Synthesis and Properties of Nucleotides Containing 4-Thio-D-ribofuranose*

Daniel J. Hoffman and Roy L. Whistler

ABSTRACT: 9-(4-Thio-β-D-ribofuranosyl)adenine 5′-phosphate was synthesized by coupling 2-cyanoethyl phosphate to 9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine with the aid of *N,N′*-dicyclohexylcarbodiimide. Sequential base and acid treatments removed the 2-cyanoethyl and isopropylidine groups, respectively, to give good yields of 4′-thioadenosine 5′-phosphate. The nicotinamide–adenine dinucleotide (NAD) analog, P^1-5′-*O*-(1-β-D-ribofuranosylnicotinamide) P^2-5′-*O*-[9-(4-thio-β-D-ribofuranosyl)adenine] pyrophosphate (IV), was obtained by coupling 4′-thioadenosine 5′-phosphate to 1-β-D-ribofuranosylnicotinamide 5′-phosphate with *N,N′*-dicyclohexylcarbodiimide. 4′-Thioadenosine 5′-phosphate reacted in the same manner as adenosine 5′-phosphate as a substrate for adenosine 5′-phosphate deaminase and as an allosteric effector of pig heart malate dehydrogenase. The NAD analog VI was active with all oxidoreductases examined. The analog VI reacted at maximum rates which were lower than the natural coenzyme with all oxidoreductases examined except liver alcohol dehydrogenase, which had a maximum rate twice that of the natural coenzyme.

The reduced analog VI exhibited fluorescence yields more than 50% greater than that of natural NADH. This was interpreted as due to an increase in the population of molecules with folded *anti* conformation which presumably provided better transfer of energy from the adenine ring to the nicotinamide ring. The greater degree of interaction between the bases of the analog was attributed to the restricted rotation of adenine about the C–N glycosyl bond caused by electronic repulsion between the nonbonding electrons of the N^3, and the ring sulfur. Optical rotatory dispersion data indicated also that the adenine ring of 9-(4-thio-β-D-ribofuranosyl)adenine has a more restricted movement than that of adenosine.

The synthesis and characterization of monosaccharides with a ring heteroatom other than oxygen has been of general interest and has been reviewed recently (Paulsen and Todt, 1968). Biological investigations of 5-thio-D-glucopyranose (Hoffman and Whistler, 1968; Shankland *et al.*, 1968) and pyrimidine nucleosides of 4-thio-β-D-ribofuranose (Whistler and Block, 1967) have indicated the potential usefulness of these sugars and sugar derivatives as possible chemotherapeutic agents and as analogs of natural metabolites.

In this report we describe the synthesis and some of the properties of 9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate and an NAD analog containing the 4′-thioadenosine moiety, P^1-5′-*O*-(1-β-D-ribofuranosylnicotinamide) P^2-5′-*O*-[9-(4-thio-β-D-ribofuranosyl)adenine] pyrophosphate.

Results and Discussion

The synthesis of 9-(4-thio-β-D-ribofuranosyl)adenine followed established routes using the procedures described by Urbas and Whistler (1966). Conversion of the nucleoside into the nucleotide in good yields was obtained by blocking the 2′- and 3′-hydroxyl groups of the nucleoside with an isopropylidene group, and phosphorylating with 2-cyanoethylphosphoric acid in the presence of *N,N′*-dicyclohexylcarbodiimide (Tener, 1961), and finally removing the 2-cyanoethyl and isopropylidene groups by sequential treatment with base and acid. This nucleoside was then condensed with 1-β-D-ribofuranosylnicotinamide 5′-phosphate through the aid of *N,N′*-dicyclohexylcarbodiimide to produce an analog of NAD (VI).

5′-Thioadenosine 5′-Phosphate (V) as an Effector of Pig Heart Malate Dehydrogenase. Kuramitsu (1966) has shown that AMP, ADP, and ATP are allosteric effectors of pig heart malate dehydrogenase. The nucleotides inhibit the reduction of oxaloacetate by NADH, but activate the oxidation of malate by NAD^+. Tables I and II clearly show that 5′-thioadenosine 5′-phosphate is just as effective a modifier of pig heart malate dehydrogenase as AMP. This suggests that the ring oxygen has little importance in the binding of the nucleotide to the allosteric site.

