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SYNTHESIS OF 4-AMINOBENZIMIDAZOLE AND 4-NITROINDOLE NUCLEOSIDES AND NUCLEOTIDES

by

Edwards Stengle Atwood

B.S., Union College, 1965

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

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PhD
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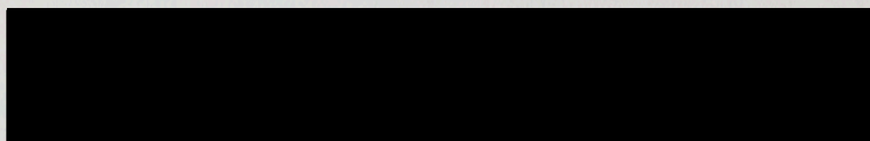
Approved by

First Reader



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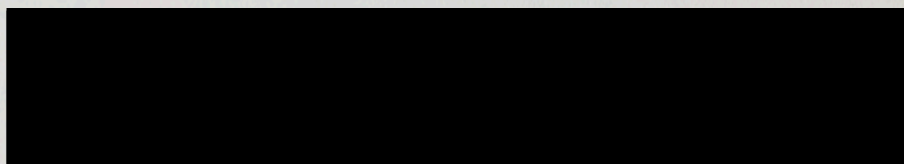

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Finally, the author wishes to express his debt to his wife, Bonnie. Without her sympathy, understanding and support when things looked bleak, he might have given up hope long ago.

Abstract

The synthesis of two analogues of adenosine, 4-amino-1-β-D-ribofuranosylbenzimidazole and 4-nitro-1-β-D-ribofuranosylindole, and their associated nucleoside-5' phosphates is described. The interaction of 4-aminobenzimidazole nucleoside-5' triphosphate with the enzyme adenylate kinase is also described.

4-Nitro-1-β-D-ribofuranosylbenzimidazole was prepared from 4(7)-nitrobenzimidazole and 2,3,5-tri-O-benzoyl-ribofuranosyl bromide by the mercuric cyanide method in 50-60% yield. After removal of the benzoyl groups, 4-aminobenzimidazole nucleoside-5' phosphate was prepared by direct phosphorylation followed by reduction of the nitro group by hydrogenation over 5% Pd/C in 60% yield. The nucleoside di- and triphosphates were prepared using the anion exchange method of Michelson.

4-Nitro-1-β-D-ribofuranosylindole was synthesized in 70% yield by condensing 4-nitroindole and 2,3,5-tri-O-benzoylribofuranosyl bromide in the presence of silver(I) oxide in benzene. The benzoyl groups were removed with methanolic ammonia. The nucleoside-5' phosphate was prepared by blocking the 2' and 3' hydroxyl groups with the ethoxyformylidene group and condensing the 5' hydroxyl with cyanoethyl phosphate in the presence of mesitylene sulfonyl

chloride. The nucleoside-5' diphosphate was prepared by the anion exchange method. An unsuccessful attempt to prepare the nucleoside triphosphate by the same method is described.

Kinetic measurements were made for the interaction of 4-aminobenzimidazole nucleoside-5' triphosphate with adenylate kinase in the presence of 1 mM adenosine monophosphate. Km for the benzimidazole nucleotide is 0.40 mM as opposed to 0.029 mM for adenosine triphosphate under the same conditions. Vmax for the benzimidazole triphosphate is 12 times that of ATP.

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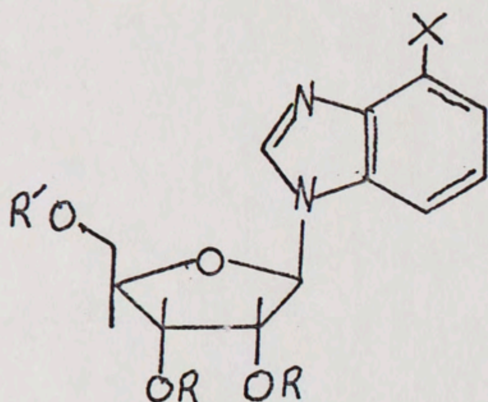
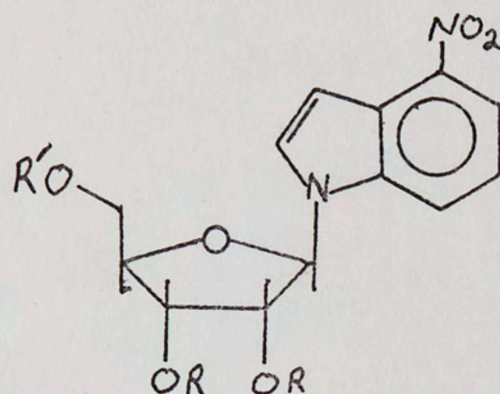
I. Introduction

Formation of an enzyme-substrate complex at the start of an enzymatic reaction requires the formation of some type of bond. These bonds may be ionic bonds, hydrogen bonds, hydrophobic bonds or a combination of bond types. An understanding of the detailed mechanism of an enzymatic reaction requires knowledge of the nature and orientation of these bonds.

Adenosine nucleotides are very common enzyme substrates. The specific requirement of an adenosine nucleotide in these reactions indicates that the adenine ring is involved in the binding. This may be by hydrophobic bonding to the purine ring or by hydrogen bonding to the ring nitrogens and/or the amino group at position 6 of adenine.

One technique for determining enzyme binding requirements is to synthesize substrate analogues that have small differences in structure. For this purpose, 4-amino-1-β-D-ribofuranosylbenzimidazole and 4-nitro-1-β-D-ribofuranosylindole have been synthesized along with their respective nucleoside-5' phosphates (Figure 1). These deaza analogues of adenosine will help determine whether ring nitrogens are required for binding in adenosine nucleotide specific enzymes.





1 R=R'=H, X=NO_2

3 R=R'=benzoyl, X=NO_2

5 R=H, R'= $-PO_3H_2$, X=NH_2

7 R=H, R'= $-P_2O_6H_3$, X=NH_2

9 R=H, R'= $-P_3O_9H_4$, X=NH_2

BENZIMIDAZOLE

2 R=R'=H

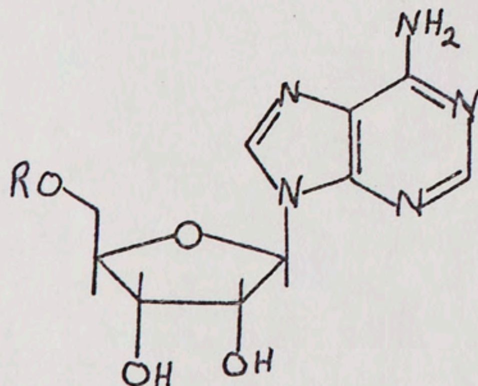
4 R=R'=benzoyl

6 R=H, R'= $-PO_3H_2$

8 R=H, R'= $-P_2O_6H_3$

10 R=H, R'= $-P_3O_9H_4$

INDOLE



11 R=H, Adenosine

12 R= $-PO_3H_2$, AMP

13 R= $-P_2O_6H_3$, ADP

14 R= $-P_3O_9H_4$, ATP

Figure 1.

II. Historical

A wide variety of enzymes require adenosine-containing compounds as substrates or cofactors in the reactions that they catalyze. Glycolysis, oxidative phosphorylation, photosynthesis, active transport and biosynthesis require adenosine compounds in one or more reactions. These may be cofactors such as Coenzyme A, nicotinamide-adenine dinucleotide, adenosine-5' diphosphate or adenosine-5' triphosphate (Lehninger, 1965). The requirement of adenosine is often specific in that other common nucleotides (uridine, guanosine, cytidine) which differ only in the base portion cannot replace adenosine.

In 1965 Leonard and Laursen (1965a) reported the synthesis of 3-β-D-ribofuranosyladenine (3-isoadenosine) by the direct alkylation of adenine with tri-O-benzoylribofuranosyl bromide. This compound has the ribose attached to the nitrogen at position 3 rather than the nitrogen at position 9 as in adenosine (see figure 2 for the numbering of adenine). To test the hypothesis that 3-isoadenosine could replace adenosine, they synthesized the 5' mono-, di- and triphosphates of 3-isoadenosine (Leonard and Laursen, 1965a; Leonard and Laursen, 1965b). These three nucleotides were found to be substitutes for the corres-

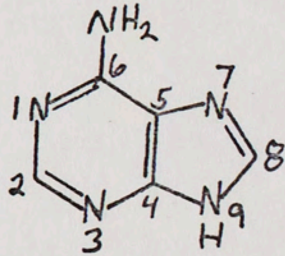
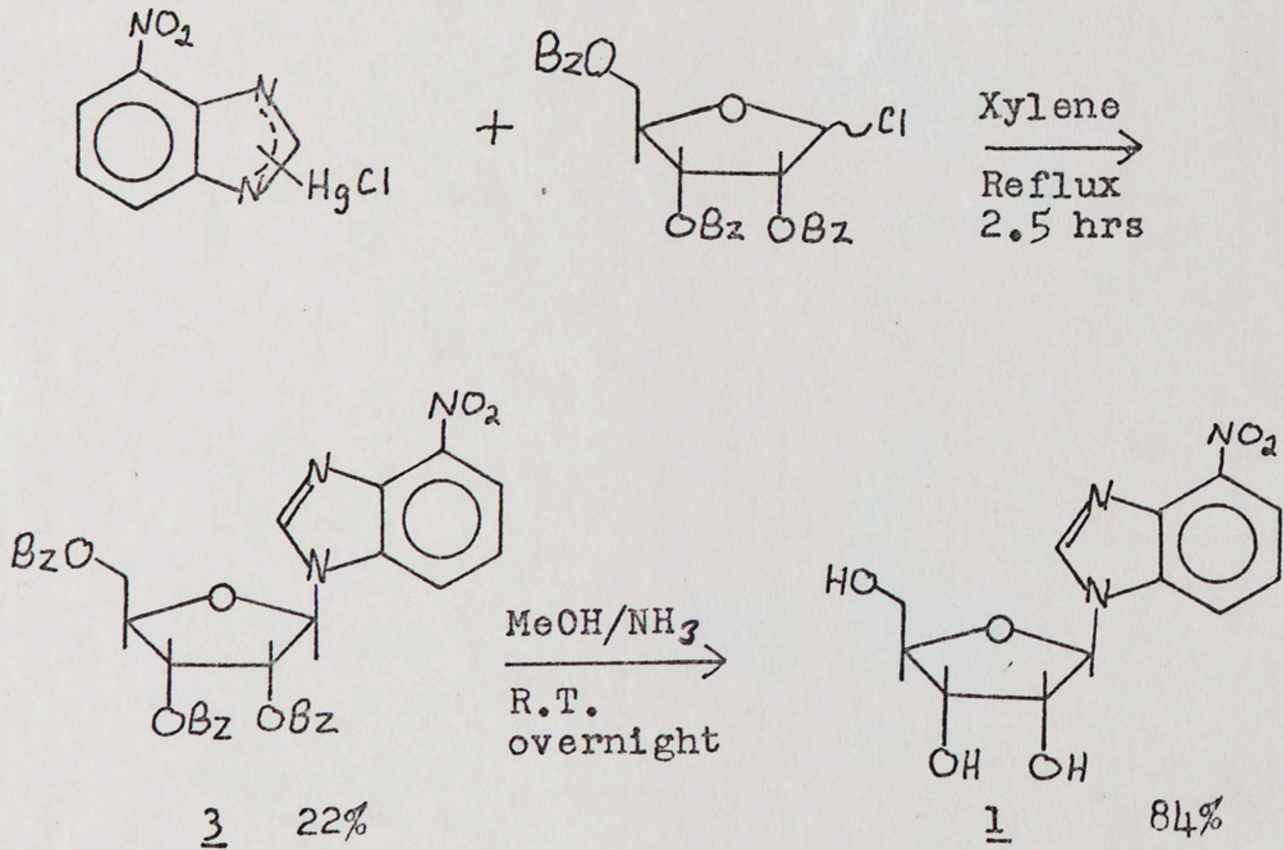


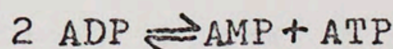
Figure 2.



(Mizuno *et al.*, 1962)

Figure 3.

ponding adenosine nucleotides in the dismutation reaction



catalyzed by the adenosine specific enzyme adenylate kinase (EC.2.7.4.3) (Leonard and Laursen, 1965b).

Other adenosine analogues found to serve as substrates for adenylate kinase are formycin (7-amino-3-(β-D-ribofuranosyl)pyrazolo-(4,3-d)pyrimidine) 5' mono-, di- and triphosphates (Ward *et al.*, 1969), adenosine-5' diphosphate N'-oxide and adenosine-5' triphosphate N'-oxide (Jebeleanu *et al.*, 1974) and 1,N^6-ethenoadenosine triphosphate (Secrist *et al.*, 1972).

4-Nitro-1-β-D-ribofuranosylbenzimidazole (1)

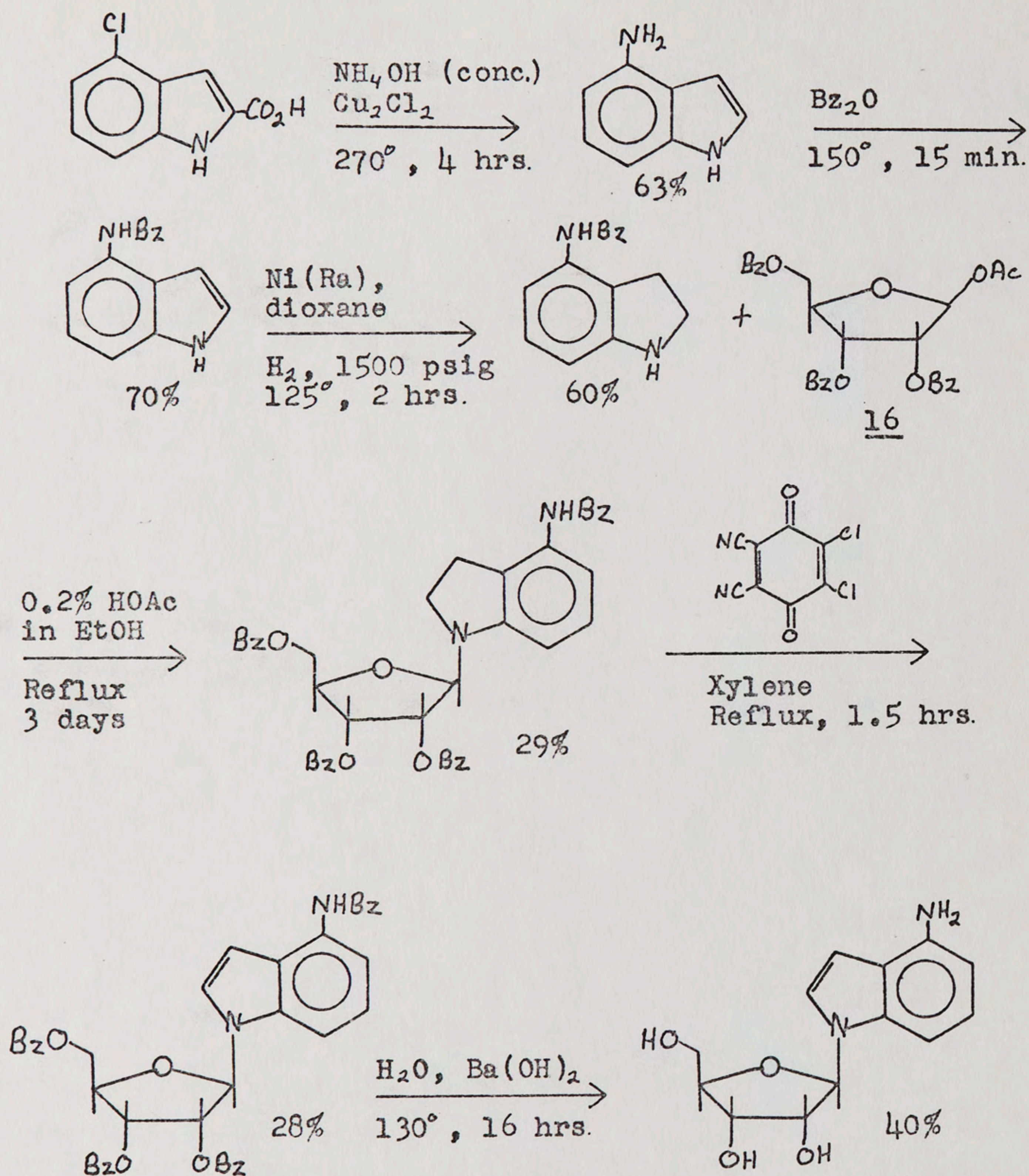
Mizuno *et al.* (1962) reported the synthesis of 4-nitro-1-β-D-ribofuranosylbenzimidazole (1) by the reaction of the chloromercuri salt of 4(7)-nitrobenzimidazole with tri-O-benzoylribofuranosyl chloride in refluxing xylene followed by removal of the of the benzoyl groups with methanolic ammonia (figure 3). They assigned the β-configuration to the compound on the basis of the specific rotation and the 1,2 trans rule for the reaction of O-acyl sugars (Howard, 1950; Baker *et al.*, 1954). The position of alkylation was deduced from the observation that 1 did not form a methiodide after heating for seven hours at 140-150° in a sealed tube with an excess of methyl iodide. Under iden-

tical conditions, 1-methyl-7-nitrobenzimidazole readily formed a methiodide.

The reduction of 4-nitro-1-β-D-ribofuranosylbenzimidazole (1) to the aminobenzimidazole nucleoside has been accomplished with hydrogen in the presence of 5% palladium on charcoal (Ikehara et al., 1964; Jenkins et al., 1968). The Japanese group synthesized the aminobenzimidazole nucleoside-5' triphosphate (9) and measured the ability of 9 to substitute for ATP (14) in reaction with actomyosin (Ikehara et al., 1964).

Indole nucleosides

A number of indoline nucleosides have been synthesized by condensing an indoline (2,3-dihydroindole) with a 1-O-acyl sugar in the presence of an acid catalyst. These have been converted to the indole nucleosides by oxidation with 2,3-dichloro-5,6-dicyanoquinone. Walton et al. (1968) used this method to synthesize 1-β-D- ribofuranosylindole, 1-β-D-ribopyranosylindole and 4-amino-1-β-D-ribofuranosylindole. The aminoindole nucleoside was obtained in 3% overall yield from 4-benzamidoindoline and 2,3,5-tri-O-benzoyl-1-O-acetyl-D-ribofuranose (16) (figure 4). Preobrazhenskaya et al. (1967) have also reported the synthesis of 1-β-D-ribofuranosylindole as well as 1-β-D-ribofuranosylindole-5' phosphate (Vigdorchik et al., 1968).



(Walton et al., 1968)

Figure 4.

A series of indole glycopyranosides has been reported which covers compounds formed with D-galactose, D-mannose, L-rhamnose, D-xylose, D-lyxose, L-arabinose and D-ribose and both substituted and unsubstituted indolines (Magnin *et al.*, 1969; Magnin *et al.*, 1972). The 100 MHz nuclear magnetic resonance spectra of the fully acetylated glycosides is described.

III. Discussion

III.1. Nucleosides

The reported syntheses of 4-nitro-1-β-D-ribofuranosylbenzimidazole (1) and 4-amino-1-β-D-ribofuranosylindole give low yields of the desired products which makes these procedures unacceptable as intermediate steps in a multistep synthesis. Since quantities of the nucleosides were required for further elaboration to the nucleotides, it was necessary to develop more efficient synthetic schemes.

