lambda_{max}^{H_2O}$ 274 mμ, $\lambda_{max}^{H^+}$ 274 mμ (no hyperchromic shift), $\lambda_{min}^{H_2O}$ 241 mμ, $\lambda_{min}^{H^-}$ 239 mμ. *p*-Toluenesulfonate ion was exchanged for chloride by
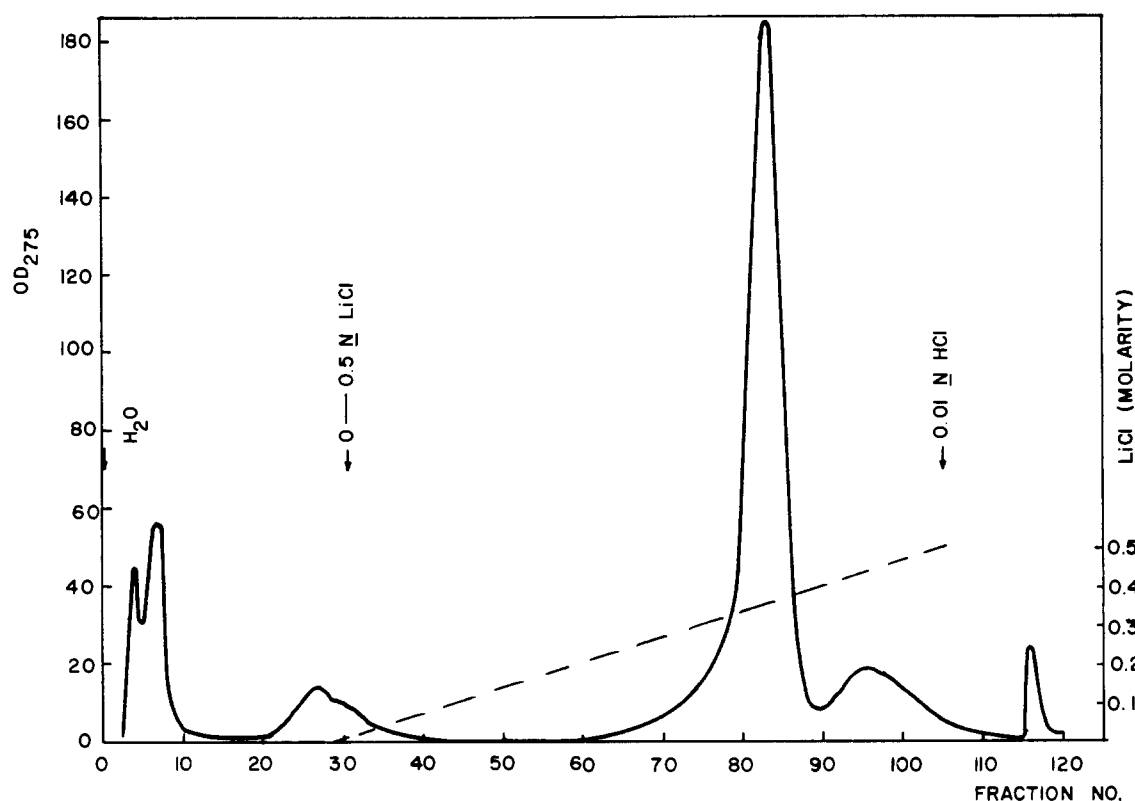
361

FIGURE 4: Purification of 3-iso-AMP, prepared via ethoxymethylene-3-isoadenosine, on a column (3.8 × 20 cm) of Dowex 1-X8 (chloride, 200-400 mesh). Eluent as shown; 25-ml fractions.

passing the aqueous solution through a column (1 × 5 cm) of Dowex 1 (chloride). The solution was evaporated to dryness and the residue (29 mg; 20%) was recrystallized from ethanol yielding a solid which decomposed over a range above 250° without melting: $\lambda_{max}^{H_2O}$ 272 mμ; λ_{min} 240; $\lambda_{max}^{H^{-3}O}$ 272; λ_{min} 240; $\lambda_{max}^{OH^-}$ 255 mμ (shoulder). There was no hypsochromic shift on going from neutral to acid solution.