Enzymatic Deamination of 4′-Thioadenosine 5′-Phosphate. Even though rabbit muscle 5′-AMP deaminase exhibits considerable specificity for adenosine 5′-phosphate the sulfur analog V has practically the same kinetic constants for the deamination. Hence it is concluded that the ring oxygen does not significantly contribute to the specificity. (The reaction conditions used were those of Nikiforuk and Colowick, 1955.)

Action of Oxidoreductases on the NAD^+ and NADH Analogs (VI and VII). The maximal rates of reduction of the NAD^+ analog VI (Table III) by rabbit muscle and beef heart lactate dehydrogenase and by pig heart malate dehydrogenase are the same as for NAD^+ reduction but their K_m's are significantly greater suggesting that the ring sulfur lowers the affinity of the coenzyme for these enzymes. The K_m's

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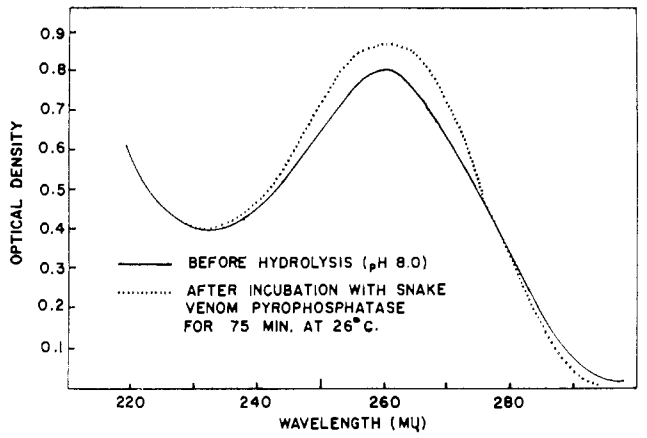


FIGURE 1: Hypochromicity of the NAD^+ analog VI.

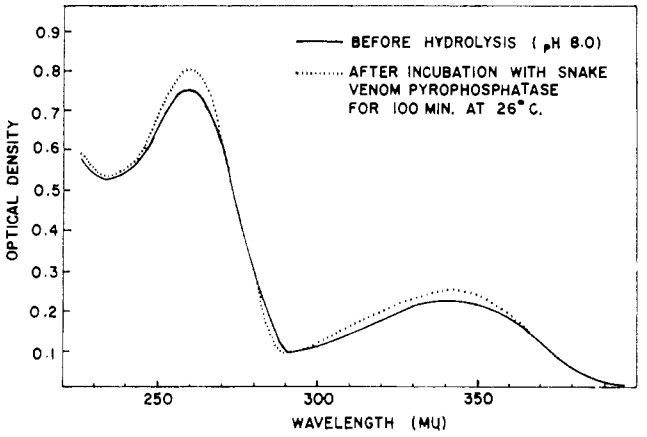


FIGURE 2: Hypochromicity of the NADH analog VII.

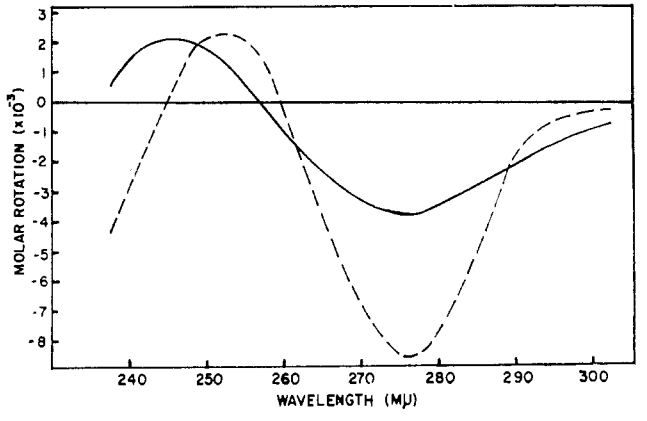


FIGURE 3: Optical rotatory dispersion of adenine nucleosides. (——) Adenosine and (- - - -) 4′-thioadenosine.

of pig heart malate dehydrogenase and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase were most effected by the sulfur analog. Maximum rates of reaction of the NAD^+ analog VI with the latter enzyme and yeast alcohol dehydrogenase were 20 and 10%, respectively, that of natural NAD^+. However, liver alcohol dehydrogenase exhibited a V_{max} for VI twice that of natural NAD. The range of K_m, K_d, and V_{max} values obtained shows that the NAD analog VI differs in the extent of its participation in O–R reactions.