III.1.1. 4-Nitro-1-β-D-ribofuranosylbenzimidazole (1)

In recent years, a number of different methods for preparing nucleosides by condensing a preformed base with an appropriately substituted sugar have been developed. A review of the subject has been published by Zorbach (1970), concentrating on reactions involving the naturally occuring purine and pyrimidine bases. Townsend and Revankar (1970) have reviewed the synthesis of benzimidazole nucleosides and nucleotides. Three of these new methods were investigated as alternatives to the chloromercuri-salt method for the synthesis of 4-nitro-1-β-D-ribofuranosylbenzimidazole (1): the fusion method, the trimethylsilyl fusion method and the mercuric cyanide-nitromethane method.

Fusion Method

The synthesis of nucleosides by fusion of the base and a 1-O-acyl sugar was developed by Sato et al. (1961). The base and sugar are ground together with a catalyst such as trichloroacetic acid and heated until a clear melt is obtained. The most common acyl group is the acetate and acetic acid is removed by evacuating the reaction flask. Whittle and Robins (1965) have used the acid fusion method to prepare the anomeric mixture of 1-(2'-deoxyribofuranosyl)benzimidazoles.

4(7)-Nitrobenzimidazole (15) and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (16) were heated with bis-(p-nitrophenyl)phosphate in an oil bath at 180° under diminished pressure. After cooling, 4-nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)benzimidazole (3) was isolated from the mixture in 25-28% yield by column chromatography on silica gel in benzene-ethyl acetate (4:1). This represented a modest increase in yield over the chloromercuri-salt method although still rather low.

A major problem with the fusion method is the volatility of 4(7)-nitrobenzimidazole. The crude material has been purified by sublimation at atmospheric pressure (Rabinowitz and Wagner, 1951). Under the conditions for fusion, the base 15 was rapidly removed from the melt.

Trimethylsilyl Fusion Method

Several benzimidazole nucleosides have been synthesized by fusing a 1-trimethylsilylbenzimidazole and a 1-halo-sugar at elevated temperatures in vacuo. Birkofer et al. (1960) and Bräuniger and Koine (1965) have used this procedure to prepare several benzimidazole pyranosides. 5,6-Dimethyl-1-β-D-ribofuranosylbenzimidazole was synthesized by this method by Revankar and Townsend (1968).

Hexamethyldisilazane and 4(7)-nitrobenzimidazole (15) were heated, with a small crystal of ammonium sulfate, at reflux for two hours. After removal of excess hexamethyldisilazane by evaporation under reduced pressure, the 1-trimethylsilyl-4-nitrobenzimidazole was mixed with 1-bromo-2,3,5-tri-O-benzoylribofuranose (17)(from one equivalent of 1-O-acetyl-2,3,5-tri-O-benzoylribofuranose(16)). The mixture was heated at 110° for 30 minutes. The nucleoside (3) was isolated in 30% yield by column chromatography on silica gel. This was still not an acceptable yield.

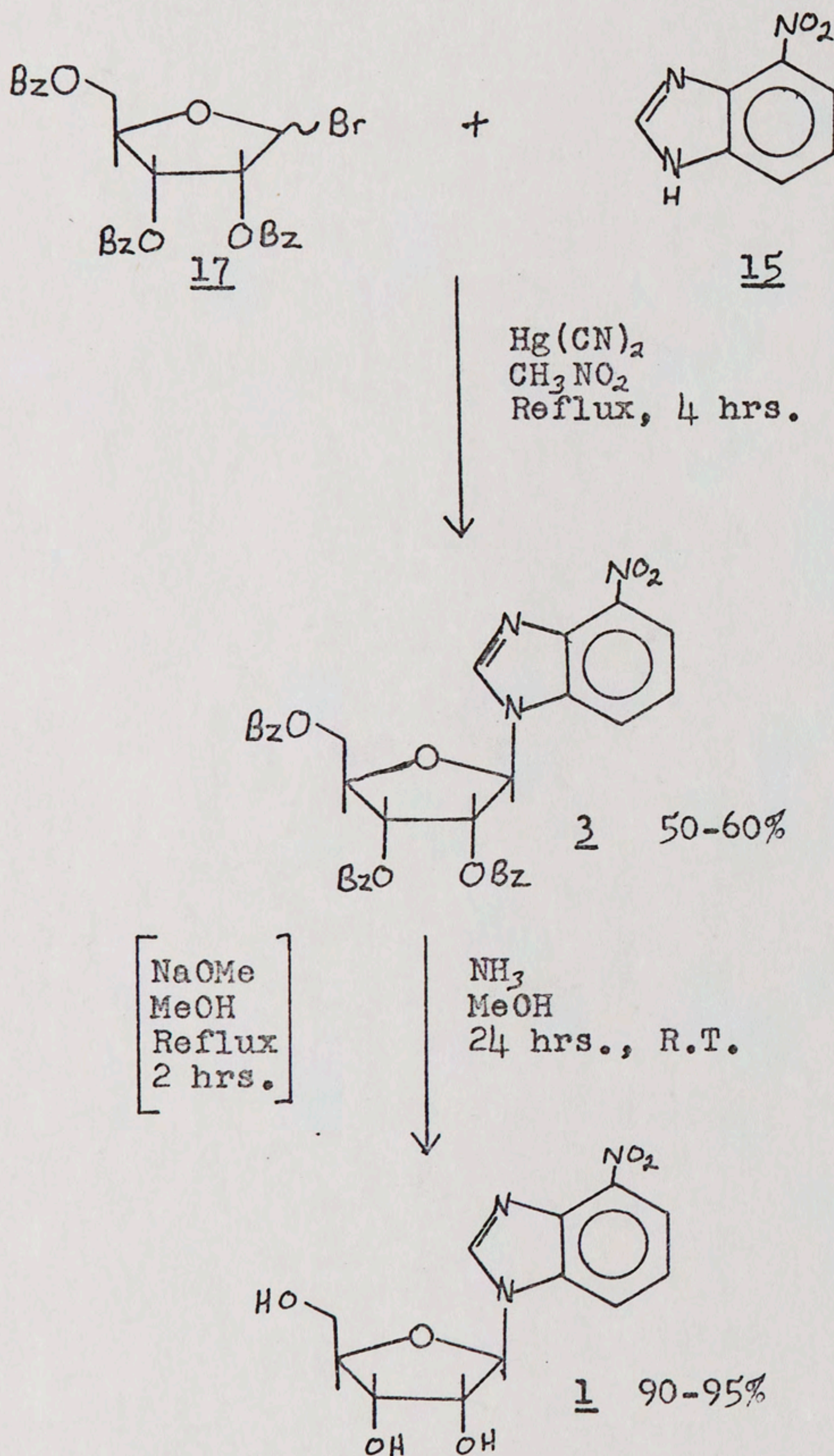
Mercuric cyanide-Nitromethane Method

The use of mercuric cyanide as an alternative to silver oxide or silver carbonate in the Koenigs-Knorr glycoside synthesis was introduced by Helferich and coworkers (Helferich and Weis, 1956; Helferich and Steinpress, 1958). In addition, they used the polar solvent, nitromethane,

in place of non-polar solvents such as benzene, with the result that the reaction took place in homogeneous solution.

The basic procedure has been applied successfully to nucleoside synthesis. Yamoka et al. (1965) have reported the synthesis of the glucosides of N-benzoyladenine, N-benzoylcytosine and benzimidazole in good yield. Watanabe and Fox (1969) have used the mercuric cyanide-nitromethane procedure to synthesize uridine in 77% yield as well as cytidine and several 5-substituted uridines.

1-Bromo-2,3,5-tri-O-benzoylribofuranose (17) in nitromethane was added over a period of 30 minutes to an azeotropically dried solution of 4(7)nitrobenzimidazole (15) and mercuric cyanide in nitromethane. The solution was refluxed for four hours with slow distillation of solvent. After filtration and evaporation of the nitromethane, the dark residue was extracted with chloroform. The chloroform solution was extracted with 30% potassium iodide (to remove mercuric ion), 5% sodium carbonate (to remove hydrogen cyanide and some unreacted 15), and water. After precipitating unreacted 15 by dissolving the residue in methylene chloride-petroleum ether, the nucleoside 3 was crystallized from methanol in 50-60% yield. This is a twofold increase in yield and will allow the preparation of the quantity of nucleoside 3 desired.



Synthesis of nitrobenzimidazole nucleoside 1

Figure 5.

The product, 4-nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)benzimidazole (3), melted at 125-127° (Mizuno *et al.*, 1962, gave 124-125° as the melting point of 3). Nucleoside 3 had an Rf on silica gel thin layer chromatography identical to that of 3 produced by the chloromercuri-salt method.

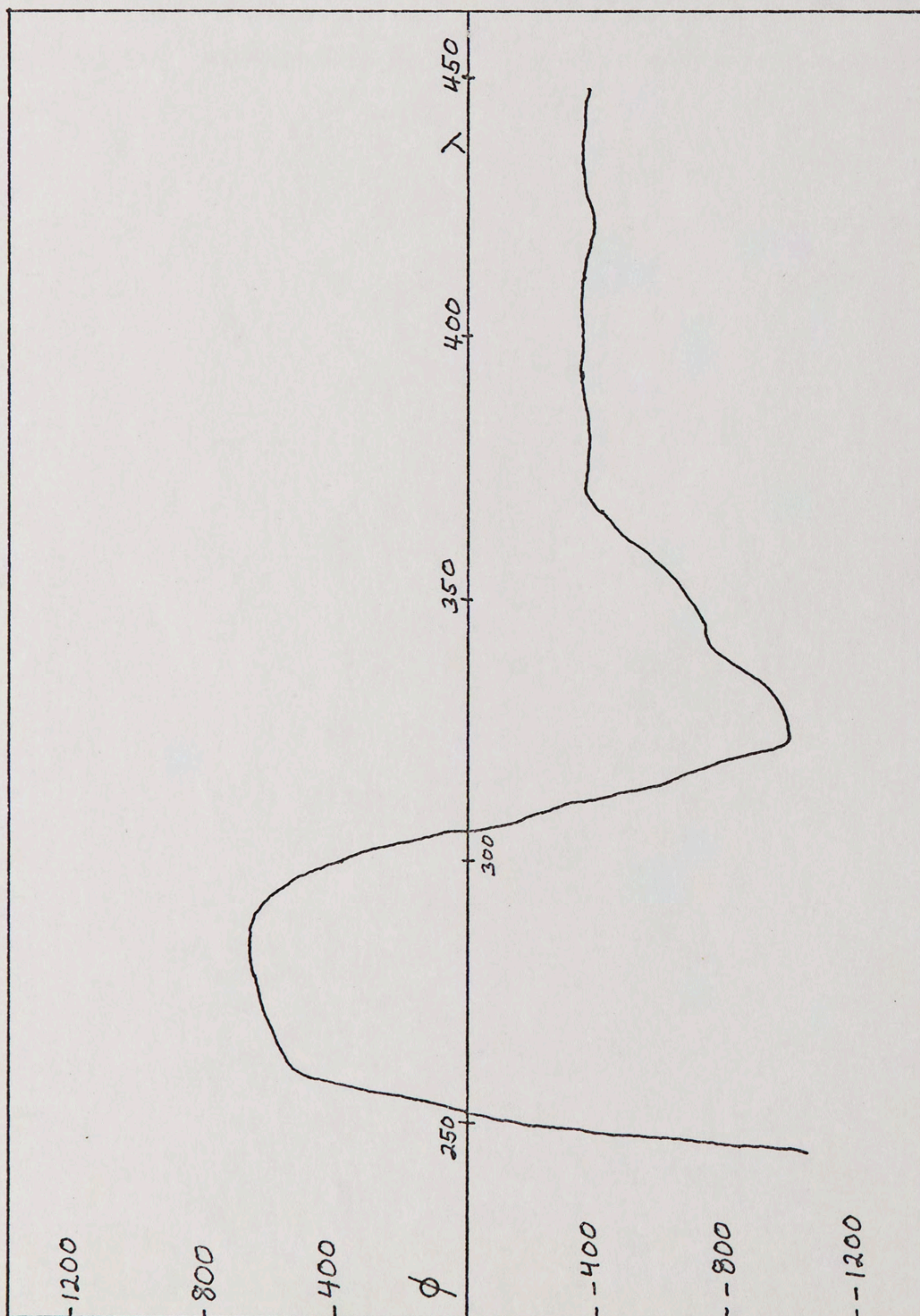
Removal of Benzoyl Groups

The benzoyl groups were removed from the nucleoside in one of two ways. Tribenzoyl nucleoside 3 was treated with methanolic ammonia for 24 hours at room temperature. Alternatively, 3 was treated with three equivalents of sodium methoxide in methanol at reflux for two hours, followed by neutralization with glacial acetic acid. Both methods gave 4-nitro-1-β-D-ribofuranosylbenzimidazole (1) in 90-95% yield after crystallization from water. The nucleoside 1 melted at 193-195° in agreement with Mizuno *et al.* (1962).

Proof of Structure

The main proof of structure consists of the correspondence between the physical properties of 1 and 3 produced by the mercuric cyanide method and those reported by Mizuno and coworkers. As stated above, the melting points of 1 and 3 agree with the published data. The ultraviolet absorption spectrum of 1 has a maximum at

Figure 6. ORD curve for 4-nitrobenzimidazole nucleoside 1



317 nm (ϵ 7700) and a minimum at 258 nm (ϵ 2300) in agreement with Mizuno et al. (1962).

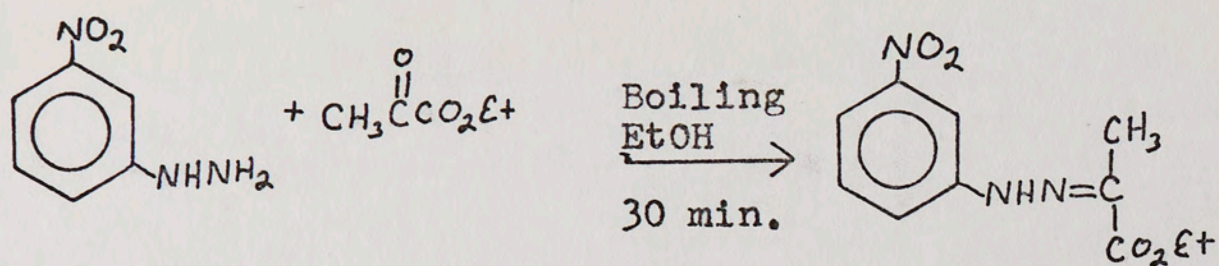
The assignment of the β configuration by Mizuno et al. has been confirmed by the optical rotatory dispersion curve obtained for 1 (figure 6). This features a negative Cotton effect at the long wavelength maximum which is indicative of a β nucleoside (Ulbricht et al., 1964; Rogers and Ulbricht, 1970).

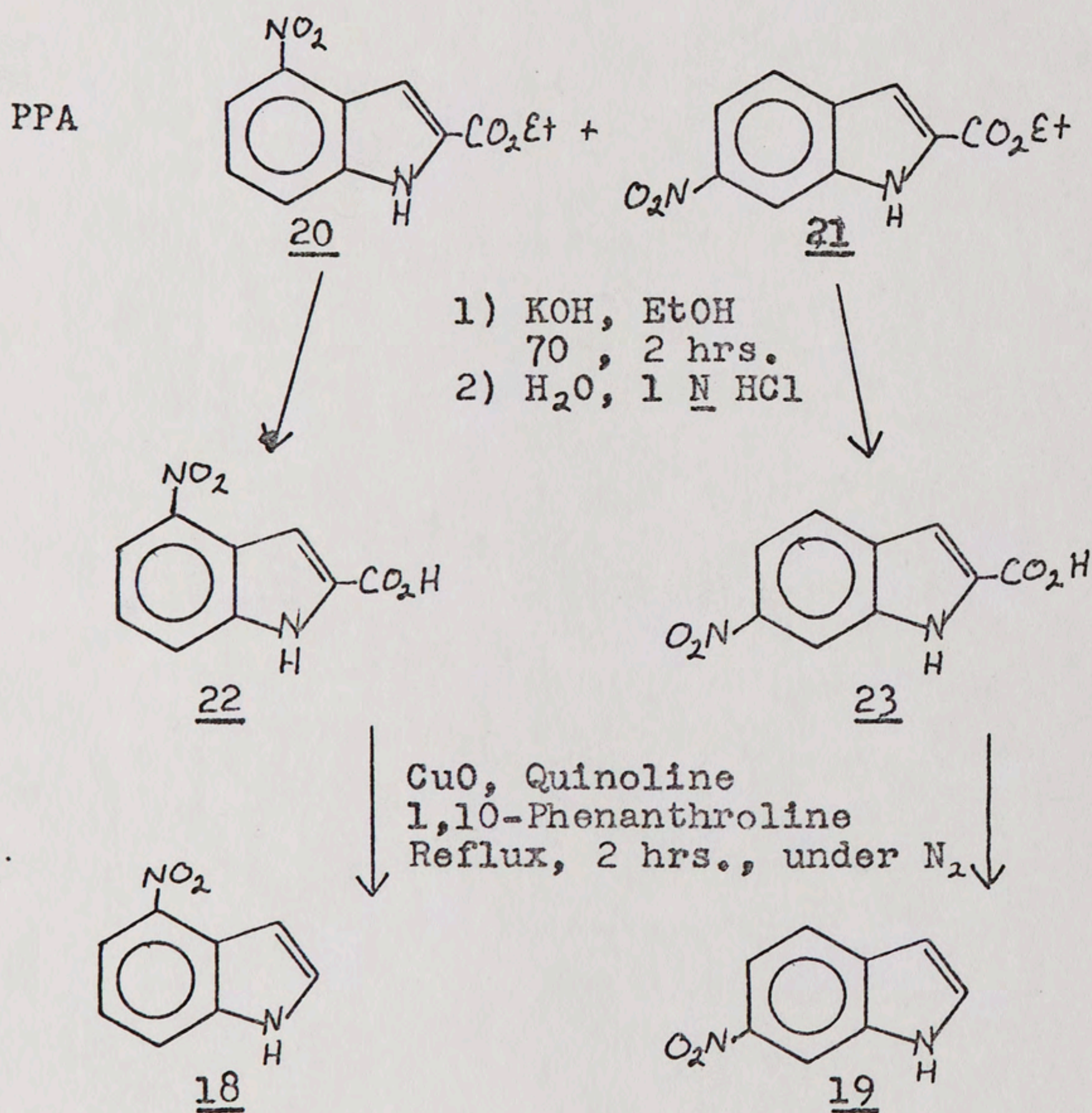
III.1.2. 4-Nitro-1-β-D-ribofuranosylindole (2)

III.1.2.1. 4-Nitroindole (18)

The proposed route for the synthesis of 4-nitro-1-β-D-ribofuranosylindole (2) was the condensation of the sodium salt of 4-nitroindole (18) with 2,3,5-tri-O-benzoylribofuranosyl bromide (17), the rationale being that the nitro group would serve to stabilize the anion used in the condensation.

4-Nitroindole was prepared in 1958 by Parmerter et al. (1958) via the Fischer indole synthesis (figure 7). The cyclization of ethyl pyruvate m-nitrophenylhydrazone in polyphosphoric acid gave two isomeric nitroindole-2-carboxylic acid ethyl esters. Separation of the isomers by fractional crystallization from benzene gave ethyl 4-nitroindole-2-carboxylate (20) in 19% yield and ethyl 6-nitroindole-2-carboxylate (21) in 8% yield. Ester 20 was





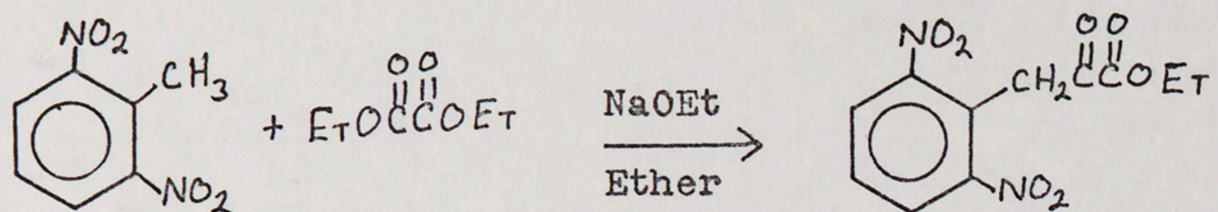
Preparation of nitroindoles 18 and 19

Figure 7.

hydrolyzed and decarboxylated to give 18 with an overall yield of 8.8% from the hydrazone.