3-β-(2',3'-Ethoxymethylene-D-ribofuranosyl)adenine (*XVI*). To a solution of 50 ml of dimethylformamide and 25 ml of ethyl orthoformate were added 3.0 g (11.2 mmoles) of 3-isoadenosine (compound I) and 4.2 g (12.3 mmoles) of di-(p-nitrophenyl) phosphate. The mixture was stirred until the solid dissolved, and the solution was allowed to stand at room temperature for 4 hours. The solvent was removed under vacuum, and the residue was dissolved in about 50 ml of ethanol-water (1:1). Di-(p-nitrophenyl) phosphate was removed by passing the solution through a short column of Dowex 1 (bicarbonate). The column was washed thoroughly with ethanol and water, and the effluent was evaporated to an oil which did not crystallize. The oil was triturated in ether, and the resulting white powder (2.97 g, mp 170-190°) was removed by filtration. The solid, which was not further purified, appeared to be free of 3-isoadenosine and adenine by thin-layer chromatography on silica gel in 1-butanol-water-boric acid (upper layer from a mixture of 1-butanol and a saturated aqueous solution of boric acid).

3-β-D-Ribofuranosyladenine- 5'-phosphate (*3-Iso-AMP*) (*XII*). METHOD A: FROM COMPOUND XVI. A solution of cyanoethyl phosphate (7 ml, 7 mmoles [Tener, 1961]) in dry pyridine was evaporated under vacuum to an oil, which was redissolved in 20 ml of dry pyridine. The solution was evaporated again, and 30 ml of dry pyridine and 1.61 g (5.0 mmoles) of crude 2',3'-ethoxy-methylene-3-isoadenosine (compound XVI) were added. The mixture was swirled until the solid dissolved, and 9.0 g of DCC was added. The solution was allowed to stand at room temperature in a stoppered flask for 50 hours, 10 ml of water was added, and the resulting mixture was stirred for 1 hour. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to an oil under vacuum. Fifty ml of water was added and the aqueous phase was extracted with 3 × 50 ml of ether to remove DCC. The solution was filtered and concentrated to 30 ml, and 20 ml of concentrated ammonium hydroxide was added. The solution was heated at 60° for 1 hour and was filtered and evaporated to an oily solid. To the residue was added 25 ml of 80% acetic acid, and the solution was kept at room temperature for 11 hours. The solvent was removed under vacuum, and excess acetic acid was largely removed by two codistillations with 25-ml portions of water. The oily residue was dissolved in 25 ml of water, and the solution was basified to pH 9 with ammonium hydroxide and applied to a column (3.8 × 20 cm) of Dowex 1-X8 (chloride, 200-400 mesh). The column was washed