TABLE I: 4′-Thioadenosine 5′-Phosphate (V) as an Inhibitor of NADH Oxidation by Pig Heart Malate Dehydrogenase.

Nucleotide Concn (mM)	Relative Activity	
	Adenosine 5′-Phosphate	4′-Thioadenosine 5′-Phosphate
0	100	100
0.25	94	97
0.50	72	75
0.75	63	60
1.00	46	45

TABLE II: 4′-Thioadenosine 5′-Phosphate (V) as an Activator of NAD^+ Reduction by Pig Heart Malate Dehydrogenase.

Nucleotide Concn (mM)	Adenosine 5′-Phosphate	4′-Thioadenosine 5′-Phosphate
0	100	100
0.25	109	105
0.50	127	124
0.75	151	148
1.00	178	182

TABLE III: Kinetic Constants of NAD^+ and NADH Analogs VI and VII with Oxidoreductases.

Oxidoreductase	K_m^{VI}/K_m^{NAD}	$V_{max}^{VI}/V_{max}^{NAD}$	K_d^{VII}/K_d^{NADH}
Yeast alcohol dehydrogenase	0.38	0.10	
Liver alcohol dehydrogenase	0.55	2.1	1.2
Rabbit muscle lactate dehydrogenase	1.8	0.97	0.30
Beef heart lactate dehydrogenase	1.6	0.97	
Pig heart malate dehydrogenase	8.3	0.96	0.80
Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase	5.3	0.20	

Hypochromicity of the NAD^+ and NADH Analogs (VI and VII). Figures 1 and 2 show that both NAD^+ and NADH analogs VI and VII exhibit a hyperchromic shift very similar to their respective natural molecules when cleaved with snake venom pyrophosphatase. The hyperchromic shift suggests that the oxidized and reduced analogs of NAD VI and VII possess a folded conformation (Weber, 1958; Fawcett and Kaplan, 1962).

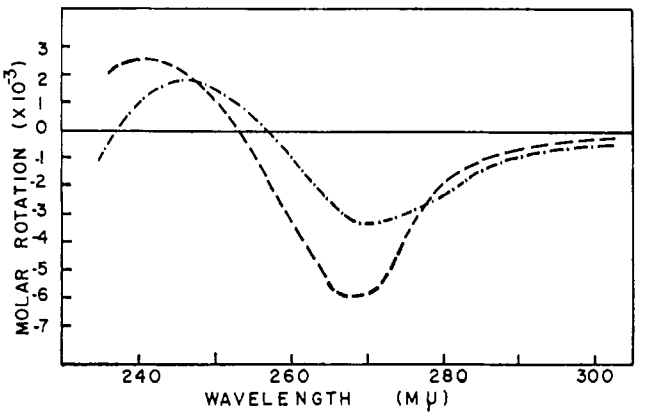


FIGURE 4: Optical rotatory dispersion of adenine nucleotides. (-·-·-) Adenosine and (- - - -) 4′-thioadenosine (5′-phosphates).

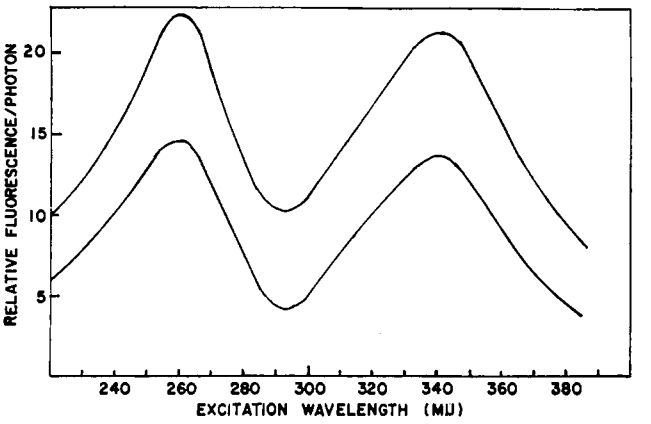


FIGURE 5: Excitation spectra of the NADH analog VII, upper curve, and natural NADH, lower curve, at 470 mμ.