Because of the low yield of 18 via the Fischer indole synthesis, an alternative preparation was sought. The first attempt was to prepare 2,6-dinitrophenylpyruvic acid following the procedure used by Uhle (1949) to prepare 2-nitro-6-chlorophenylpyruvic acid. The reaction sequence, dinitrophenylpyruvic acid to aminonitrophenylpyruvic acid to nitroindole carboxylic acid, is shown in figure 8. However, the condensation of 2,6-dinitrotoluene, as the sodium salt, with ethyl oxalate did not occur. In all cases, either 2,6-dinitrotoluene was recovered unchanged or polymeric material was formed. This is most likely the result of steric hindrance by the two nitro groups ortho to the site of reaction. The formation of a planar enolate in conjugation with the aromatic ring is prevented, with the result that a second carbonyl group can react. This leads to polymeric products. When ethyl formate was substituted for ethyl oxalate, the same results were obtained. Walton et al. (1968) reported the same results for the attempted synthesis of 2,6-dinitrophenylpyruvic acid from 2,6-dinitrotoluene and ethyl oxalate.

Reduction of one nitro group with ammonium sulfide gave 2-methyl-3-nitroaniline in good yield. The amino group



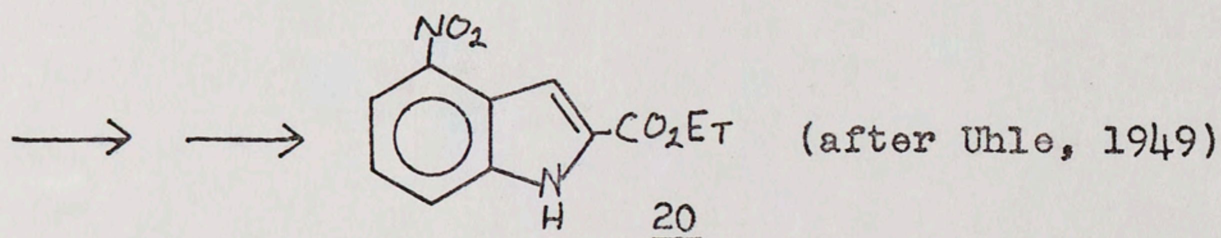


Figure 8.

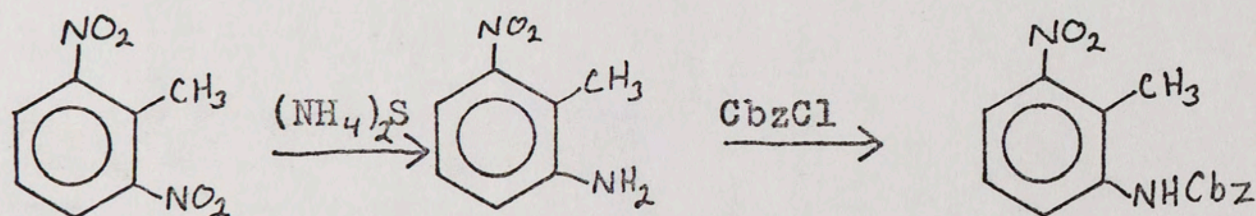


Figure 9.

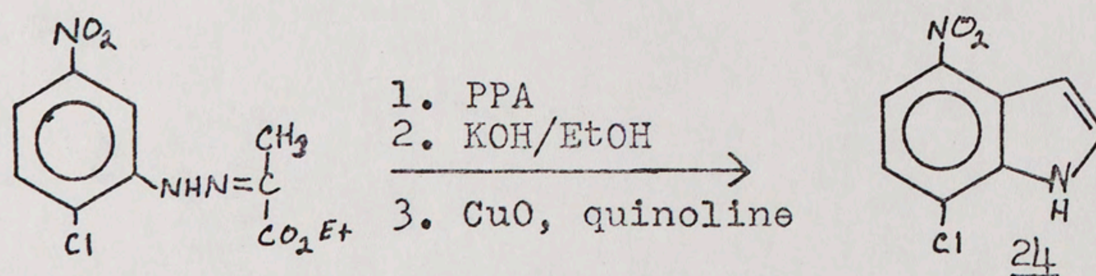


Figure 10.

was blocked with carbobenzyloxy (Cbz) chloride (figure 9). The 2-Cbz-amino-6-nitrotoluene was subjected to the same conditions as 2,6-dinitrotoluene in the hope that the steric effects would be reduced by the presence of the Cbz-amino group instead of the nitro group. Again, the desired reaction did not occur.

An indole compound with the proper nitrogen substitution and a chlorine in the 7 position was reported by Ainsworth and Suschitzky (1967). With the assumption that the chlorine could be removed by hydrogenolysis using palladium on charcoal, it was decided to use 4-nitro-7-chloroindole (24) as the base in the synthesis of the indole nucleoside. Compound 24 was prepared in good yield by the Fischer indolization of ethyl pyruvate 2-chloro-5-nitrophenylhydrazone in polyphosphoric acid, followed by hydrolysis and decarboxylation (figure 10). However, 24 could not be condensed with ribosyl bromide 17 to form the nucleoside (see section III.1.2.2.).

Reexamination of the Parmerter *et al.* (1958) paper showed that the cyclization of ethyl pyruvate *m*-nitrophenylhydrazone had produced some 15 g of mixed indole esters 20 and 21, a 64% yield. Most of this material had been discarded during the fractional crystallization as an inseparable mixture. Since the Fischer indolization appeared to

produce sufficient material, it was decided to investigate the possibility of obtaining a more efficient separation of the isomers 20 and 21.

Ethyl pyruvate m-nitrophenylhydrazone was cyclized to a mixture of 20 and 21 in polyphosphoric acid. The mixture was found to separate into two distinct yellow spots on silica gel thin layers with benzene as solvent. The maximum separation on thin layers was achieved by elution with benzene-acetone (95/5 by volume). Separation of the isomers on a column of silica gel using 5% acetone in benzene as the eluant resulted in an improved resolution of the mixture, although the separation was still incomplete. For a yield of mixed indole esters of 75-80%, roughly half would remain unseparated following column chromatography. The yields of pure materials were: ethyl 6-nitroindole-2-carboxylate (21) (eluted first) 20% of theory and ethyl 4-nitroindole-2-carboxylate 25-30%.

The esters were each hydrolyzed to the acids 22 and 23, and then decarboxylated to the indoles 18 and 19. The decarboxylation was carried out according to the recommendations of Cohen and Schambach (1970). This procedure uses a complexing agent (1,10-phenanthroline) and a nitrogen atmosphere for copper oxide catalyzed decarboxylations in quinoline. Under these conditions, yields of both 4-

nitroindole (18) and 6-nitroindole (19) were 70-80% in comparison to a yield of 51% for the decarboxylation of 4-nitroindole-2-carboxylic acid 22 and a yield of 38% for the 6-nitro isomer 23 (Parmerter et al., 1958).

Greater resolution of the 4- and 6-nitroindole isomers was obtained by delaying the separation until after the decarboxylation step. The mixture of nitroindole esters 20 and 21 was hydrolyzed to the acids 22 and 23. The mixed acids were decarboxylated in the same way as the pure substances. The resulting mixture of 4-nitroindole and 6-nitroindole was separated on a column of silica gel packed in petroleum ether (60-110°) and eluted with ethyl acetate-petroleum ether (1/3 by volume). The 6-nitro isomer eluted first, followed by a narrow overlap (approximately 10% of the total indoles) and 18.

III.1.2.2. 4-Nitro-1-β-D-ribofuranosylindole 2

The alkylation of the sodium salt of an indole usually gives a mixture of N and C-3 alkylated products. With N,N-dimethylformamide as solvent, indole sodium salt alkylates only on nitrogen (Cardillo et al., 1967). A preliminary experiment with benzyl chloride as the alkylating agent and the sodium salt of 4-nitro-7-chloroindole (24) in dimethylformamide produced a single product. This was identified as 1-benzyl-4-nitro-7-chloroindole on the basis of the infrared spectrum (no N-H absorption at 3300 cm^{-1}) and

the ultraviolet spectrum (no shift of the long wavelength peak to the visible region, ~500nm, on addition of strong base (Berti et al., 1960)). On this basis, the alkylation of the sodium salt of 24 with ribosyl bromide 17 was attempted. Under the conditions used for the successful alkylation with benzyl chloride, the sodium salt of 24 and 17 produced no identifiable products, and no starting materials could be recovered.

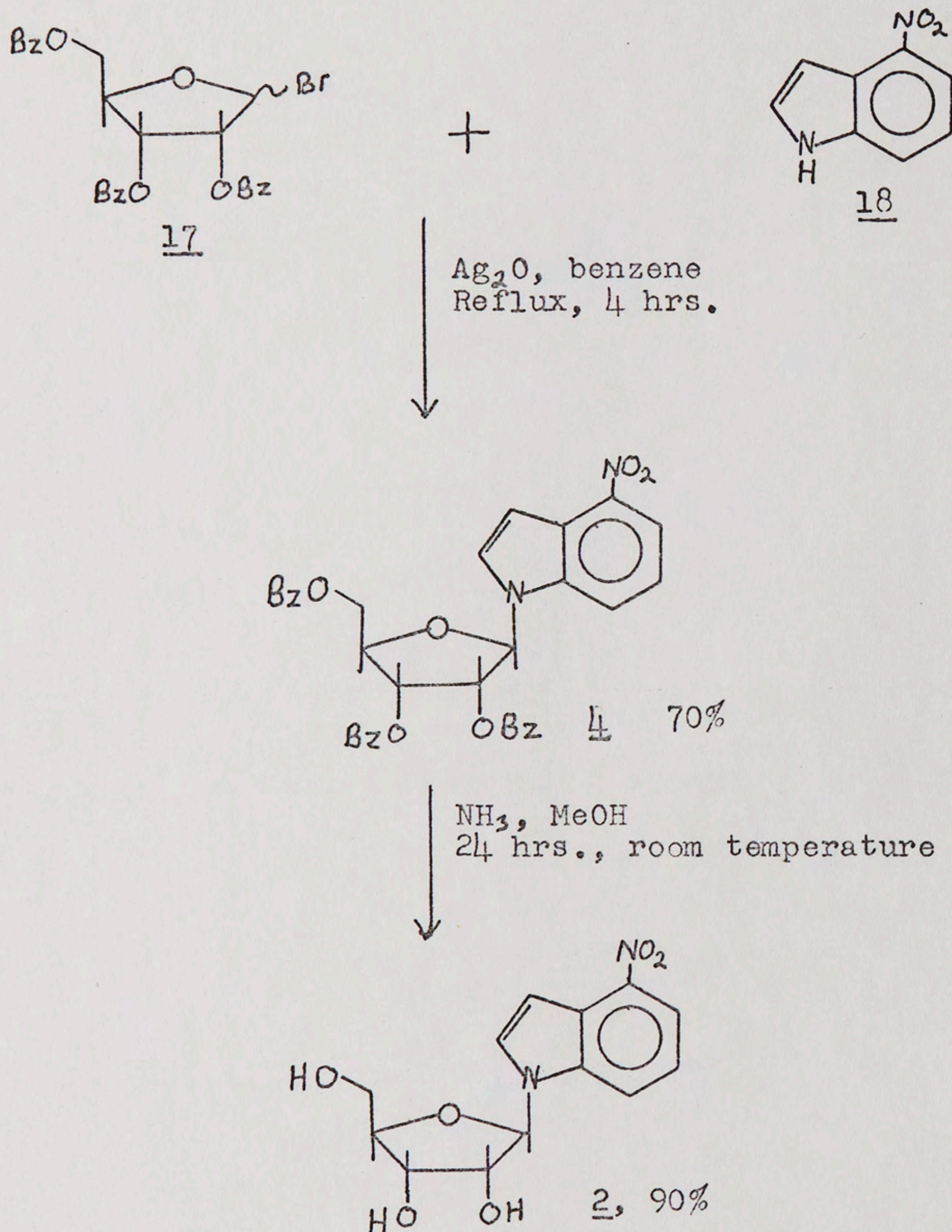
The failure of 4-nitro-7-chloroindole (24), sodium salt, to react with 2,3,5-tri-O-benzoylribofuranosyl bromide (17) in the desired manner has two possible causes. Either the chlorine in the 7 position hinders the approach of the bulky ribose or the materials are not stable under the reaction conditions. Since 4-nitroindole (18) also failed to react under these conditions as well as indole (Walton et al., 1968), it must be concluded that the reaction is not feasible because the reactants are not stable to the conditions.

Other possible condensation methods were investigated for use with 4-nitroindole. The chloromercuri-salt method Davoll and Lowy, 1951), The mercuric cyanide-nitromethane procedure (Helferich and Weis, 1956), the cadmium carbonate catalyzed procedure (Conrow and Bernstein, 1971) and the silver salt procedure (Davoll et al., 1948) produced

only traces of nucleoside product.

Since the condensation methods investigated were all basically variations of the Koenigs-Knorr glycoside synthesis (Koenigs and Knorr, 1901), the original conditions of silver oxide in benzene were investigated. When 18 was refluxed with silver oxide and ribosyl bromide 17 in benzene, the desired condensation occurred. The reaction required anhydrous conditions (crushed molecular sieves in the flask and azeotropic drying of the silver oxide and nitroindole by distillation of solvent), a nitrogen atmosphere, and slow addition of 17 in a volume of dry benzene equal to the volume in the flask, to produce good yields of 4-nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)indole (4). Nucleoside 4 was isolated by chromatography on silica gel in benzene along with unreacted 18.

The nucleoside 4 from the initial reactions was contaminated by an impurity which produced ribose when the benzoyl groups were removed. The mixture was analyzed by high pressure liquid chromatography. Comparison of the chromatogram of the mixture with those of the starting materials showed that the impurity was part of the 2,3,5-tri-O-benzoyl-1-O-acetylribofuranose 16 used to prepare ribosyl bromide 17. Recrystallization of 16 from 2-pro-



Synthesis of Nitroindole Nucleoside 2

Figure 11.

panol followed by washing the crystals with cold 2-pro panol and ether to remove the impurity.

4-Nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-indole (4), prepared using the purified 16, was isolated by column chromatography as a foamed yellow glass in 70% yield. Analysis of the glass in the liquid chromatography system used above gave a chromatogram with a single symmetrical peak, indicating a pure compound.

Removal of the Benzoyl Groups

The benzoyl groups of 4 were removed by treatment with methanolic ammonia at room temperature. When the resulting oil was mixed with ether to remove methyl benzoate, benzoic acid and benzamide, it was discovered that nucleoside 2 was soluble in ether. As a result, methyl benzoate had to be removed by shaking the oil with petroleum ether. 4-Nitro-1-β-D-ribofuranosylindole 2 was obtained as bright yellow blades from ether-methylene chloride-petroleum ether. The crystals melted at 164-165°.

Proof of Structure

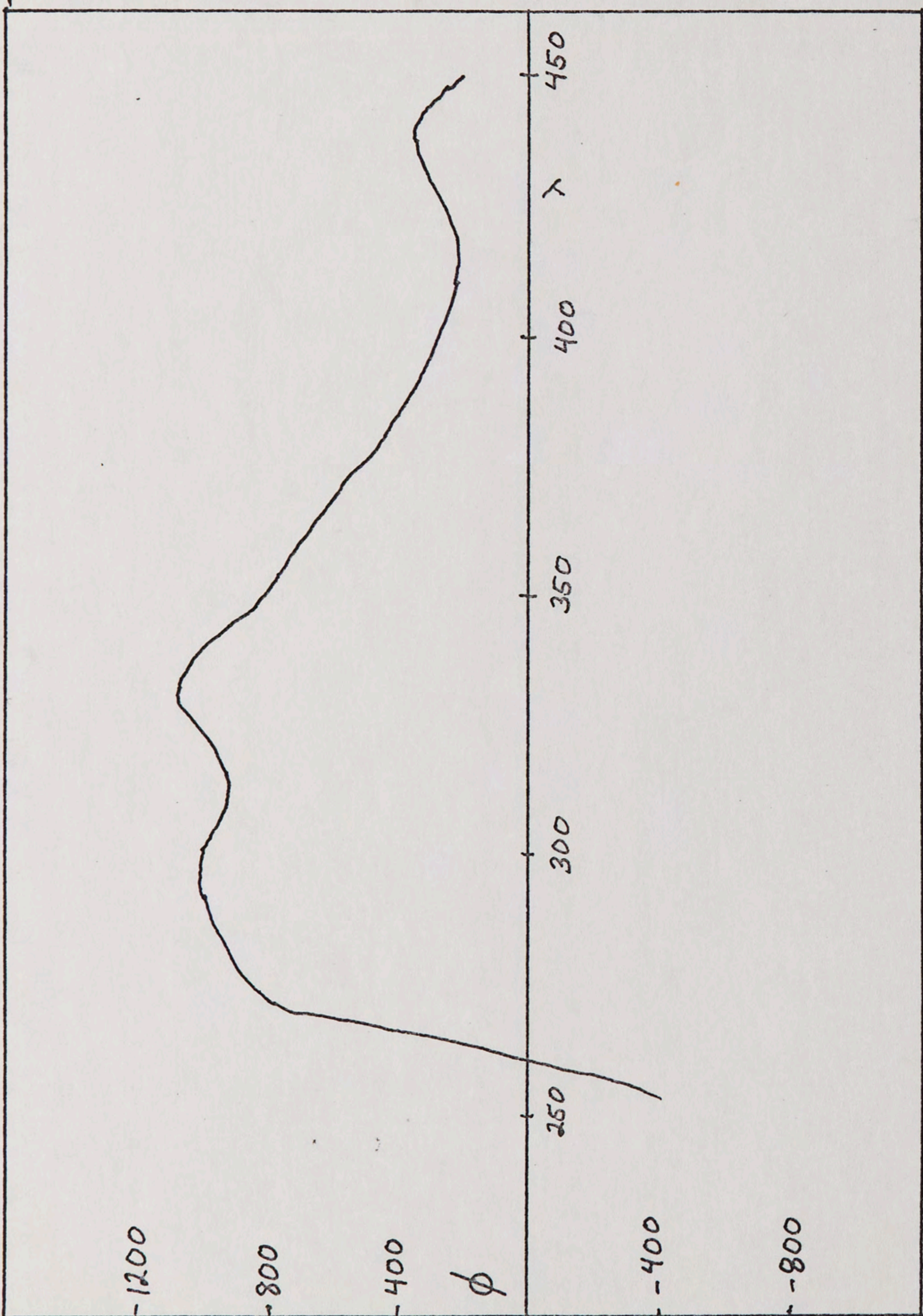
The site of ribosylation is assigned as N-1 on the basis of the infrared, ultraviolet and nuclear magnetic resonance spectra. The infrared spectra of 2 and 4 have no absorbance at 3300 cm^{-1} characteristic of N-H stretching vibrations in the parent compound 18. The ultraviolet

absorbance spectrum of 2 is similar to that of 18 in the position of the maximum (380 nm for 18, 370 nm for 2). This is in agreement with the spectral evidence for 1-benzyl-4-nitro-7-chloroindole and 1-benzyl-4-nitroindole.