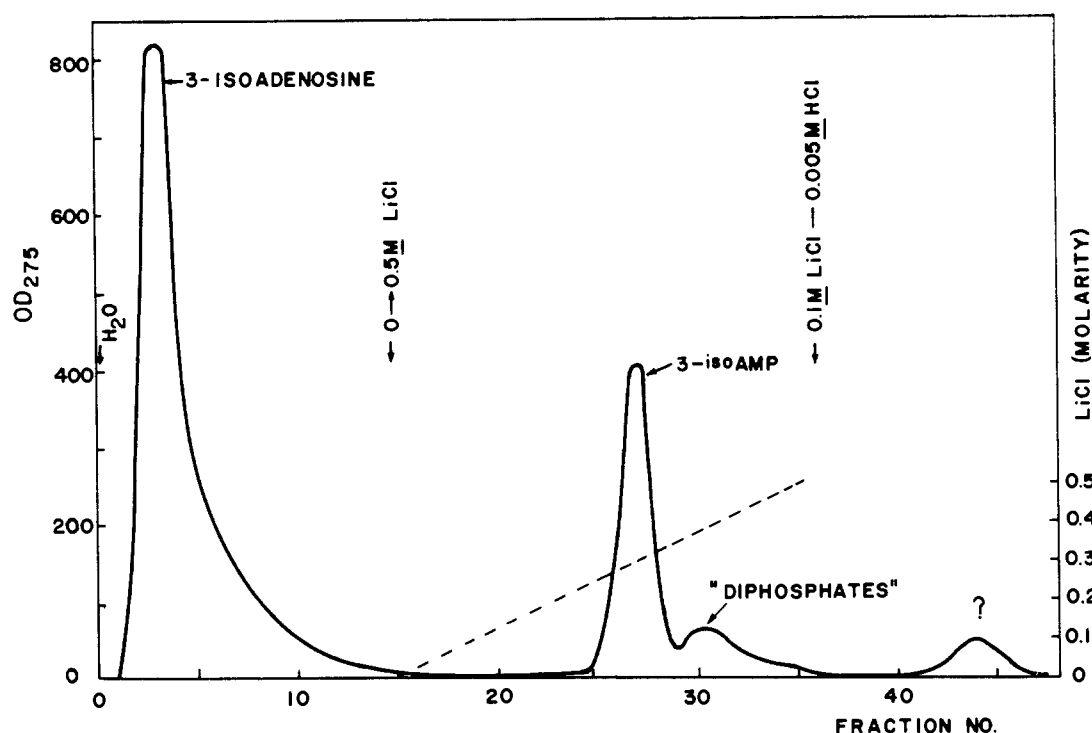FIGURE 5: Purification of 3-iso-AMP, prepared by phosphorylation of 3-isoadenosine, on a column (3.8 × 15 cm) of Dowex 1-X8 (chloride, 200–400 mesh). Eluent as shown; 100-ml fractions.

with 500 ml of water and was eluted with a linear gradient of 1 liter of 0.5 M lithium chloride into 1 liter of water; 25-ml fractions were collected, and elution was followed by measuring the absorbance at 275 mμ (Figure 4). The product was eluted in fractions 70–86 (980 OD units at 275 mμ, ca. 1.8 mmoles). Fractions 77–86 were combined and evaporated to dryness; traces of water were removed by codistillation with 50 ml of absolute ethanol. Methanol (25 ml), followed by 200 ml of acetone, was added, and the precipitate was removed by filtration, washed thoroughly with acetone and ether, and dried under vacuum, 730 mg. The salt was dissolved in 15 ml of water and was acidified to pH 3.8 with hydrochloric acid. The solution was allowed to crystallize in the refrigerator overnight. The first crop weighed 410 mg (1.18 mmoles, 24%). Concentration of the mother liquors yielded 96 mg (0.28 mmole, 5.6%) more. This material was identical with that obtained by method B (vide infra).

METHOD B: DIRECT PHOSPHORYLATION OF COMPOUND I. Dry 3-isoadenosine (compound I) (10.52 g, 26.5 mmoles) was partially dissolved in 300 ml of dry dimethylformamide by heating the mixture on the steam bath. The suspension was cooled rapidly to room temperature, and 25 ml of a solution of cyanoethyl phosphate (25 mmoles) in dry pyridine (Tener, 1961) and 50 ml of DCC were added. The solution was stirred in a sealed flask at room temperature. After 3 days, 25 ml of water was added and the mixture was stirred for 1 hour. The solvent was removed under vacuum and 150 ml of water was added to the residue. The aqueous