Optical Rotatory Dispersion. Comparisons of the optical rotatory dispersion of adenosine, 4′-thioadenosine and their 5′-phosphate are shown in Figures 3 and 4. Both sulfur-containing analogs exhibit a larger negative Cotton effect than the natural metabolites. This difference can be explained by assuming that the sulfur-containing analogs have an increased population of molecules in the *anti* conformation; a state which would be promoted by electronic repulsion between the nonbonding electrons of the N^3 and the sulfur and thus inhibiting the molecules from assuming a *syn* conformation. The enhanced rotatory power is not due to excitation of the sulfur electrons since methyl 4-thio-D-ribofuranosides do not exhibit a Cotton effect in the range 230–300 mμ in the concentration range of about 10^{-4} M, used for the nucleosides. Also the ultraviolet spectra of adenosine and 4′-thioadenosine are very similar. Thus, their results support the view of others (Emerson *et al.*, 1966) that negative Cotton effects in adenine nucleosides result from *anti* conformations. Schweizer *et al.* (1968), reasoning from proton magnetic resonance data, conclude that in solution, adenosine 5′-phosphate exists mainly in the *anti* conformation.

Fluorescence. An interesting property of the NADH analog VII is its greater fluorescence yield compared with natural NADH (Figure 5). The excitation spectrum of the analog VII shows an efficient energy transfer from the adenine moiety to the nicotinamide moiety by the coupled oscillator mechanism (Weber, 1958; Miles and Urry, 1968). This energy transfer is about 56% more efficient for the analog VII than for natural NADH. The greater fluorescence could possibly arise in part from the greater population of analog molecules in the *anti* conformation. Bender and Grisolia (1968) recently presented evidence that the adenine moiety in the folded conformation of NADH (Velick, 1961) exists in the *anti* conformation. Therefore, the greater the population of adenine moieties in the *anti* conformation of the coenzyme, the greater the probability that the molecule exists in the folded conformation required for transfer of energy from the adenine to the nicotinamide moieties.

The emission spectrum of the NADH analog VII (Figure 6) is also enhanced over that of natural NADH. A similar enhancement is observed when the dielectric constant of an NADH solution is lowered (Velick, 1961). Therefore, the nicotinamide moiety in the NADH analog VII may have a more hydrophobic environment than that of natural NADH.

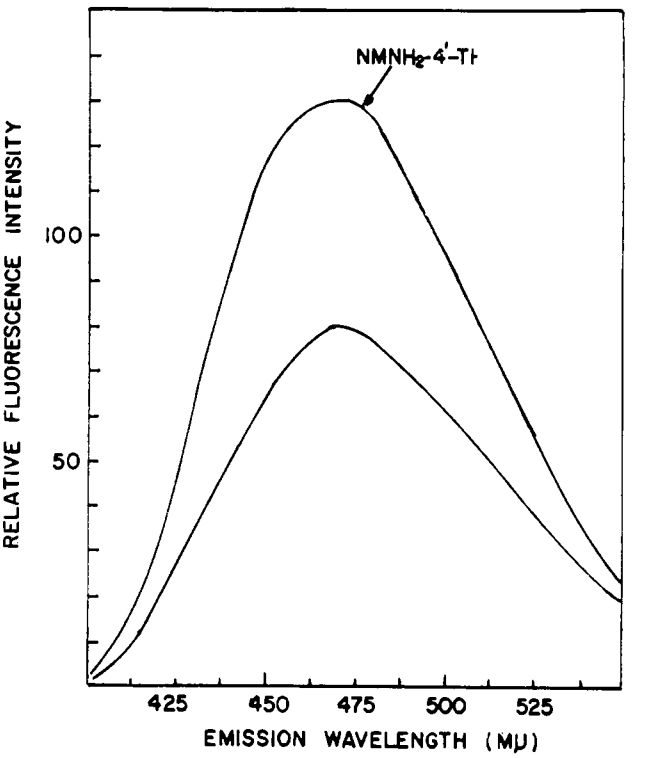
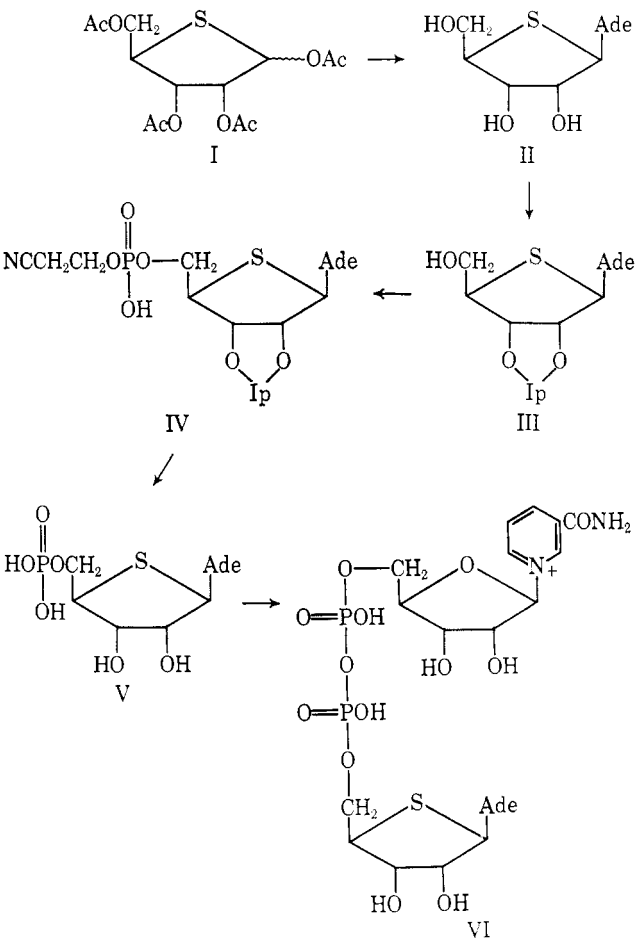