The nuclear magnetic resonance spectra showed identical multiplets in the region δ 8.2-7.8 ppm for 2, 4, and 18 as well as 1-benzyl-4-nitroindole indicating that the C-3 proton of the indole is present in all three substituted indole compounds.

The configuration at C-1 of the ribose moiety is designated β for several reasons. Condensations of acyl substituted glycosyl halides and bases catalyzed by heavy metal salts, such as mercuric ion or silver ion, form products with the substiuents at C-1 and C-2 trans to each other. This is the "trans rule" (Howard, 1950; Baker et al., 1954) which is derived from the effect of participation of the acyl group on the C-2 hydroxyl. Secondly, the optical rotatory dispersion curve (figure 12) for 2 shows a broad trend toward more positive values of molecular rotation before decreasing rapidly below 270 nm. This is interpreted as a negative Cotton effect distorted by several unresolved peaks and troughs and, as such, is indicative of the β configuration for the nucleoside. The ORD curve for 2 is somewhat similar in overall shape to the ORD curve for 1

Figure 12. ORD curve of nitroindole nucleoside 2



(figure 6) which is known to have the β configuration.

The NMR coupling constant for H-1'-H-2', 4.5 Hz, provides no additional information for assigning the configuration at C-1'. When the coupling constant is small enough, (less than 2-3 Hz), the dihedral angle between the protons can be determined (Karplus, 1959; Karplus, 1963; Lemieux and Lown, 1963) which limits the possible configurations. In this case, that is not possible.

Elemental analysis of 4-nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)indole 4 was consistant with the formula $C_{34}H_{26}N_2O_9$. The solubility of 4-nitro-1-β-D-ribofuranosylindole 2 in organic solvents caused great difficulty in purification with the result that no satisfactory elemental analysis could be obtained. The byproducts of the deblocking reaction, benzoic acid and benzamide, are normally removed by triturating the residue with ether. These are carried along with nucleoside 2 in the crystallization from ether-petroleum ether. Two separate elemental analyses gave results consistant with contamination by benzoic acid and/or benzamide.

Mass spectra of nucleosides 2 and 4 did not show an identifiable molecular ion. The molecular ion may be present in the spectrum of 4 but, in the absence of a mass marker, the exact value of m/e could not be determined.

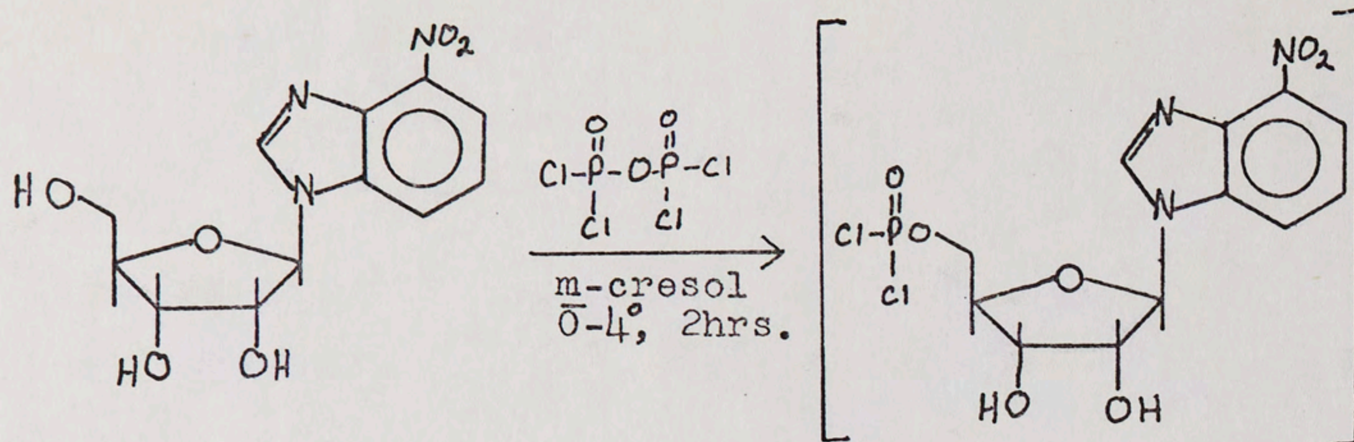
The spectrum of 2 does not show the molecular ion at ionization energies of 70 ev, 20 ev, or 10 ev. This is not unusual because the molecular ions of nucleosides are known to be of low abundance or entirely absent (Bieman and McCloskey, 1962; Brown *et al.*,1969).

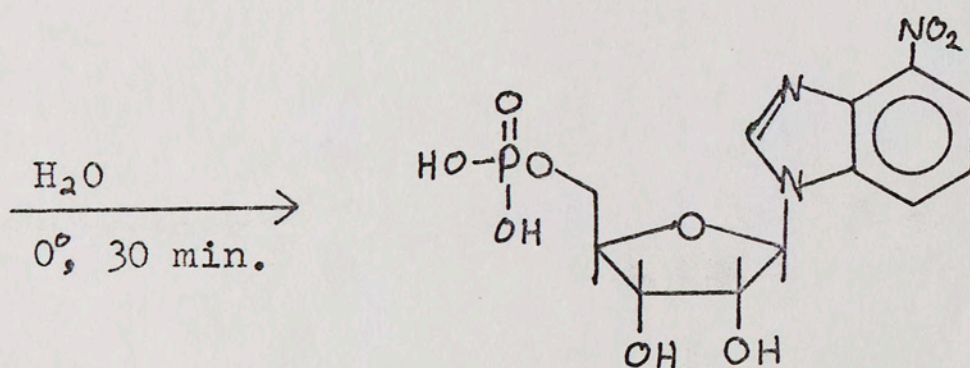
The spectrum of 2 shows prominent peaks at m/e 162 and 163 representing B+H and B+2H which are characteristic of nucleosides (Bieman and McCloskey, 1962). These peaks arise from the transfer of one and two protons respectively from the sugar hydroxyls to the base. Ribose was not detected because the peak at m/e 133 was masked by the ion derived from the B+2H peak at m/e 163 by loss of NO.

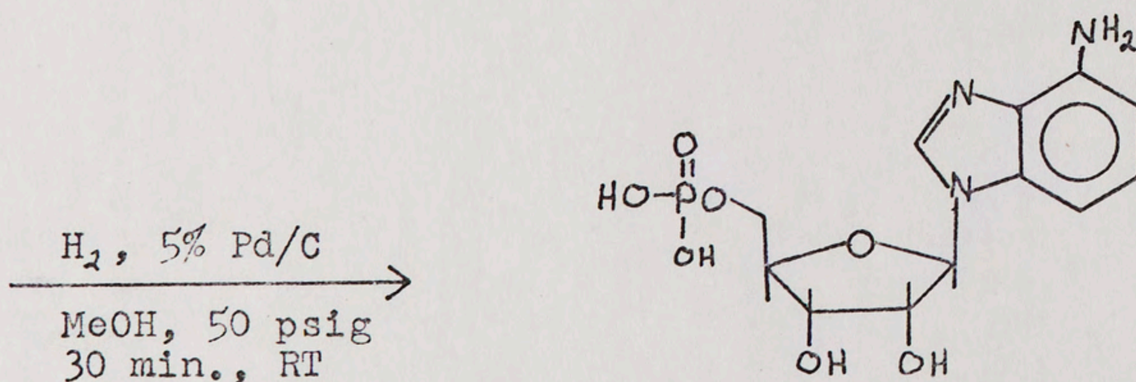
III.2. Nucleotides

III.2.1. Phosphorylation of Nucleosides

Phosphorylation methods have been developed for use with protected and unprotected nucleosides. Protected nucleosides have the 2' and 3' hydroxyl groups blocked with an easily cleaved group (or groups) such as isopropylidene (Michelson, 1963; Zorbach and Tipson, ed., 1968), ethoxyformylidene (Leonard and Laursen, 1965a), diacetate (Ikehara *et al.*, 1964b), and borate (Ikehara *et al.*, 1964a). In this way, reaction with the phosphorylating agent is limited to the 5' hydroxyl. Unprotected nucleosides have been selectively phosphorylated using pyrophosphoryl







Phosphorylation and reduction of nucleoside 1

Figure 13.

chloride in _m_-cresol or acetonitrile (Imai _et al._, 1969) or phosphorus oxychloride in trimethyl phosphate (Yoshikawa _et al._, 1967; Yoshikawa _et al._, 1969). The reaction conditions in these cases have been carefully selected to enhance the difference in reactivity between primary and secondary hydroxyl groups.

III.2.1.1. 4-Amino-1-β-D-ribofuranosylbenzimidazole-5'-phosphate (5)

The original proposal called for the phosphorylation of 4-nitro-1-β-D-ribofuranosylbenzimidazole as the isopropylidene derivative. When the isopropylidene derivative of 1 proved difficult to prepare in good yield (50% at best), the direct phosphorylation of 1 was investigated. Nucleoside 1 was reacted with pyrophosphoryl chloride in _m_-cresol at 0-4° according to the method of Imai _et al._ (1969). The product was freed of salts (mostly phosphate) and separated from unreacted 1 by chromatography on charcoal. Elution with ethanol-ammonia-water gave 4-nitro-1-β-D-ribofuranosylbenzimidazole-5' phosphate as the ammonium salt in 76% yield.

The nitro group was converted to the amine by catalytic hydrogenation in the manner reported for 1 (Jenkins _et al._, 1968). The attempted purification of the aminonucleotide 5 on Dowex-1-X8 (anion exchange resin, in the

formate form) with ammonium formate-formic acid buffer at pH 3.75 failed when 5 crystallized, completely blocking the column. Thereafter, 4-amino-1-β-D-ribofuranosylbenzimidazole-5' phosphate (5) was isolated without chromatography. The crude nucleotide from the phosphorylation reaction was reduced by catalytic hydrogenation (5% palladium on charcoal). The amino-nucleotide 5 was dissolved in a small amount of distilled water and precipitated by adjusting the pH to 3.75 with ammonium formate. 4-Amino-1-β-D-ribofuranosylbenzimidazole-5' phosphate was isolated as a pinkish powder, after drying *in vacuo* for two days, in 60% overall yield.

Proof of Structure

Nucleotide 5 had a UV maximum (pH 7) at 265 nm (ε 7700) and a shoulder at 290 nm in agreement with the values for 4-amino-1-β-D-ribofuranosylbenzimidazole (Jenkins *et al.*, 1968).

The location of the phosphate moiety was determined by incubation of nucleotide 5 with *Crotalus adamanteus* venom. One component of the venom is a 5'-nucleotidase Richards *et al.*, 1965; Hurst and Butler, 1951). After two hours at room temperature, 5 was completely cleaved to inorganic phosphate and 4-amino-1-β-D-ribofuranosylbenzimidazole (identified by TLC in comparison with an auth-

entic sample) which confirms that the phosphate is on the 5'-hydroxyl of the ribose.

III.2.1.2. 4-Nitro-1-β-D-ribofuranosylindole-5' phosphate (6)

The phosphorylation of the indole nucleoside 2 presented a different problem from the benzimidazole compound 1. Nucleoside 2 is unstable to strong acid. As a result, it could not be phosphorylated without prior protection as was 1 since the workup of the direct method includes stirring in 0.5 M acid as the pyrophosphoryl chloride is decomposed.

The choice of protecting group was also concerned with the instability to acid shown by 2. The isopropylidene group was deemed unsatisfactory since it requires heating to 100° in 70% acetic acid for cleavage. The ethoxyformylidene group, which can be cleaved in 80% acetic acid at room temperature (Leonard and Laursen, 1965a), was selected as the blocking group.

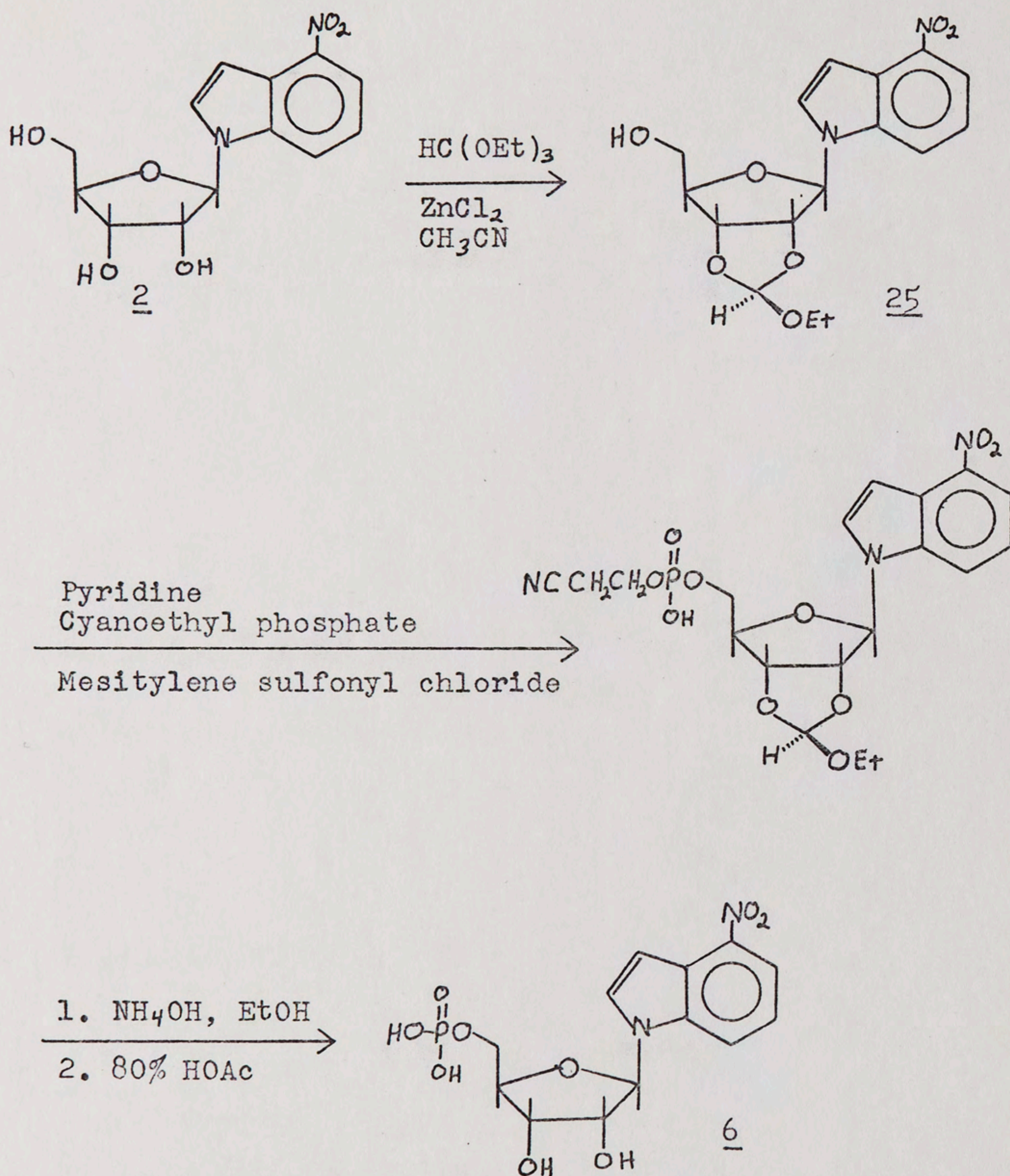
Reaction of 4-nitroindole nucleoside 2 with ethyl orthoformate and bis(p-nitrophenyl)phosphate in dry acetonitrile (after Leonard and Laursen, 1965a) led to the discovery that 2 was not stable to the acid catalyst used. Anhydrous zinc chloride (Smith et al., 1962) was found to be sufficiently acidic to catalyze the formation of the ethoxyformylidene derivative of 2 without causing cleavage

of the indole-ribose bond so long as anhydrous conditions were maintained.

Attempts to purify the ethoxyformylidene derivative 25 by column chromatography on silica gel were unsuccessful. Although two clearly separated yellow bands could be seen on the column, TLC analysis of the effluent showed that the leading band always contained some 2 as well as the desired 25 while the second band contained only 2. When the NMR spectrum of the crude product confirmed that 25 was indeed formed in the reaction, it was decided to use the protected nucleoside in the phosphorylation reaction without purification (figure 14. Only one of two possible isomers of compound 25 is shown.).

Phosphorylation of 25 was accomplished with cyanoethyl phosphate (Tener, 1961) using dicyclohexylcarbodiimide (Tener, 1961) or mesitylene sulfonyl chloride (Narang et al., 1968) as the dehydrating agent. Mesitylene sulfonyl chloride was the preferred reagent in terms of time (16 hours rather than 3 days in the case of DCC) and ease of workup (no dicyclohexylurea). The cyanoethyl group was removed by 7 M ammonia in 50% ethanol at 60° and the ethoxyformylidene group was removed in 80% acetic acid at room temperature.

Purification of 4-nitro-1-β-D-ribofuranosylindole-5'



Phosphorylation of nucleoside 2

Figure 14.

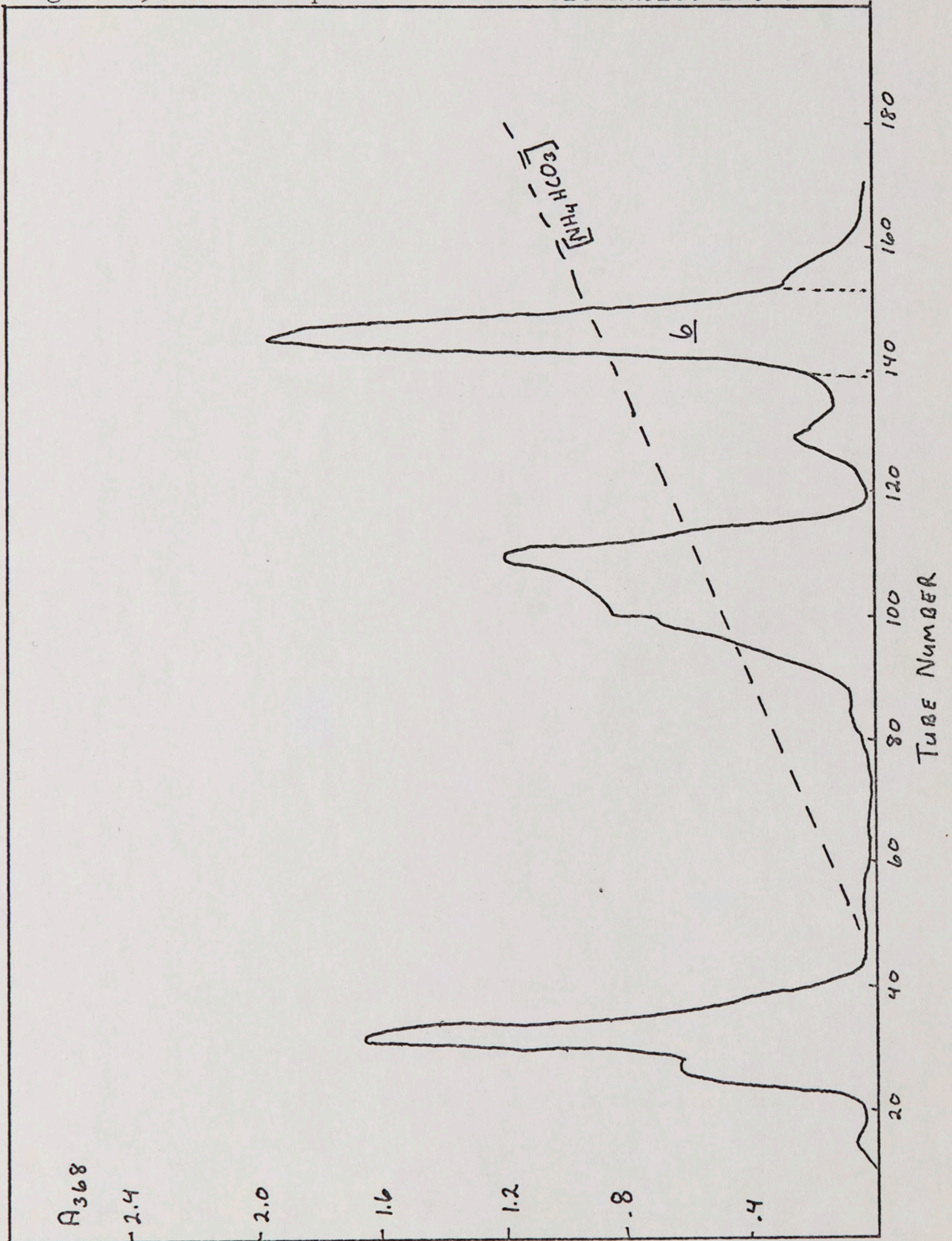
phosphate by chromatography on Dowex-1-X8 (formate) resin failed completely when the nucleotide could not be eluted from the resin. Even drastic conditions of 2 M acid or base failed to elute 6 from the resin. Partial purification was achieved by gel filtration using Biogel P-2 (R) which included removal of the polyacrylonitrile formed in the removal of the cyanoethyl group. That 6 could be eluted from Biogel P-2 (R) indicated that the nucleotide might be purified on an anion exchange resin that did not have a polystyrene backbone. Diethylaminoethylcellulose was chosen because it had been used previously to separate nucleotides (Tener et al., 1958) and does not have the aromatic properties of the Dowex resins.