solution was extracted (decantation) with four 100-ml portions of ether. Concentrated ammonium hydroxide (90 ml) was added and the mixture was heated at 65–70° for 1 hour. Dicyclohexylurea was removed by filtration and was washed with two 25-ml portions of hot water. The filtrate and washings were concentrated to about half their volume, and the solution was filtered and applied to a column (3.8 × 15 cm) of Dowex 1-X8 (chloride, 200–400 mesh). The column was washed with 1.5 liters of water and was eluted with a linear gradient of 1 liter of 0.5 M lithium chloride into 1 liter of water and finally with 0.1 M lithium chloride–0.005 N hydrochloric acid (Figure 5); 100-ml fractions were collected. Fractions 26–28 were combined and evaporated to dryness. The residue was triturated in a mixture of 20 ml of methanol and 200 ml of acetone, and the solid was removed by filtration. The solid was dissolved in 60 ml of water, and the solution was acidified to pH 3.8 with hydrochloric acid. The product crystallized as clusters of fine white needles (1.79 g). The crude product was recrystallized twice by dissolving the solid in water with the aid of lithium hydroxide and reacidifying to pH 3.8 with hydrochloric acid; yield, 1.45 g (14.3%). The mother liquors were saved for recovery of 3-iso-adenosine-2'(3')-phosphate (vide infra). Samples of 3-iso-AMP decomposed over a range of 170–210°, depending on the degree of hydration. The product consumed 1.03 moles of periodate and was completely hydrolyzed to 3-isoadenosine and inorganic phosphate by crude Crotalus adamanteus venom. For analysis, a

363

sample was recrystallized once from water and was dried at 100°.

Anal. Calcd for $C_{10}H_{14}N_5O_7P$: C, 34.58; H, 4.06; N, 20.17. Found: C, 34.85; H, 4.18; N, 19.89.

Optical rotation, $[\alpha]_D^{26}$ −6.6° (*c*, 0.910 in 0.1 N NaOH). 3-Isoadenosine was recovered by combining and evaporating fractions 2–13 to dryness and recrystallizing the residue from water; yield 4.77 g (45%).

3-β-D-Ribofuranosyladenine-2'(3')-phosphate (*XIII*). A mixture of (3-isoadenosine)-5'-, -2'-, and -3'-phosphates (*ca*. 1.0 mmole), from which most of the 5' isomer had been removed by crystallization (mother liquors from the previous reaction), was dissolved in 25 ml of 0.1 M Tris-HCl buffer (*p*H 7.6, 0.1 M in KCl, and 0.01 M in $MgCl_2$). Lyophilized *Crotalus adamanteus* venom (20 mg, obtained from Sigma Chemical Co.) was added, and the solution was kept at room temperature. More venom (20 mg) was added after 6 hours. The course of the reaction was followed by measuring the liberation of inorganic phosphate (Flynn *et al*., 1954) from 3-isoadenosine-5'-phosphate. After 10 hours the solution was basified to *p*H 9.5 with ammonium hydroxide. The solution was filtered to remove magnesium ammonium phosphate, diluted to 50 ml with water, and applied to a column (1.8 × 9 cm) of Dowex 1-X8 (formate, 200–400 mesh). The column was washed with water (75 ml) to remove 3-isoadenosine and was eluted with 0.005 N formic acid (650 ml), which removed about 0.1 mmole (estimated spectrophotometrically) of unidentified material, and with 0.015 N formic acid, which removed about 0.49 mmole of 3-isoadenosine-2'(3')-phosphate; 25-ml fractions were collected. The fractions containing the 2'(3')-phosphate were combined and evaporated to about 2 ml, at which point the product (56 mg) crystallized. The product, which was not further characterized, showed a single spot (R_F 0.50; R_F 3-iso-AMP-5'-phosphate 0.42) in isobutyric acid–water–ammonium hydroxide (66:33:1) (Pabst, 1961). The mother liquors, which also showed a single spot (R_F 0.50), were saved for conversion to the cyclic phosphate (*vide infra*).

3-β-D-Ribofuranosyladenine-2',3'-cyclicphosphate (*XV*). The mother liquors from the previous experiment, containing about 0.2 mmole of 3-isoadenosine-2'(3')-phosphate, were evaporated to dryness. The residue was dissolved in a mixture of 1.0 ml of water and 2.0 ml of pyridine. A solution of 2.0 g of DCC in 5.0 ml of pyridine was added and the solution was kept at room temperature for 35 minutes (Dekker and Khorana, 1954). Water (25 ml) was added and the mixture was extracted with three 50-ml portions of ether. The solution was filtered, adjusted to *p*H 9.0 with ammonium hydroxide, and applied to a column (1.8 × 6 cm) of Dowex 1-X8 (formate, 200–400 mesh). The column was washed with water (200 ml) and was eluted with 0.005 N formic acid (800 ml), which eluted the product (*ca*. 0.17 mmole), and with 0.015 N formic acid, which removed a small amount of starting material; 25-ml fractions were collected. The fractions containing the product were combined and evaporated to dryness, and the residue was recrystallized from water; 36 mg; mp

230 (darkens) −240° (decomp). The product showed a single spot (R_F 0.48; R_F 3-iso-AMP-2'(3')-phosphate 0.14) on chromatography in isopropyl alcohol–ammonium hydroxide–water (70:5:25).