FIGURE 6: Emission spectra of the NADH analog, upper curve, and natural NADH, lower curve.

Experimental Section

Yeast and horse liver alcohol dehydrogenase (EC 1.1.1.1), rabbit muscle and beef heart lactate dehydrogenase (EC 1.1.1.27), pig heart malate dehydrogenase (EC 1.1.1.37), and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) were purchased from Worthington Biochemical Corp. NAD, NADH (NMN), NADase (EC 3.2.2.5), 5′-nucleotidase (EC 3.1.3.5), *Crotalus adamanteus* venom,

SCHEME I



adenosine deaminase (EC 3.5.4.4), and 5′-AMP-deaminase (EC 3.5.4.6) were purchased from Sigma Chemical Co. The snake venom pyrophosphatase (EC 3.6.1.1) was isolated from *C. adamanteus* venom according to the procedure of Leonard and Laursen (1965). All other chemicals were reagent grade.

Ultraviolet spectra were obtained with a Cary Model 14 recording spectrometer. Fluorescence data were obtained on a Aminco–Bowman spectrophotofluorometer. Optical rotations were determined with a Perkin-Elmer Model 141 automatic polarimeter. Optical rotatory dispersions were made with a Bendix Model 460-C spectropolarimeter. The measurements were taken in 1-cm cylindrical cells from Optical Cell Co., Brentwood, Md. The water solutions had optical densities between 1 and 2.

Chromatography. Paper chromatography was performed by the ascending method on Whatman No. 1 filter paper using the following irrigants: (A) 1-butanol–acetic acid–water (20:3:7, v/v); (B) 1-propanol–ammonium hydroxide–water (7:1:2, v/v).

Paper electrophoresis was conducted for 6 hr on Whatman No. 54 paper in 0.05 M disodium hydrogen phosphate buffer (pH 8.0) at a potential gradient of about 5 V/cm (200 V applied).

Nucleosides and nucleotides on chromatograms were detected by irradiating with ultraviolet light. Quaternary pyridinium derivatives were detected by the fluorescent method of Kodicek and Reddi (1951).

Qualitative thin-layer chromatography was performed by the ascending method on silica gel G coated microscope slides irrigated with (C) hexane–ethyl acetate (7:3, v/v) or (D) chloroform–methanol (3:1, v/v). Compounds were located on the chromatograms by spraying with 5% sulfuric acid in ethanol and charring over a hot plate.