Chromatography on DEAE-cellulose in an ammonium bicarbonate gradient easily separated the 4-nitroindole nucleotide 6 from various impurities which included partially deblocked nucleotide (figure 15). Ammonium bicarbonate was removed from the solid remaining after the fractions containing 6 were pooled and evaporated by sublimation at reduced pressure in a heated desiccator. Yields, estimated from optical density measurements at 368 nm, using an ϵ of 4700, were 18-25% for the four reactions.

Proof of Structure

The UV spectrum of 6 is the same as that of 2.

Figure 15. Elution profile for indole nucleotide 6



Incubation of 4-nitro-1-β-D-ribofuranosylindole-5' phosphate with lyophilized *Crotalus adamanteus* venom in pH 8 Tris buffer gave no indication of cleavage after 24 hours at room temperature. In order to determine if the lack of reaction was the result of inhibition of the 5'-nucleotidase, a sample of indole nucleotide 6 was added to a mixture of adenosine-5' monophosphate and venom which had been incubated for 15 minutes at room temperature. The course of the reaction was followed by thin layer chromatography over a period of two hours. While AMP is completely cleaved to adenosine and inorganic phosphate in this time, the mixture of AMP, venom and 6 contained a substantial amount of AMP at the end of two hours (approximately half the initial amount of AMP).

Björk (1964) has shown that venom 5'-nucleotidase requires a nitrogenous base and ribose (as opposed to ribitol) for hydrolysis of the phosphate ester bond on the 5' carbon. Ribose-5' phosphate and nucleoside-2'(3') phosphates do not interfere with the process. Therefore, the inhibiting species must be a nucleoside-5' phosphate.

The actual species inhibiting the 5'-nucleotidase has not been determined. The very hydrophobic nucleoside 2 may well be the actual inhibitor because the enzyme-product complex would not decompose. Attempts to determine

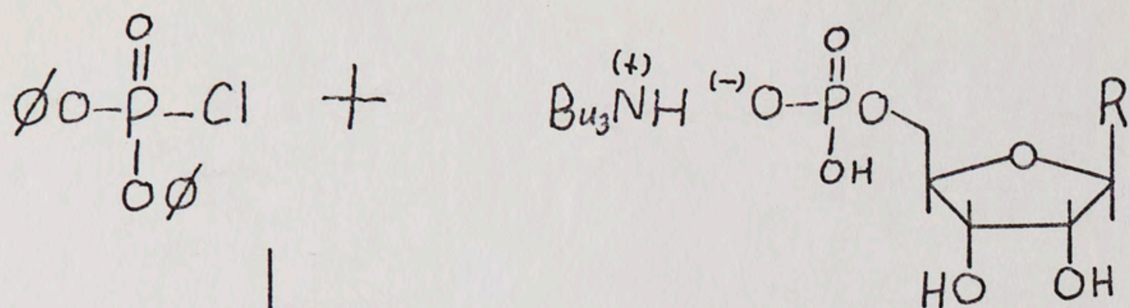
if inorganic phosphate was released during the incubation of venom and 6 or the 4-aminoindole nucleotide gave no clear results, possibly because the amount of phosphate in the reaction mixture was below the 0.1 micromoles needed to produce a visible color with the Fiske-Subbarow reagent (Gunsalus, 1959).

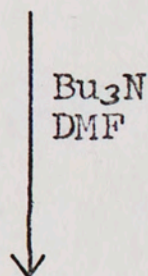
III.2.2. Nucleoside di- and triphosphates

The method chosen for elaboration of the nucleoside-5' monophosphates 5 and 6 was the anion exchange method (Figure 16)(Michelson, 1958; Leonard and Laursen, 1965b). Reaction of the nucleotide with diphenylphosphoryl chloride gives a P^1-diphenyl-P^2-(nucleoside)pyrophosphate. The diphenyl phosphate is a good leaving group in a displacement reaction with phosphate or pyrophosphate.

III.2.2.1. 4-Amino-1-β-D-ribofuranosylbenzimidazole-5' diphosphate 7

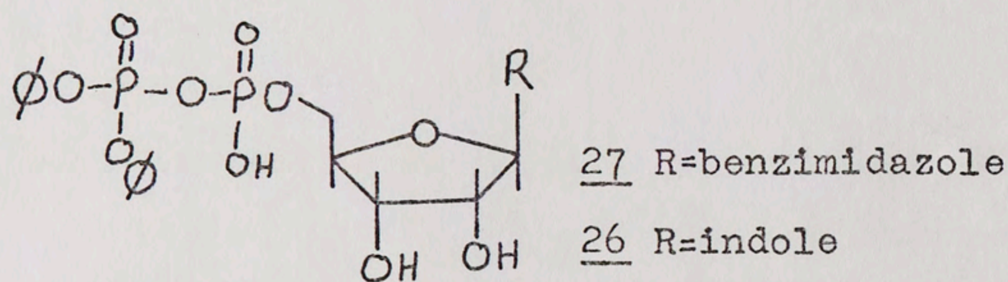
Aminobenzimidazole nucleotide 5 was converted to the tributylammonium salt to increase its solubility in organic solvents. The salt was mixed with diphenylphosphoryl chloride and tributylamine in dimethylformamide and allowed to react at room temperature. The product, P^1-diphenyl-P^2-(4-aminobenzimidazole-1-ribofuranosyl)pyrophosphate, 27, was not isolated but carried immediately into the exchange reaction.





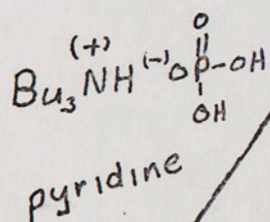
5 R=benzimidazole

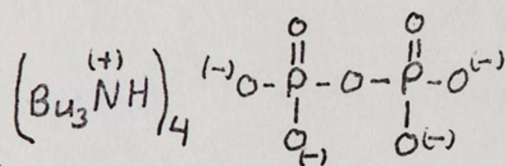
6 R=indole



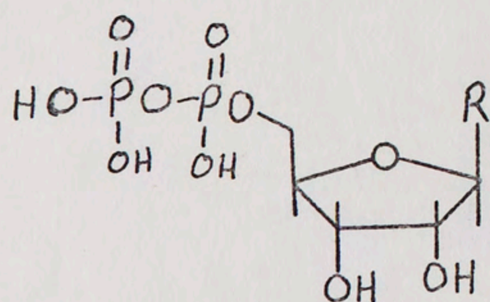
27 R=benzimidazole

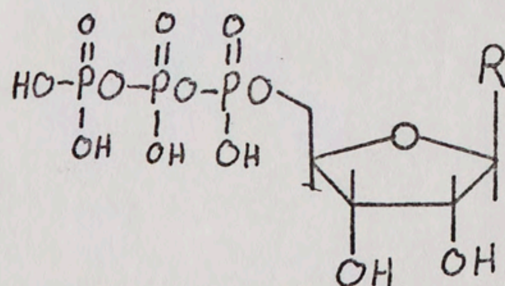
26 R=indole





DMF





7 R=benzimidazole

8 R=indole

9 R=benzimidazole

10 R=indole

Figure 16. Anion exchange method

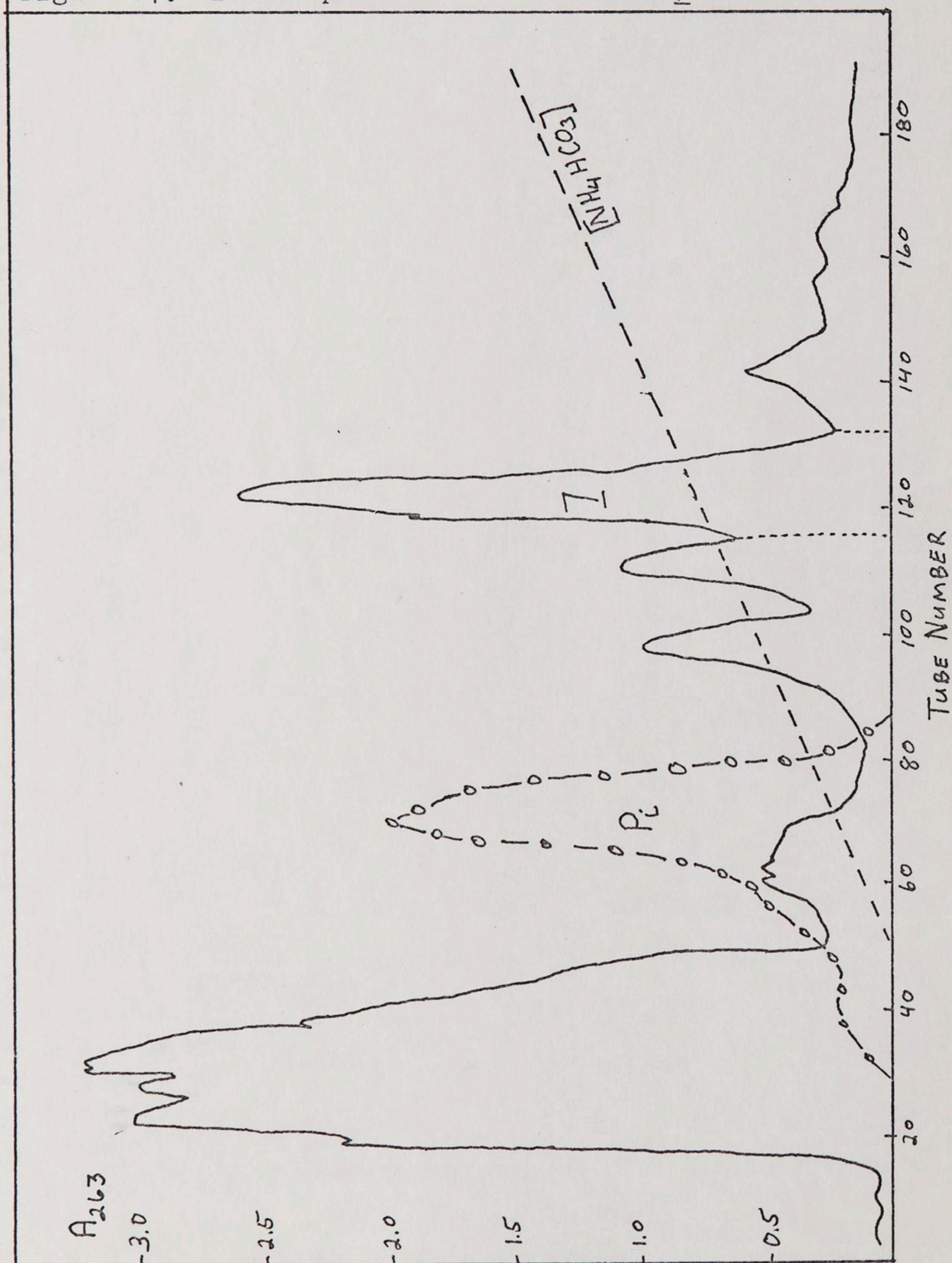
Pyrophosphate 27 was mixed with tributylammonium phosphate in pyridine and kept at room temperature. The product was isolated by chromatography on DEAE-cellulose in ammonium bicarbonate. Increasing concentration of ammonium bicarbonate (a linear gradient) provided a smooth elution of the nucleotide material. Diphosphate 7 eluted in tubes 116-132, well separated from inorganic phosphate which was located in tubes 60-80 (Figure 17). Benzimidazole- containing fractions were located by measuring the optical density at 263 nm. 4-Amino-1-β-D-ribofuranosylbenzimidazole-5' diphosphate 7 was isolated as the ammonium salt in 56% yield by evaporation and sublimation of the residual ammonium bicarbonate.

Diphosphate 7 had a UV maximum at 274 nm, a shift of 11 nm from the maximum for the monophosphate 5. The compound showed a single spot on polyethyleneimine-cellulose (PEI-cellulose) with an Rf of 0.43.

III.2.2.2. 4-Amino-1-β-D-ribofuranosylbenzimidazole-5' triphosphate 9

The triphosphate 9 was prepared by reaction of pyrophosphate 27 with tetrakis(tributylammonium)pyrophosphate in dimethylformamide. The reaction was performed in dimethylformamide to avoid loss of product. Adenosine triphosphate has been reported to undergo a dismutation reaction

Figure 17. Elution profile of nucleotide 7



in pyridine, yielding mono-, di-, tetra- and higher phosphates (Wehrli et al., 1964). As a result, reaction time was increased to two days.

The desired triphosphate 9 was separated from other nucleotides by chromatography on DEAE-cellulose in an ammonium bicarbonate gradient (Figure 18). The yield of 4-amino-1-β-D-ribofuranosylbenzimidazole-5' triphosphate was estimated from optical density measurements at 263 nm as 38% of theory.

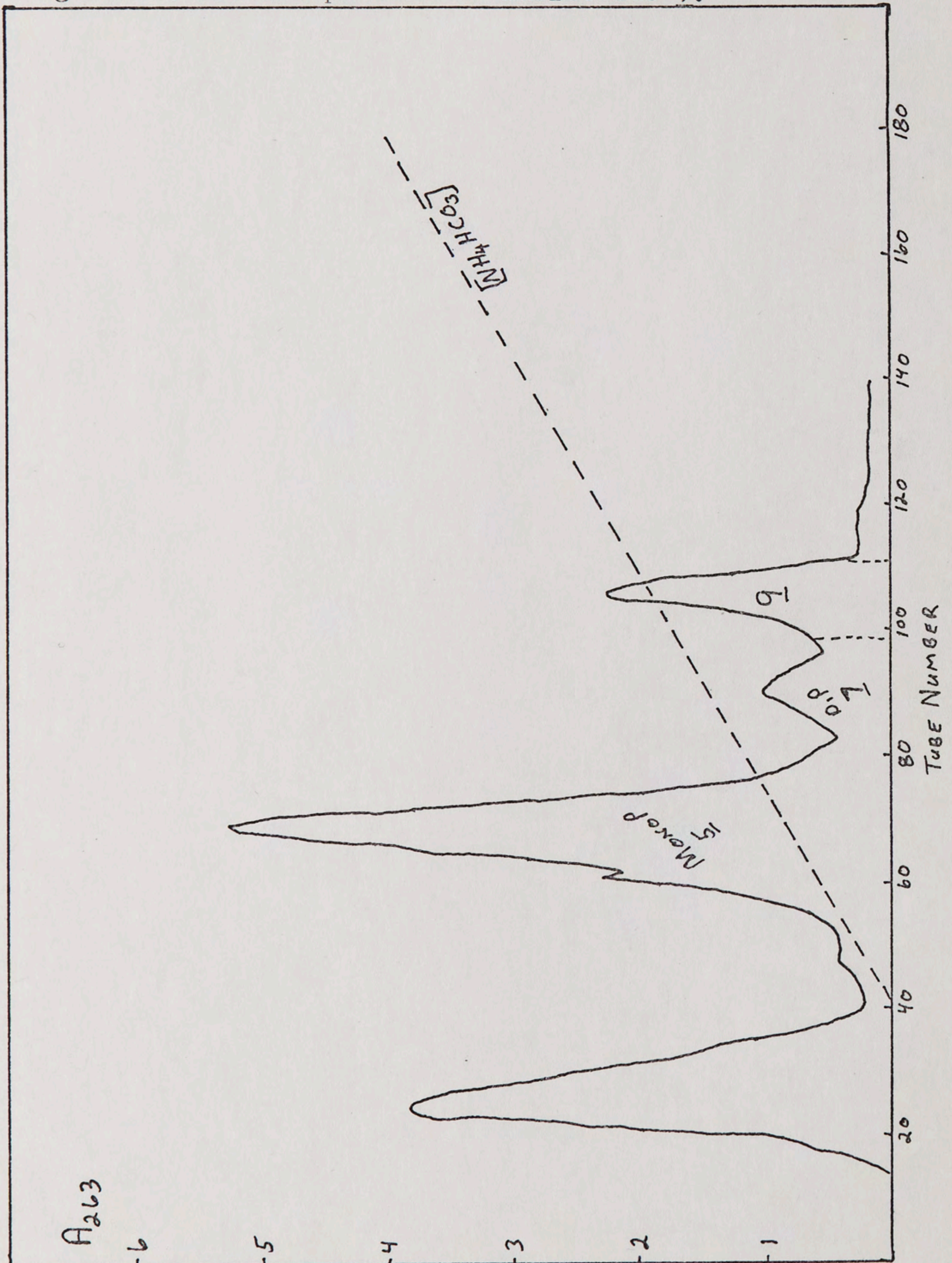
After evaporation and sublimation of ammonium bicarbonate, nucleoside-5' triphosphate 9 was isolated as a tan powder. The compound had a UV maximum at 274 nm and gave a single spot on PEI-cellulose, Rf 0.21.

III.2.2.3. 4-Nitro-1-β-D-ribofuranosylindole-5' diphosphate 8

The synthesis of the di- and triphosphates of the indole nucleoside 2 followed the same procedure as used for the benzimidazole nucleotides 7 and 9, with one exception. P^1-Diphenyl-P^2-(nitroindole-1-ribofuranosyl)pyrophosphate 26 had a moderate solubility in ether with the result that excess diphenylphosphoryl chloride had to be removed by shaking with ether-petroleum ether.

Nitroindole nucleoside-5' diphosphate 8, prepared by reaction of pyrophosphate 26 and tributylammonium phosphate

Figure 18. Elution profile of nucleotide 9.



in pyridine, was separated from unreacted monophosphate 6 by chromatography on DEAE-cellulose in ammonium bicarbonate (Figure 19). The second peak which contained the desired product was evaporated to dryness and residual ammonium bicarbonate removed as before. The yield of 8 was estimated from optical density measurements at 368 nm to be 38%.

4-Nitro-1-β-D-ribofuranosylindole-5' diphosphate 8 had a UV spectrum identical to monophosphate 6.

III.2.2.4. 4-Nitro-1-β-D-ribofuranosylindole-5' triphosphate 10

Diphenylpyrophosphate 26 was reacted with tetrakis-(tributylammonium)pyrophosphate in dimethylformamide as before. Chromatography on DEAE-cellulose (Figure 20) failed to separate triphosphate 10 from other nucleotides because the gradient chosen was too steep. The overlapping peaks, tubes 92-126, were pooled and stripped of solvent and ammonium bicarbonate. The mixture was redissolved in 0.1 M ammonium bicarbonate and rechromatographed on DEAE-cellulose using a shallower gradient (Figure 21). Optical density measurements at 368 nm showed the loss of at least 80% of the indole absorbance.