Anal. Calcd for $C_{10}H_{12}N_5O_6P$: C, 36.48; H, 3.68; N, 21.28. Found: C, 36.54; H, 3.85; N, 21.52.

Periodate Oxidations. The method of Dixon and Lipkin (1954) was modified by using buffered (0.002 M sodium acetate, *p*H 5.2) solutions and correcting for the absorption of iodate.

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Synthesis and Properties of Analogs of Adenosine Diphosphate, Adenosine Triphosphate, and Nicotinamide-Adenine Dinucleotide Derived from 3-β-D-Ribofuranosyladenine (3-Isoadenosine) *

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ABSTRACT: Coenzyme analogs ([3-isoadenosine]-5'-di- and triphosphates [3-iso-ADP and 3-iso-ATP], and the oxidized form of the 3-isoadenosine analog of nicotinamide-adenine dinucleotide [NMN-3-iso-AMP]) derived from 3-isoadenosine were synthesized from P^1-diphenyl P^2-([3-β-D-ribofuranosyladenine]-5')-pyrophosphate by the anion-exchange method and were examined for their ability to replace the corresponding natural coenzymes in enzymatic reactions. Mixtures of 3-iso-AMP, 3-iso-ADP, and 3-iso-ATP were equilibrated by myokinase. 3-Iso-ATP replaced ATP in the hexokinase-catalyzed phosphorylation of glucose and in the production of light in the luciferin-luciferase system. 3-Iso-ADP was polymerized (A. M. Michelson, personal communi-

cation) by polynucleotide phosphorylase. The nicotinamide-adenine dinucleotide (NAD) analog, NMN-3-iso-AMP, was examined in six dehydrogenase systems and was found to be reduced from 6 to 140% as rapidly as NAD, depending upon the dehydrogenase. The hypochromicity of both the oxidized and reduced forms of NMN-3-iso-AMP suggests an interaction between the purine and pyridine rings; however, no energy transfer from the dihydronicotinamide to the adenine moiety was observed by fluorescence methods. The results suggest that 3-isoadenosine possesses many of the structural features of adenosine and that 3-isoadenosine derivatives may be useful in studies of the binding sites of certain enzymes.

I n the preceding article (Leonard and Laursen, 1965) we described the synthesis of an isomer of adenosine, 3-isoadenosine,[1] and of (3-isoadenosine)-5'-phosphate (compound I). The biological activity (Leonard and Laursen, 1963; Gerzon et al., 1964) of 3-isoadenosine in certain cell cultures suggested that 3-isoadenosine may

have been metabolized in place of adenosine. This was not unanticipated in view of the gross structural similarities that exist between adenosine and 3-isoadenosine. In order to investigate some of the possible modes of action of 3-isoadenosine in living organisms and to explore the possibilities for using 3-isoadenosine

[1] Abbreviations used in this paper are: 3-isoadenosine, 3-β-D-ribofuranosyladenine; 3-iso-AMP, 3-iso-ADP, and 3-iso-ATP, (3-isoadenosine)-5'-mono-, di-, and triphosphates, respectively. Other compounds are abbreviated in accordance with the recommendations of the International Union of Pure and Applied Chemistry (1963; cf. Dixon, 1960). NMN-3-iso-AMP and NMNH₂-3-iso-AMP, the oxidized and reduced forms of the 3-isoadenosine analog of nicotinamide-adenine dinucleotide, respectively (cf. Walter and Kaplan, 1963).

365