9-(4-Thio-β-D-ribofuranosyl)adenine (III). Chloromercuri-6-benzamidopurine (14 g, 29.6 mmoles) and Celite (14 g) were refluxed in 1 l. of sodium-dried benzene. The apparatus was equipped with a trap by which the benzene–water azeotrope (100 ml) was removed. After the azeotrope was removed, 6.7 g (21.3 mmoles) of 2,3,5-tri-*O*-acetyl-4-thio-D-ribofuranosyl chloride (Urbas and Whistler, 1966) in 450 ml of benzene was added. After 72-hr refluxing, the mixture was poured into 2 l. of hexane with stirring. The precipitate was filtered and the organic matter dissolved in 800 ml of methylene chloride. This solution was washed twice with 200 ml of water, then dried over anhydrous sodium sulfate. Removal of solvent gave 6.2 g of 9-(2,3,5-tri-*O*-acetyl-4-thio-D-ribofuranosyl)6-benzamidopurine as a dark syrup. Deacetylation was accomplished by dissolving the syrup in 100 ml of anhydrous methanol containing 500 mg of sodium methoxide and heating on a steam bath for 45 min. The reaction mixture was evaporated to dryness under diminished pressure. The residue was mixed with 10 ml of water and 7 ml of 25% acetic acid and heated to 60°. To this solution was added 20 ml of a boiling solution of picric acid in ethanol (1:9, w/w). The solution was then cooled and allowed to stand at 5° for 20 hr, whereupon the salt was filtered and recrystallized from water to give 2.4 g of the picrate salt of III, mp 194–196° dec.

The picrate was dissolved in hot water (200 ml) and IR-400 (carbonate) resin was added until the solution was colorless. The resin was removed by filtration and the filtrate cooled to 5° whereupon crystals of III formed. Recrystallization from water gave 1.82 g of III: mp 246–248°; $[\alpha]_D^{25}$ −41° (*c* 0.54, 50% aqueous pyridine); $\lambda_{max}^{pH\,2}$ 259 mμ (ε 14,-310); λ_{max}^{pH7} 261 (ε 14,810). These constants agree with those of Reist *et al.* (1964). *Anal.* Calcd for $C_{10}H_{13}N_5O_3S$: S, 11.75. Found: S, 11.92.

9-(2′,3′-O-Isopropylidene-4′-thio-β-D-ribofuranosyl)adenine (IV). To 800 mg of III in 32 ml of anhydrous acetone and 2.4 ml of 2,2-dimethoxypropane was added 1.56 g of bis-(*p*-nitrophenyl)phosphoric acid. After 6-hr stirring at 25°, the reaction was complete as determined by thin-layer chromatography (solvent C). The reaction was terminated by adding 85 ml of 0.1 M sodium bicarbonate solution. The mixture was concentrated to 50 ml under reduced pressure and cooled to 5° for 12 hr. The resulting crystals were filtered and recrystallized from 500 ml of water to give 605 mg of IV (see Scheme I): mp 288–289° dec; $[\alpha]_D^{25}$ 144° (*c* 0.608, dimethyl sulfoxide). The product was homogeneous on thin-layer chromatography using solvents C and D. *Anal.* Calcd for $C_{13}H_{17}N_5O_3S$: C, 48.3; H, 5.27; N, 21.7; S, 9.9. Found: C, 48.3; H, 5.38; N, 21.6; S, 9.7.

4′-Thio-β-D-adenosine 5′-Phosphate (V). Phosphorylation of IV was accomplished by following the method used by Tener (1961) for esterifying 2′,3′-*O*-isopropylidene-β-D-ribofuranosyladenine. The final product behaved exactly like adenosine 5′-phosphate during paper chromatography (sol-

vents A and B) and paper electrophoresis. Paper chromatography of the product from hydrolysis of V by 5′-nucleotidase showed that the nucleotide was completely hydrolyzed to P_i and III (mp 247°). Titration curves were performed by dissolving about 0.15 mequiv of mononucleotide in carbonate-free water and titrating with 0.031 N barium hydroxide. Titration curves for V and AMP were indistinguishable.

Both V and AMP gave identical ultraviolet spectra; $\lambda_{max}^{pH\,1}$ 259 mμ (ε 14,2000), $\lambda_{max}^{pH\,7}$ 260 (15,100).