The loss of indole from the mixture was not the result of mechanical losses such as a spilling of the pooled fractions or bumping during evaporation. Nor was there any in-

Figure 19. Elution profile of indole nucleotide 8

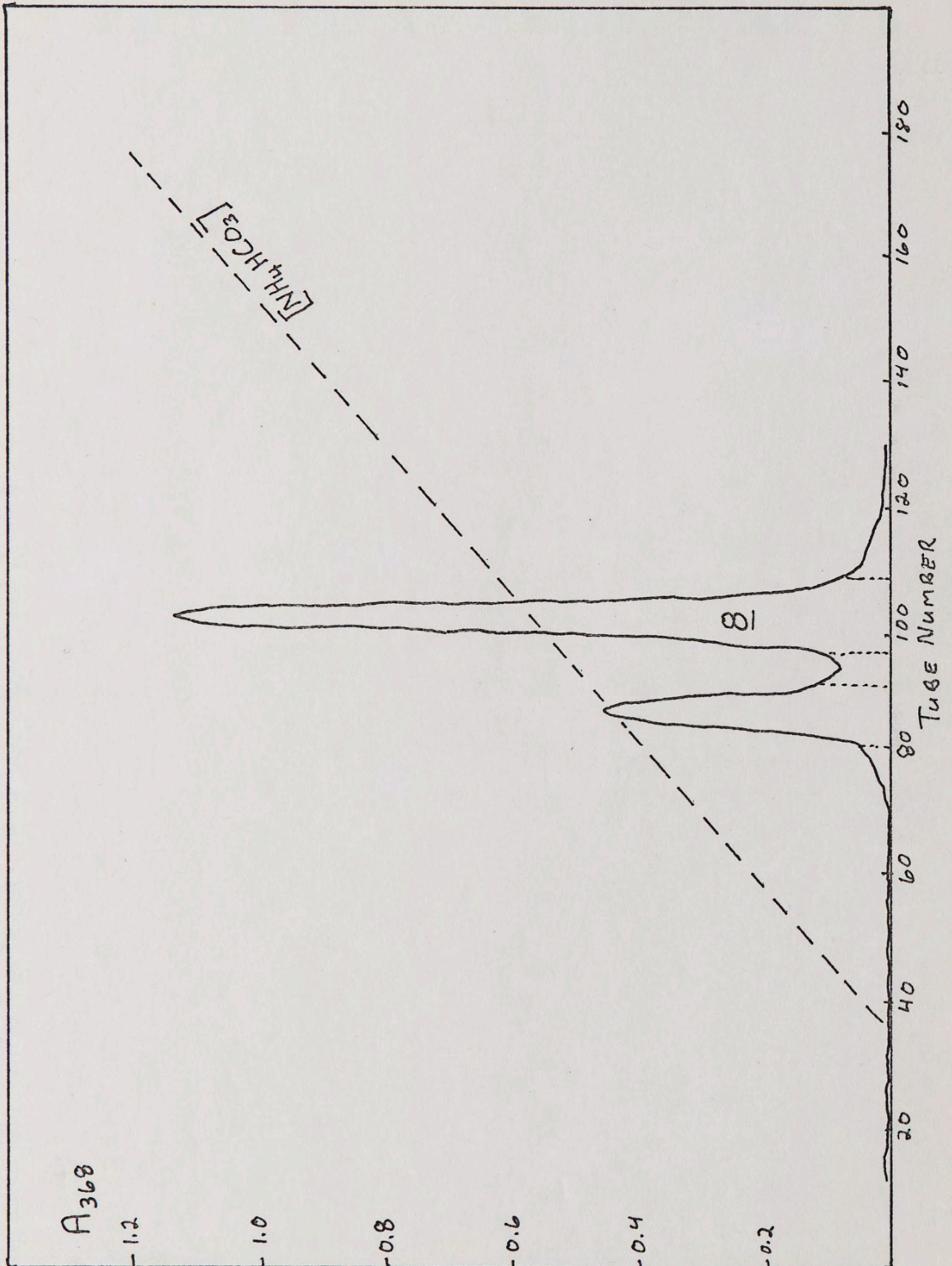


Figure 20. Elution profile I for indole nucleotide 10

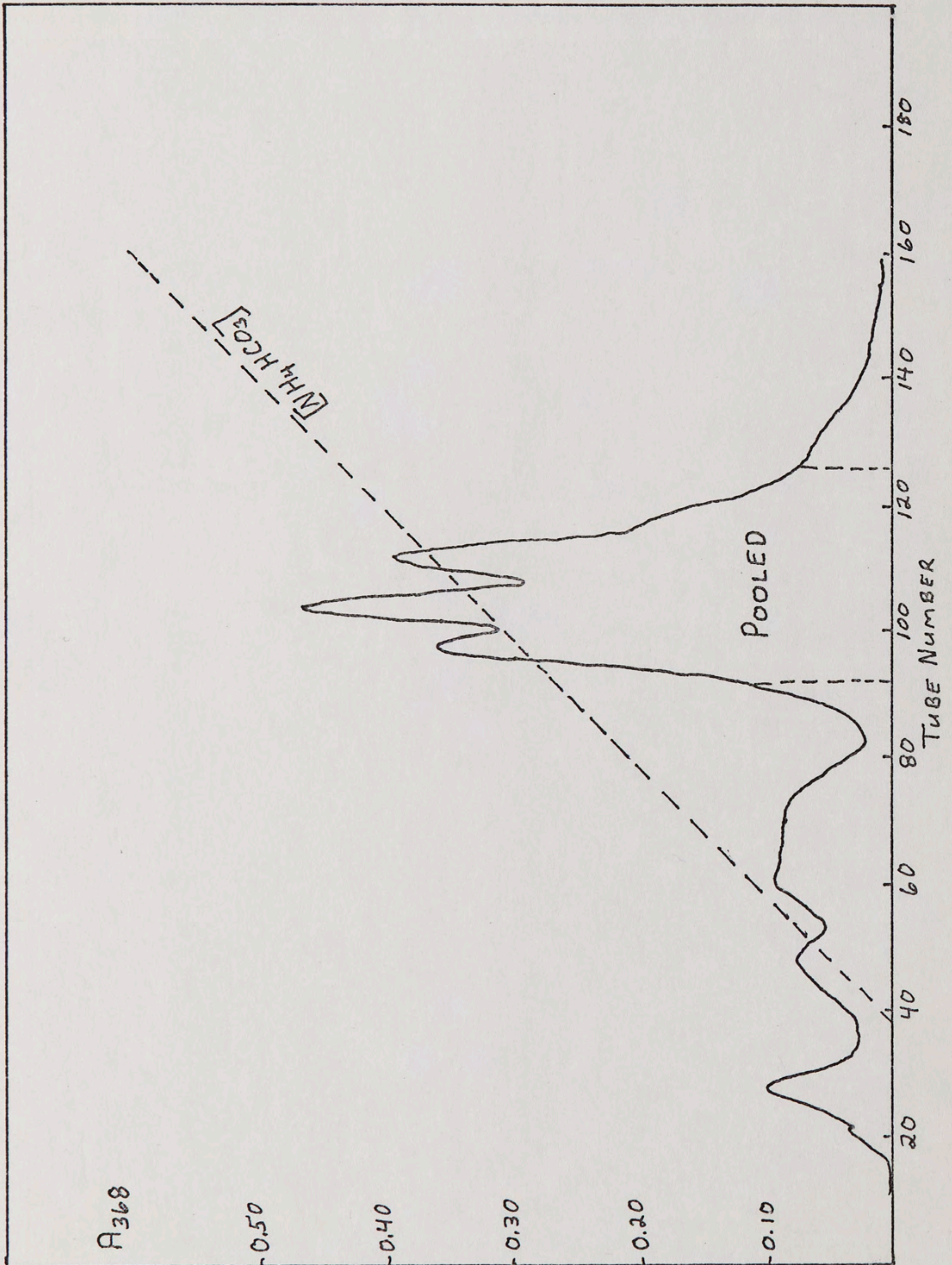
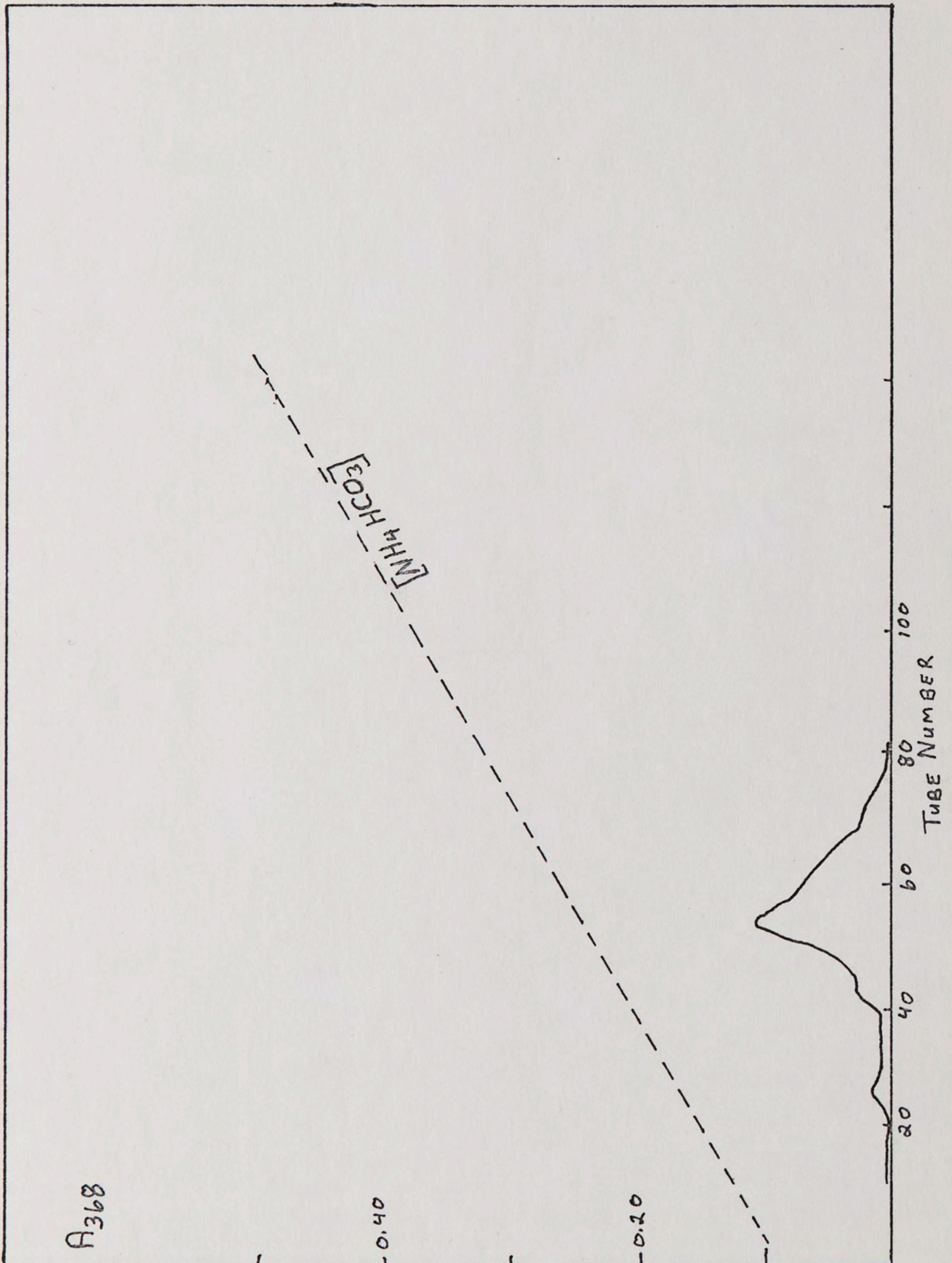


Figure 21. Elution profile II for indole nucleotide 10



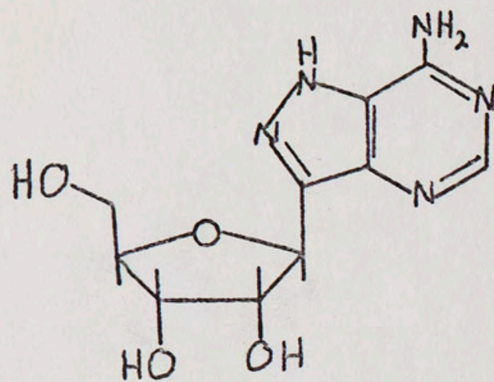
dication that the nucleotide was adsorbed on the column. The conclusion was that the nucleotide had decomposed during the removal of ammonium bicarbonate.

This result calls into question the status of the other indole nucleotides 6 and 8, which were also subjected to the same conditions of ammonium bicarbonate removal. Alternatives to sublimation as a means of removing the excess ammonium bicarbonate from the nucleotides 6, 8 and 10 are lyophilization and/or gel filtration with Biogel P-2 which has a low exclusion limit.

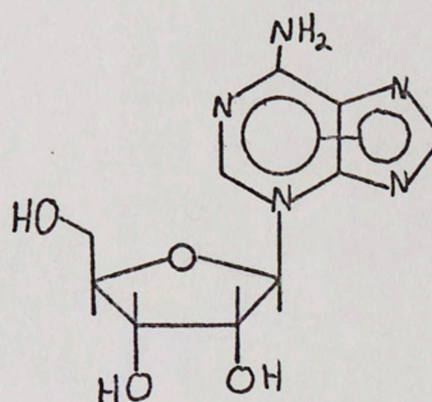
III.3. Enzyme Reactions

The overall intent of this research has been to synthesize analogues of the adenosine nucleotides (12, 13, 14, figure 1) and to determine if these analogues can replace the natural substrate in adenosine specific enzymes. The enzyme chosen for initial studies was adenylate kinase, EC 2.7.4.3, (myokinase; ATP:AMP phosphotransferase) which catalyzes the transfer of the terminal (γ) phosphate of ATP to AMP forming two molecules of ADP. The kinetics of this enzyme have been investigated (Callaghan and Weber, 1959; Rhoads and Lowenstein, 1968) and it is commercially available.

Rhoads and Lowenstein claim the kinetics are consistent with a reaction mechanism in which binding to each

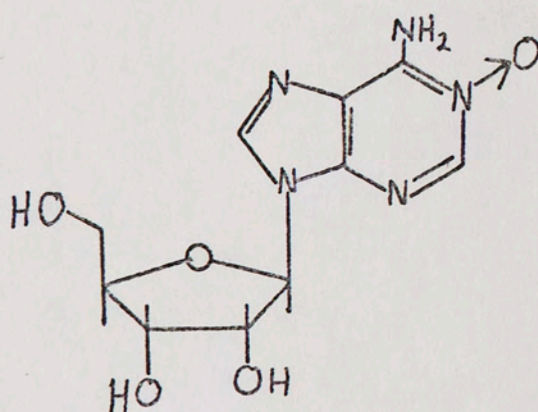


Formycin

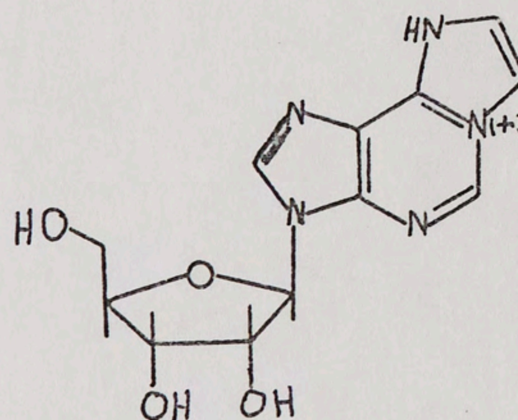


3-Isoadenosine

Analogues which bind to both sites on adenylate kinase



Adenosine N'-oxide



1,N^6-ϵ-adenosine

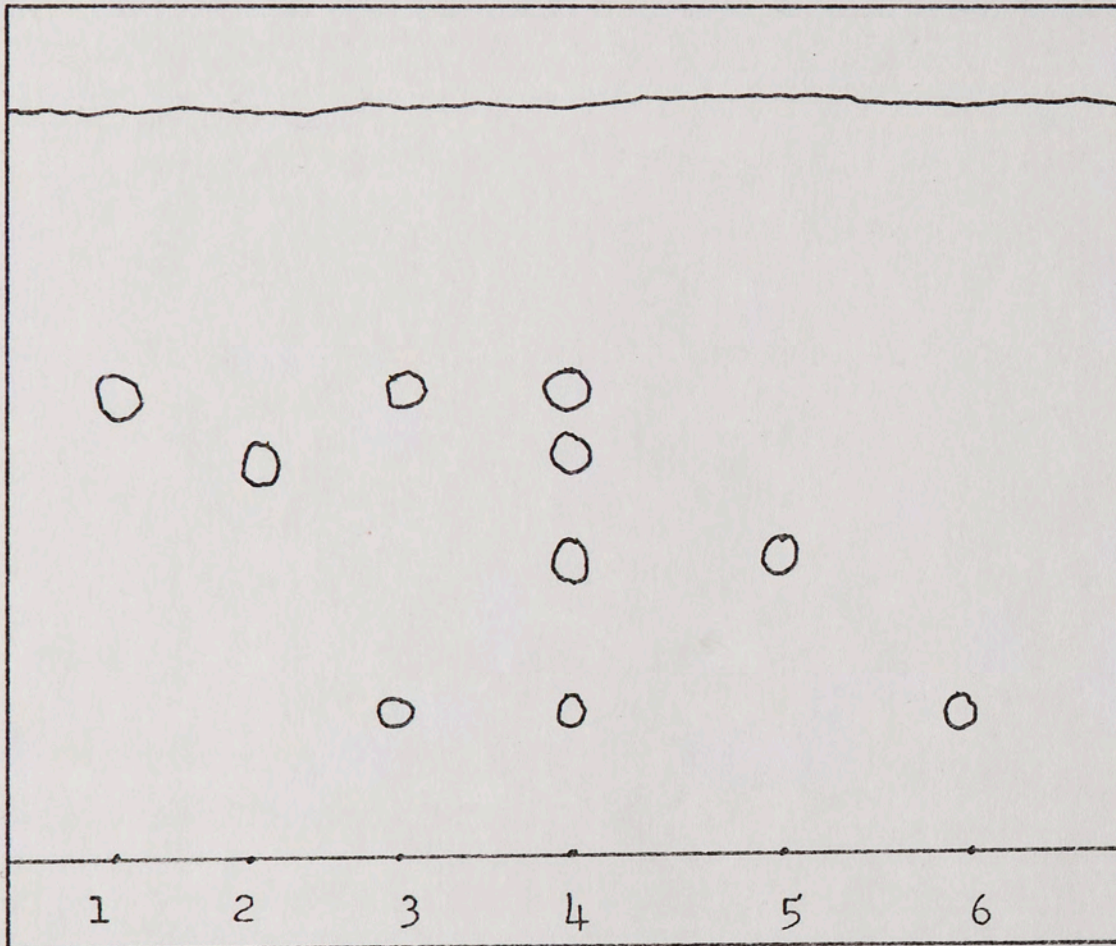
Analogues which bind to the ATP site only

Figure 22.

of two sites is random. One site is specific for magnesium chelated nucleotides (the ATP site); the other site binds only nonchelated nucleotides (the AMP site). This mechanism is supported by experiments involving substrate analogues. The 5'-mono, di- and triphosphates of formycin, (8-aza-9-deaza-adenosine, figure 22), a C-nucleoside antibiotic, are all substrates for adenylate kinase (Ward et al., 1969). Also, the mono-, di-, and triphosphates of 3-isoadenosine are substrates for the enzyme (Leonard and Laursen, 1965b). On the other hand, adenosine-N'-oxide (Jebeleanu et al., 1974; McCormick, 1966) and 1-N^6-ethenoadenosine (Secrist et al., 1972) function as substrates for adenylate kinase only as the 5'-triphosphates in conjunction with AMP.

The preliminary investigation was a determination of the interaction of benzimidazole nucleoside diphosphate 7 with adenylate kinase. The nucleotide was incubated with the enzyme and an aliquot was analyzed by thin layer chromatography on PEI-cellulose. (PEI-cellulose is an anion exchange medium which provides a good separation of nucleotides (Randerath, 1963; Randerath and Randerath, 1964).) The diphosphate was unchanged after 100 minutes.

When the triphosphate 9 was incubated with adenylate kinase and AMP (12), TLC analysis showed four spots, identified by comparison with authentic material as ABDP 7,



1. AMP
2. ADP
3. 0 time; no enzyme added
4. 100 minutes, AMP, ADP, ABDP (7), ABTP (9)
5. ABDP (7)
6. ABTP (9)

Figure 23. TLC analysis of ABTP-AMP reaction with adenylate kinase.

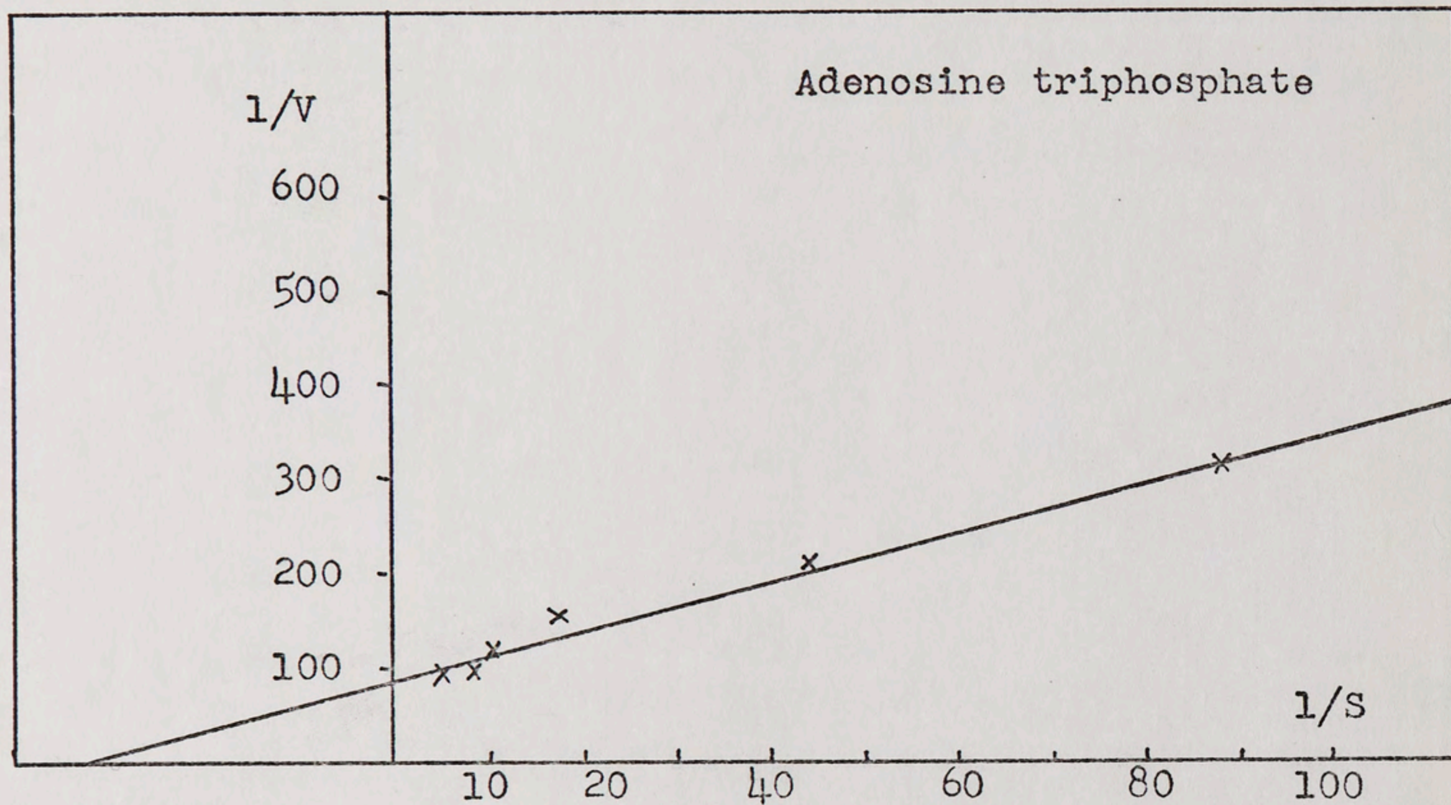
ABTP 9, AMP 12 and ADP 13.(Figure 23). Clearly 4-amino-benzimidazole nucleoside-5' triphosphate 9 is a substrate for adenylate kinase.

The kinetics of the adenylate kinase reaction were determined by coupling the system to pyruvate kinase and lactate dehydrogenase (Rhoads and Lowenstein, 1968; Secrist et al., 1972). In this way, the products of the adenylate kinase reaction are converted into a compound (NADH) which can be determined by its UV absorbance. The actual measurement is the decrease in absorbance at 340 nm which occurs as NADH is oxidized to NAD. The kinetics of the pyruvate kinase reaction were determined in the same manner. The kinetic values were calculated from measurements of the initial velocities only.

The Michaelis constants and maximal velocities for the enzyme reactions were calculated from double reciprocal plots of the initial velocities and substrate concentrations using the slope and intercept values derived from the least squares treatment of the data (Figures 24, 25 and 26).

The pyruvate kinase kinetics were examined to determine if the binding properties and maximal velocities for ABDP 7 and ADP 13 were roughly equilvalent. The adenylate kinase experiments, in any case, contained sufficient pyruvate kinase and lactate dehydrogenase to insure that any

Figure 24. Adenylate kinase kinetics



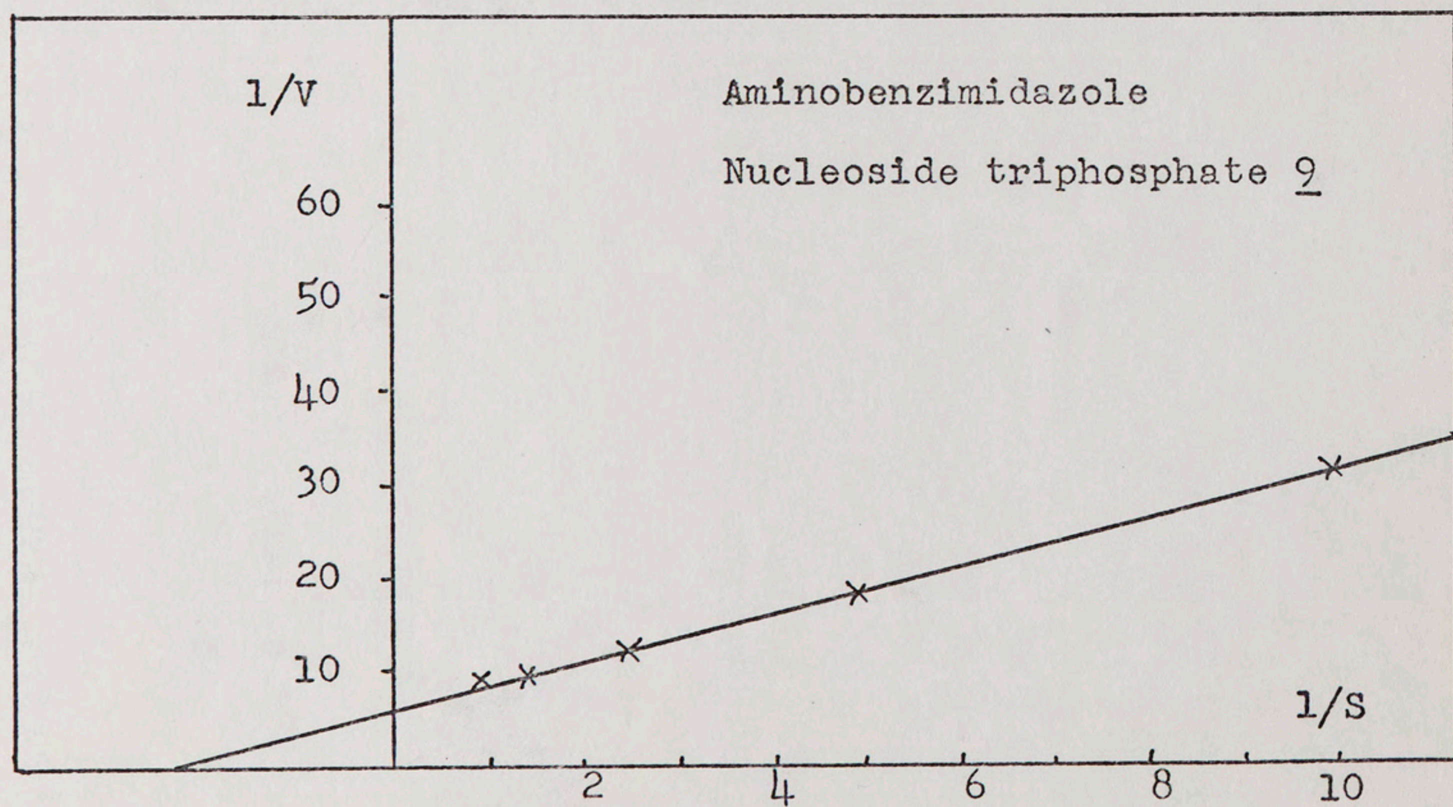
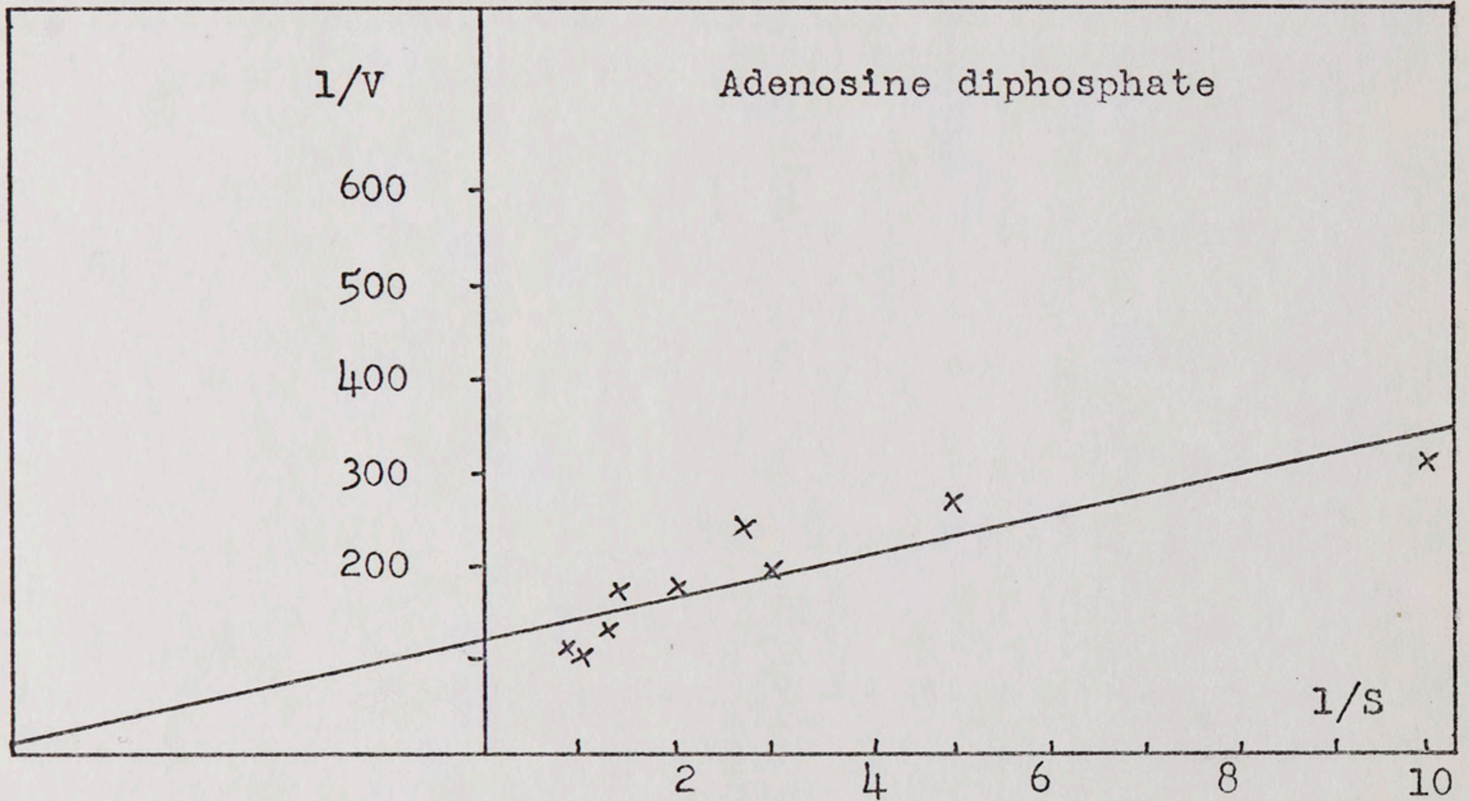
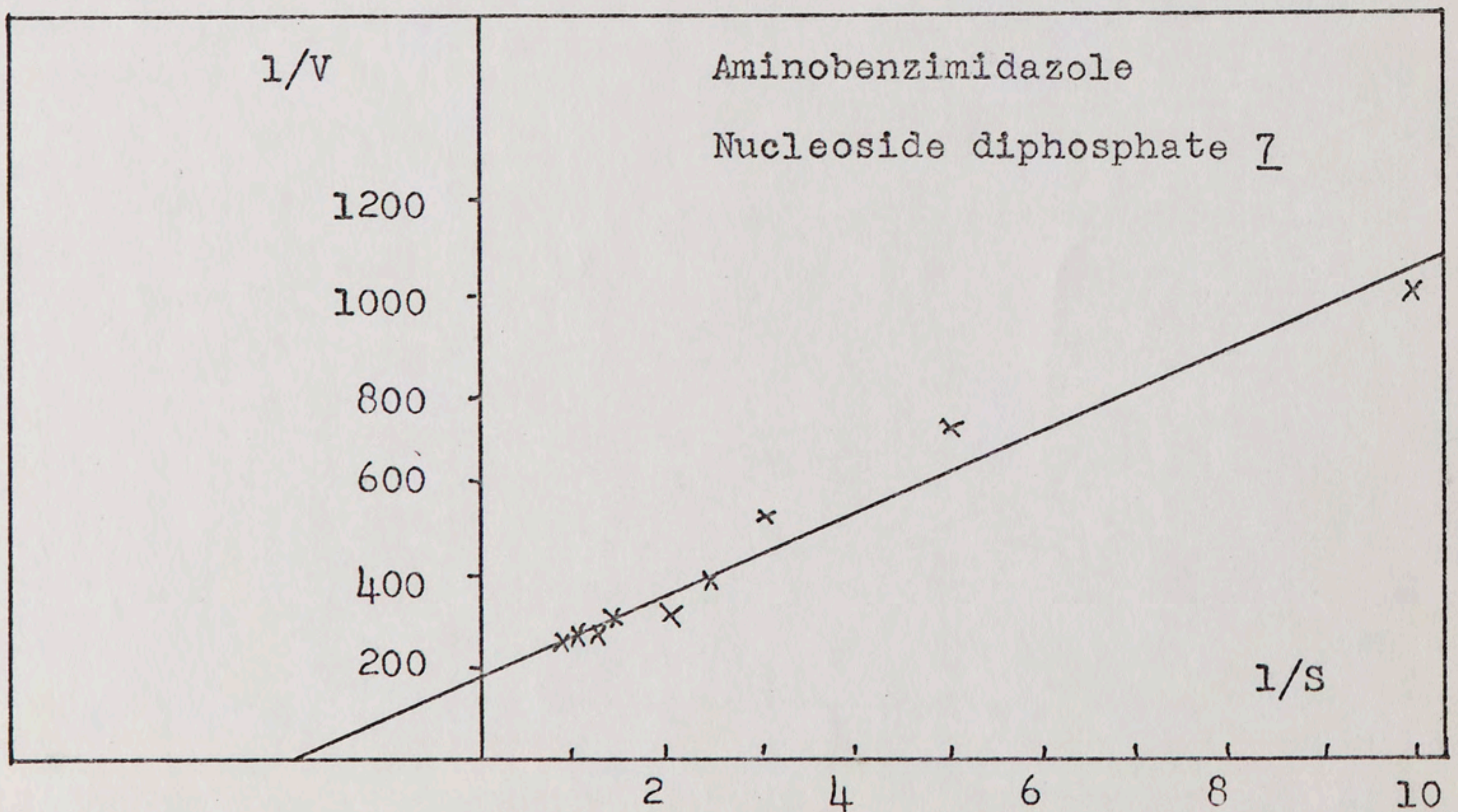


Figure 25. Pyruvate kinase kinetics





Adenylate kinase

Triphosphate	K_M (mM)	V_{max} (μmoles/min)
ABTP (9)	0.40	0.157
ATP	0.029	0.0125

Pyruvate kinase-Lactate dehydrogenase

Diphosphate	K_M (mM)	V_{max} (μmoles/min)
ABDP (7)	0.47	0.0055
ADP	0.17	0.0080

Kinetic parameters for adenylate kinase and
pyruvate kinase-lactate dehydrogenase
Figure 26.

diphosphate produced was immediately converted to triphosphate.

The observation that the Km and Vmax for ABTP 9 as a substrate for adenylate kinase are both larger than the values obtained for ATP is consistant with the proposed mechanism in which the dissociation of the enzyme-product complex is rate limiting (Rhoads and Lowenstein, 1968).

The binding requirements of adenylate kinase at the AMP site appear to involve N-1, the amine at position 6, and possibly N-7 of adenosine. All substrates which bind at the AMP site have N-1 available for hydrogen bond formation whereas those that bind only at the ATP site do not. (The need for the amine at C-6 is easily deduced from the fact that guanosine-5' phosphates, which differ in that there is a carbonyl at C-6, are not substrates.) There is no evidence for or against the requirement of N-7 in binding. This might be probed by the use of the nucleoside antibiotic tubercidin (7-deaza-adenosine). The 5' phosphate of tubercidin has been prepared (Hanze, 1968). The indole nucleoside-5' phosphate could not be used since it lacks the required nitrogen in the six-membered ring.

The requirements for the magnesium chelate which binds at the ATP site of adenylate kinase are not readily apparent. The common factors in all the structures are the

amino group at C-6 and a nitrogen at position 7. These could be involved in the formation of the magnesium chelate or in hydrogen bonding to the enzyme. Tubercidin and/or 4-aminoindole nucleotides could be used to investigate this requirement. 4-Nitroindole nucleoside-5' triphosphate would probably not be a substrate for adenylate kinase because the C-6 amino group is required for binding.

Rate measurements for the interaction of nitroindole nucleoside-5' diphosphate 8 and pyruvate kinase-lactate dehydrogenase were attempted but no results were obtained. One problem is that the absorbance at 340 nm due to the nitroindole moiety is substantial at a concentration of 0.1 mM. This would raise the total absorbance of the test solution to the point that the decrease due to oxidation of NADH would be small. Concentrations of 8 higher than 0.1 mM would have optical density readings greater than 2 and would be beyond the range of the instrument in measuring the change.