NAD Analog VI. The procedure reported in the literature (Hughes *et al.*, 1957) for the synthesis of NAD was followed except that after the last addition of *N,N′*-dicyclohexylcarbodiimide the reaction was allowed to stand at room temperature for 24 hr before purification by ion-exchange chromatography. From 45 mg of V, 44 mg of VI was obtained. Compound VI was 98% pure as shown by the NADase–cyanide assay of Colowick *et al.* (1951) for NAD. Paper electrophoresis indicated that the NAD analog VI was the only ultraviolet-absorbing compound present. The product was stored dry at −5°.

NADH Analog VII. To 5.6 mg of VI in 0.8 ml of 0.5 M solution of ethanol in water was added 30 μg of yeast alcohol dehydrogenase. After incubation for 1 hr at 26°, 25 μl of 25% barium acetate and 0.25 ml of 95% ethanol were added. The precipitate that formed was discarded after centrifugation. To the supernatant was added 2.5 ml of 95% ethanol, and the mixture allowed to stand at 0° for 2 hr. The precipitate was collected by centrifugation, washed with absolute ethanol, a 1:1 ethanol-ether mixture, and finally with ether. The product was dried in a vacuum desiccator and stored as the barium salt at −5°. A purity of 96.1% was calculated on the basis of the optical density at 340 mμ of a weighed sample (the extinction coefficient was assumed to be that of NADH; 6220).

Barium was removed by adding a slight excess of sodium sulfate and removing the precipitated barium sulfate by centrifugation at 5°.

Analysis of VI. Compound VI was analyzed according to the procedure of Leonard and Laursen (1965). The analyses support the structure assigned to the NAD analog VI. Incubation of the analog VI (2.0 mg) with snake venom pyrophosphatase (10 μg) in 0.25 ml of 0.1 M Tris-HCl buffer (pH 8.0)–0.04 M magnesium chloride for 4 hr at 25° produced only nicotinamide mononucleotide and 4′-thioadenosine 5′-phosphate (V) as indicated by paper electrophoresis.

Enzymatic Reduction of NAD and the NAD Analog VI. The K_m's and V_{max}'s of coenzyme reduction by various oxidoreductases were determined by measuring the initial rate of fluorescence produced by the reduced coenzyme. The excitation radiation was peaked at 360 mμ by an Aminco 4-7113 filter and an Aminco 4-7116 filter allowed emission wavelengths above 415 mμ. The beef heart and rabbit muscle lactate dehydrogenase reactions were performed in 0.2 M Tris-HCl buffer (pH 8.8) containing 3.6 mM lactate. Yeast alcohol dehydrogenase reactions were conducted in a buffer (pH 7.9) containing 0.053 M sodium pyrophosphate, 14 mM glycine, 12 mM semicarbazide, and 0.2 M ethanol. Horse liver alcohol dehydrogenase assays were made in a glycine–sodium hydroxide buffer (pH 9.0) containing 10 mM ethanol. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase assays were conducted in a 0.012 M sodium pyrophosphate buffer (pH 8.5) containing 1.2 mM DL-glyceraldehyde 3-phosphate, 3 mM cysteine, and 15 mM disodium arsenate. Pig heart malate dehydrogenase studies were performed in a buffer (pH 9.2) containing 8 mM Tris, 6 mM ammonium sulfate, 7.5×10^{-5} M glycylglycine, and 20 mM sodium L-malate. In all cases, 10 μl of enzyme was added to initiate the reaction.

Dissociation Constants of Reduced Coenzyme–Enzyme Complexes. Use was made of the fluorescence enhancement of NADH that accompanies the binding of the reduced coenzyme to enzyme (Boyer and Theorell, 1956). The methods and calculations of Theorell and Winer (1959) were used to determine the dissociation constants of the NAD analog VI with horse liver alcohol dehydrogenase, rabbit muscle lactate dehydrogenase, and pig heart malate dehydrogenase.

4′-Thioadenosine 5′-Phosphate as an Allosteric Effector of Pig Heart Malate Dehydrogenase. The reaction conditions to evaluate the effectiveness of V as an inhibitor of the reverse (oxidation of NADH) or activator of the forward (reduction of NAD) reactions were those previously described by Kuramitsu (1966).

Fluorescence Spectra. The fluorescence emission spectra of the reduced analogs were obtained at 26° on solutions having an absorbance of 0.45 at 340 mμ. The emission wavelength was 450 mμ. The spectra were corrected for variations in the intensity of the arc source.

Optical Rotatory Dispersion Measurements. Optical rotatory dispersion of the nucleoside analogs was obtained by using absorbancies between 1 and 2 at 260 mμ. A cylindrical cell with a 1-cm light path length gave the best results.

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Chemical Modification of Yeast Alanine Transfer Ribonucleic Acid with a Radioactive Carbodiimide*

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ABSTRACT: 1-Cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide [^{14}C]methiodide reacts with the base in inosinic acid and dihydrouridylic acid.

In yeast alanine transfer ribonucleic acid, one of the first sites of chemical attack by the carbodiimide is the inosinic acid of the anticodon.

1-Cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate (CMCMT)[1] was first shown by Gilham and coworkers to form addition products with guanylic acid, uridylic acid, pseudouridylic acid, and thymidylic acid but not with adenylic acid or cytidylic acid (Gilham, 1962; Ho and Gilham, 1967). The addition products were found to be resistant to enzymatic cleavage by pancreatic ribonuclease, which normally cleaves ribonucleotides at the 3′-phosphoryl bond of pyrimidines, or by ribonuclease T_1, which cleaves at the 3′-phosphoryl bond of G and I (Ho and Gilham, 1967). In addition the carbodiimide reaction was found to be reversible under conditions of mild alkali (Gilham, 1962).

Other workers in the field have used 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide methiodide (CMCMI) or CMCMT to investigate the structural properties of nucleic acids. Augusti-Tocco and Brown (1965) used [^{14}C]CMCMI to investigate the extent of nonhydrogen-bonded or randomly coiled regions in various polynucleotides. Knorre and his coworkers (Knorre *et al.*, 1966; Drevitch *et al.*, 1966) also investigated the reaction of [^{14}C]CMCMT with tRNA and DNA and reported the effect of this reaction on the ability of yeast to accept amino acids (Girshovich *et al.*, 1966).

In a previous paper we reported the reaction of [^{14}C]-CMCMI with $tRNA^{Ala}$ purified from yeast (Brostoff and Ingram, 1967). It was demonstrated that [^{14}C]CMCMI attaches to mononucleotides in the sequences ψpGp, UpCp, and UpUpIpGpCp of that molecule, but does not form addition products with the expected bases in the sequence TpψpCpGpApUp. Further evidence will now be presented for the reaction of this reagent with the mononucleotide Ip, and with Ip in the proposed anticodon of the $tRNA^{Ala}$ molecule. Preliminary evidence will also be presented which suggests that the mononucleotide diHUp may also react with CMCMI.

Materials and Methods

Mononucleotides and tRNA. Mononucleotides were obtained from Calbiochem, Inc. (Los Angeles, Calif.). Yeast tRNA was obtained from the General Biochemicals Corp. (Chagrin Falls, Ohio). $tRNA^{Ala}$ was purified from the commercial yeast tRNA by two successive countercurrent distributions (Apgar *et al.*, 1962; Armstrong *et al.*, 1964).

Preparation of [^{14}C]*CMCMI.* [^{14}C]CH_3I (1.5 mCi, 128 mg, New England Nuclear Corp., Boston, Mass.) was added to excess (1–2ml) 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]-carbodiimide (redistilled, Aldrich Chemical Co.) and the mixture was allowed to stand in the dark for at least 15 hr at room temperature in a glass-stoppered tube. The precipitate formed was taken up in chloroform and filtered. The chloroform was removed from the filtrate by evaporation, and the residue was taken up in acetone and again filtered. Anhydrous ether (two volumes) was added to the filtrate and crystals were allowed to form at room temperature. The white crystals were collected, washed with ether, and dried. The yield was 60% and the purity was comparable with commercially available CMCMT (Aldrich Chemical Co.) as determined by infrared spectrophotometry. After electrophoresis of [^{14}C]CMCMI in pH 1.9 buffer, more than 99% of the radioactivity present on the paper was located in

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[1] Abbreviations used are: CMCMT, 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate; CMCMI, 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide methiodide; DiHU, 5,6-dihydrouridine; DiMeG, N^2-dimethylguanosine; MeG, 1-methylguanosine; MeI, 1-methylinosine; Ψ, pseudouridine; T, ribothymidine; U*, a mixture of uridine and dihydrouridine.