III.4. Possible Future Studies

Certain possible future studies are suggested. The phosphorylation process for the 4-nitroindole nucleotides warrants further investigation. As noted in section III.2.3.4., a mild method of removing ammonium bicarbonate such as lyophilization or gel filtration should produce

the pure nucleotides and avoid the decomposition caused by sublimation.

4-Nitro-1-β-D-ribofuranosylindole-5' phosphate has potential as a tool for the investigation of enzyme mechanisms where a very hydrophobic nucleotide such as 6 might be useful as an inhibitor. A case in point is the snake venom 5'-nucleotidase.

In addition, the nitroindole compounds should be investigated as possible fluorescent probes of enzyme structure. The nitroindole compound have all exhibited fluorescence under long wavelength UV light on thin layer plates. (Indeed, the fluorescence provided a good method for locating nitroindole-containing spots.)

The preparation of the 4-aminoindole nucleotides will be necessary in order to investigate any enzyme system in which kinetic measurements involve the oxidation or reduction of NADH (NAD). Considering that 4-aminoindole is air-sensitive (Walton et al., 1968) and that the aminoindole compounds may well be as acid sensitive as the nitroindole compounds, this may prove to be more difficult than the synthesis of 4-nitro-1-β-D-ribofuranosylindole (2).

Several other enzyme systems which require adenosine nucleotides should be examined to determine if the corresponding benzimidazole and/or indole compounds can be used

as substrates. ABTP 9 is also known to be able to replace ATP as a substrate for actomyosin (Ikehara *et al.*, 1964b). Enzyme systems such as hexokinase and the aminoacyl-tRNA synthetases which require ATP are other systems in which ABTP 9 might prove to be a substitute. Hexokinase, which is commercially available, would be the easiest to investigate. Adenosine kinase, which catalyzes the conversion of adenosine to AMP with ATP as the source of phosphate, could be investigated using both ABTP 9 and the 4-aminobenzimidazole nucleoside.

IV. Experimental

IV.1. General

Solvents used were reagent grade. In instances where moisture was a problem, solvents were stored over Lindé Type 3A molecular sieves. Acetonitrile and dimethyl formamide were distilled from phosphorus pentoxide. Pyridine was distilled from phthalic anhydride.

Melting points were determined on a Büchi melting point apparatus and are uncorrected. Ultraviolet absorption spectra were recorded using either a Cary model 14 recording spectrophotometer or a Beckmann model 24 recording spectrophotometer. Nuclear magnetic resonance spectra were determined using either the JEOLCO CH-60 at 60 MHz or the Varian A-60, also at 60 MHz. Infrared spectra were recorded using the Perkin-Elmer model 237 grating spectrophotometer. Optical rotatory dispersion values were measured using the Cary model 60 recording spectropolarimeter. Continuous monitoring of column effluent was accomplished with an Isco model 222 UV monitor. Elemental analyses were performed by Galbraith Laboratories, Inc.

IV.2. Reagents

IV.2.1. 1-O-Acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose 16

D-ribose (75 g, 0.50 mole) was dissolved in 1600 ml of methanol and 5.1 ml of concentrated sulfuric acid added.

The solution was stirred at room temperature until an aliquot gave a negative test for reducing sugar (Benedict's reagent). The acid was neutralized with 50 ml of pyridine. The methanol was removed under reduced pressure on the rotary evaporator, and the residual yellow oil was evaporated twice with pyridine (50 ml) to insure neutralization of the acid.

The yellow oil was dissolved in 400 ml of chloroform and 880 ml of pyridine. The solution was cooled to 0° in an ice bath. Benzoyl chloride (292 ml, 2.53 moles) was added slowly, with stirring. The resulting solution was kept overnight at 0°. In the morning, the reaction mixture was poured onto 2 liters of ice and water. The organic layer was separated and the aqueous layer was extracted with 400 ml of chloroform. The chloroform solutions were combined, washed with water (400 ml) and dried over anhydrous sodium sulfate. The chloroform was evaporated under reduced pressure with external heating. Heating was continued under high vacuum until benzoic acid started to sublime into the steam duct of the evaporator.

The red oil, free of traces of pyridine and water, was dissolved in a mixture of acetic anhydride (280 ml) and glacial acetic acid (120 ml). The solution was cooled to 15° in an ice bath and concentrated sulfuric acid (40 ml)

was added slowly, with stirring, to prevent the temperature from rising above 15°. After standing overnight at 4°, the solution was poured, with stirring, into 2 l of water and ice. The precipitate which formed, an off-white gum, was washed several times with distilled water by decantation. After the final washing, the product was collected by suction on a Buchner funnel and pressed dry.

1-O-Acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose 16 crystallized from hot 2-propanol on standing at room temperature. After one recrystallization from 2-propanol, the white crystals melted at 128-129° (lit. 131-132°, Recondo and Rinderknecht, 1959); 130 g, 52% of theory, based on D-ribose.

IV.2.2. 2,3,5-Tri-O-benzoylribofuranosyl bromide 17

1-O-Acetyl-2,3,5-tri-O-benzoylribofuranose 16 (0.504 g, 1 mmole) was placed in a 25-ml round-bottomed flask and dissolved in 1 ml of dry methylene chloride and 6 ml of 30% hydrogen bromide in glacial acetic acid. The flask was stoppered and let stand at room temperature for 75 minutes. The solvents were removed under reduced pressure at a water bath temperature below 30°. The residual red oil was evaporated 4-5 times with 5 ml of dry toluene to remove traces of hydrogen bromide and acetic acid. The product 17 was used immediately without characterization.

IV.2.3. Silver(I) oxide

A solution of silver nitrate was treated with sodium hydroxide solution until precipitation was complete. The brown-black precipitate was collected in a sintered glass filter funnel and washed with water (4 times), 95% ethanol (3 times), acetone (twice) and ether (twice). The silver oxide was transfered to a brown glass screw-cap bottle and dried under high vacuum for 24 hours. The resulting black powder was used without characterization to prepare the nitroindole nucleoside 4.

IV.2.4. Pyrophosphoryl chloride

In a 100-ml round-bottomed flask were placed phosphorus pentoxide (7.1 g, 0.05 mole) and phosphorus pentachloride (20.8 g, 0.10 mole). Carbon tetrachloride (50 ml) was added to the flask, a reflux condenser fitted, and the mixture heated to reflux.

After 4 hours at reflux, the mixture was cooled to room temperature and filtered to remove solid material. The solids were washed with a small amount of carbon tetrachloride. The filtrate and washings were combined and distilled under reduced pressure.

Pyrophosphoryl chloride distilled at 103-108°/26 mm Hg (lit. 105°/18 mm Hg, Crofts *et al.*, 1960). The water-white liquid weighed 5.25 g, 41.5% of theory.

IV.3. 4-Nitro-1-β-D-ribofuranosylbenzimidazole 1

IV.3.1. 4(7)-Nitrobenzimidazole 15

2-Amino-3-nitroaniline (25 g, 0.16 mole), in a 100-ml round-bottomed flask, was dissolved in 50 ml of 72% formic acid. A reflux condenser was fitted to the flask and the solution heated at reflux for 2 hours. The hot reaction mixture was poured into 750 ml of ice and water and stirred until the ice melted. The yellow solution was made basic with concentrated ammonium hydroxide. The yellow precipitate was collected by suction and pressed free of water.

The filter cake was broken up and dissolved in an excess of hot 95% ethanol (approximately 100 ml excess). Decolorizing charcoal was added to the hot solution. The mixture was filtered while hot, cooled to room temperature and finally cooled overnight at 4.

4(7)-Nitrobenzimidazole 15 crystallized as yellow needles. The crystals were collected by suction on a buchner funnel and air-dried. 19.0 g of crystals were obtained (73%), melting at 240-241° (lit. 240-241°, Rabinowitz and Wagner, 1951).

IV.3.2. 4-Nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)benzimidazole 3

4(7)-Nitrobenzimidazole 15 (0.833 g, 5.0 mmoles) and

mercuric cyanide (1.239 g, 5.0 mmoles) were suspended, with stirring (magnetic), in 500 ml of nitromethane in a 1000 ml three-necked flask fitted with a condenser, a distilling head and a dropping funnel. The mixture was heated to reflux and approximately 50 ml of nitromethane were removed by distillation during which time the suspended material dissolved. The solution was cooled prior to addition of the tri-O-benzoylribofuranosyl bromide 17.

2,3,5-tri-O-benzoylribofuranosyl bromide 17 (5.0 mmoles) in 25 ml of nitromethane was added dropwise as heating and stirring were resumed. After 30 minutes, all the ribosyl bromide 17 had been added and the solution was again heated at reflux with slow distillation of solvent. Approximately 100 ml of nitromethane were removed by distillation during the four hour reflux period.

The solution was cooled to room temperature and filtered to remove material which had precipitated on cooling. The flask and solids were washed with 25 ml of nitromethane. The filtrate and washings were combined and evaporated under reduced pressure. The residue, a dark red oil, was dissolved in chloroform with the exception of some gummy solid. The chloroform solution and the solid were transfered to a 2-liter separatory funnel. The flask was rinsed once with 30% potassium iodide solution and

once with chloroform, and the washings added to the separatory funnel. The chloroform solution was extracted 4 times with 30% potassium iodide solution to remove mercuric cyanide, twice with 5% sodium carbonate solution and once with water. The chloroform solution was dried over anhydrous sodium sulfate.

The chloroform was evaporated and the residue dissolved in methylene chloride. Petroleum ether (30-60°) was added to faint turbidity. A yellow powder precipitated which was removed by filtration and identified as 4-nitrobenzimidazole 15. The filtrate was stripped of solvent and the residue dissolved in hot methanol. After standing overnight at room temperature and several hours at 4°, 4-nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)benzimidazole 3 crystallized as yellow prisms. The crystals, after air-drying, weighed 1.83 g, (60% of theory) and melted at 125-127° (lit. 124-125°, Mizuno *et al.*, 1962).

IV.3.3. 4-Nitro-1-β-D-ribofuranosylbenzimidazole 1

Tribenzoyl nitrobenzimidazole nucleoside 3 (602 mg, 1 mmole) in a 50-ml round-bottomed flask was heated briefly with 25 ml of anhydrous methanol to promote solution, then cooled to 0° in an ice bath. Ammonia was passed through the cold solution for 5 minutes. The flask was

sealed and the mixture was kept at room temperature for 24 hours.

Methanol and ammonia were removed under reduced pressure. The residual gum was triturated with three portions of ether to remove methyl benzoate. The solid residue was dissolved in a minimum of boiling water and filtered through a glass wool plug to remove a small amount of insoluble material. Crystals of 1 appeared as the solution cooled. The product was isolated after standing for several hours at 4°, 264 mg, 90% of theory.

4-Nitro-1-β-D-ribofuranosylbenzimidazole 1 has two crystal forms, light yellow needles and pale yellow plates. Both melt at 193-195°, separately or in admixture (lit. 192-194°, Mizuno *et al.*, 1962).

Alternatively, the benzoyl groups of 3 were removed by treatment with three equivalents of sodium methoxide in methanol for two hours at reflux. After neutralization with glacial acetic acid, the product was isolated as above. Yields were 90-95%.

IV.4. 4-Nitro-1-β-D-ribofuranosylindole 2

IV.4.1. 4-Nitroindole 18

IV.4.1.1. Ethyl 4-nitroindole-2-carboxylate 20

Ethyl pyruvate *m*-nitrophenylhydrazone (5 g, 19.9 mmoles) and 25 g of polyphosphoric acid in a 100-ml

beaker were heated in an oil bath to an internal temperature of 90-100°. Heating was discontinued when the temperature reached 105°. The exothermic reaction reached temperatures of 120-130°. When the temperature dropped to 90°, heating was resumed so that the mixture had been at a temperature of greater than 90° for a total of 20 minutes. The hot mixture was poured onto 200 ml of ice and water. Ice water was added to the beaker to decompose the polyphosphoric acid mixture remaining in the beaker.

When all the polyphosphoric acid had been decomposed, the yellow precipitate was collected on a sintered glass funnel and pressed dry. The filter cake was dissolved in hot benzene and the benzene solution filtered into a flask. Silica gel (50 g) was added to the flask and the solvent evaporated slowly to adsorb the indole mixture on the silica gel. When dry, the silica gel was added to the top of a column of silica gel (900 g) packed in benzene-acetone (95/5). The column was eluted with the same solvent and twenty-milliliter fractions were collected. Fractions containing the products were determined by thin layer chromatography on silica gel plates (benzene-acetone, 95/5). Ethyl 6-nitroindole-2-carboxylate <u>21</u> eluted first (0.90 g, 19.3%, fractions 145-185) followed by a mixture of 6- and 4-nitro isomers (1.55 g, 33.2%, fractions 186-210). Pure

ethyl 4-nitroindole-2-carboxylate 20 (1.25 g, 26.8%, fractions 211-290) followed; total yield 3.70 g, 77.3%.

The pure materials were each recrystallized from benzene. Ethyl 6-nitroindole-2-carboxylate melted at 194-195° and ethyl 4-nitroindole-2-carboxylate melted at 226-228° (lit. 6-nitro 21, 195-197° and 4-nitro 20, 228-230°, Parmerter *et al*., 1958).

A more effective procedure was to dissolve the crude mixture from the Fischer indole reaction in chloroform and pass the solution through a small silica gel column (50 g) to remove highly colored impurities. The mixture was not separated into isomers but carried through to the next step as a mixture. Three separate reactions using 10 g (39.8 mmoles) of ethyl pyruvate *m*-nitrophenylhydrazone gave crude yields of 93.3, 90.9 and 91.9% mixed indoles.

IV.4.1.2. 4-Nitroindole-2-carboxylic acid 22

Ethyl 4-nitroindole-2-carboxylate 20 (4.01 g, 17.1 mmoles) was dissolved, with stirring, in 150 ml of 95% ethanol at the boiling point. A solution of potassium hydroxide (3.4 g, 60.7 mmoles) in 6 ml of water was added to the hot ethanol solution. The dark red solution was kept at the boiling point for 1.5 hours during which time a copious dark red precipitate formed. Warm water (150 ml) was added to dissolve the precipitate.

The clear red solution was made acid with 3 M hydrochloric acid (color changed from red to yellow). The yellow solid formed was collected by suction filtration and recrystallized from dilute aqueous ethanol. 4-Nitroindole-2-carboxylic acid 22, 3.37 g, 95.7% of theory, melted at 310-312° (lit. 317-318°, Parmerter et al., 1958).

In the same manner, ethyl 6-nitroindole-2-carboxylate 21 was hydrolyzed to 6-nitroindole-2-carboxylic acid 23, mp 296-298° (lit. 304-305°, Parmerter et al., 1958).

The mixed nitroindole esters 20 and 21 (25.74 g, 0.11 mole) were hydrolyzed in the same way, yielding 21.20 g, 93.6%, of the mixed nitroindole acids 22 and 23. Again, the isomers were not separated but carried as a mixture into the next step.

IV.4.1.3. 4-Nitroindole 18

4-Nitroindole-2-carboxylic acid (3.00 g, 14.6 mmoles) was placed in a 100-ml round-bottomed flask along with cupric oxide (0.308 g) and 1,10-phenanthroline (0.176 g) and freshly distilled quinoline (50 ml). A magnetic stirring bar was added and the flask fitted with a nitrogen inlet and a reflux condenser. The reaction mixture was heated slowly under nitrogen until carbon dioxide evolution commenced and was held at that temperature for 2 hours.

After cooling to room temperature, the reaction

mixture was diluted with 500 ml of ether containing 5 ml of glacial acetic acid. The ether solution was filtered into a 2-l separatory funnel and extracted with 3 M hydrochloric acid (5 X 400 ml), 5% sodium bicarbonate solution (2 X 250 ml) and water (2 X 250 ml). The yellow ether solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The oily light brown solid was dissolved in a minimum of hot methanol, decolorized with charcoal, and filtered. Water was added to produce a faint turbidity, and 4-nitroindole 18 crystallized as yellow plates, mp 205-207° (lit. 203-204°, Parmerter *et al.*, 1958). The crystals weighed 2.01 g, 84.6%.

6-Nitroindole-2-carboxylic acid 23 was decarboxylated by the same process to 6-nitroindole 19 in 65% yield, mp 142-143° (lit. 144-145°, Parmerter *et al.*, 1958).

The mixed indole acids 22 and 23 were decarboxylated in the same way as the pure isomers. The crude mixture of 4- and 6-nitroindoles 18 and 19, after evaporation of the ether, was dissolved in acetone and adsorbed on 200 g of silica gel by slow evaporation of the acetone. The dry silica gel with indoles adsorbed was slurried with petroleum ether (60-110°) and the slurry added to the top of a column of silica gel (1300 g) packed in the same solvent. The column was eluted with ethyl acetate-petroleum ether

(1/3, v/v). Twenty-milliliter fractions were collected. Appropriate fractions (determined by TLC on silica gel with benzene-ethyl acetate (95/5) as solvent) were pooled and evaporated to dryness. A total of 15 liters of solvent was required to elute all the indoles. As in the case of the indole esters, the order of elution is 6-nitroindole 19 followed by 4-nitroindole 18.

In a typical reaction, mixed nitroindole acids (6.52 g, 31.65 mmoles) were decarboxylated and chromatographed to yield 6-nitroindole 19 (2.13 g, 41.3%) and 4-nitroindole 18 (2.27 g, 44.0%).

IV.4.2. 4-Nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)indole 4

4-Nitroindole 18 (163 mg, 1 mmole), silver(I) oxide (232 mg, 1 mmole) and crushed molecular sieves (Lindé Type 3A) were placed in a 100-ml 3-necked round-bottomed flask along with a magnetic stirring bar. Benzene (50 ml) was added and the flask fitted with a nitrogen inlet, a condenser topped with a distilling head and a dropping funnel. The mixture was heated to reflux under a constant nitrogen flow and approximately 10 ml of solvent removed by distillation.

2,3,5-Tri-O-benzoylribofuranosyl bromide 17 (1.5 mmoles) in 15 ml of dry benzene was added dropwise to the refluxing

mixture over a period of 20 minutes. The mixture was kept at reflux for 4 hours with very slow distillation of solvent (about 15 ml). After cooling to room temperature, the mixture was filtered to remove solids. The flask and solids were washed with benzene and ethyl acetate. The filtrate and washings were combined and evaporated under reduced pressure to a heavy red oil.

The oil was dissolved in a small amount of benzene (about 5-10 ml) and applied to the top of a column of silica gel packed in benzene. The column was eluted with benzene and 15-ml fractions were collected. Unreacted 18 eluted first, followed by the desired product. 4-Nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)indole 4 (0.462 g, 76.1%) was isolated as an analytically pure foamed yellow glass which resisted all attempts at crystallization.

Analysis: Calculated for $C_{34}H_{26}N_2O_9$,

C, 67.32 ; H, 4.32 ; N, 4.62

Found: C, 67.11 ; H, 4.41 ; N, 4.44.

IV.4.3. 4-Nitro-1-β-D-ribofuranosylindole 2

4-Nitro-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-indole 4 (606 mg, 1 mmole) was dissolved in 600 ml of dry methanol and the solution cooled to 0° in an ice bath. Ammonia was passed through the solution for ten minutes.

The flask was sealed and kept at room temperature for 24 hours.

The methanol and ammonia were removed under reduced pressure. The oily red residue was shaken with several portions of petroleum ether. The residue was dissolved in ether-methylene chloride and petroleum ether added to faint turbidity. 4-Nitro-1-β-D-ribofuranosylindole crystallized on standing at room temperature. The crystals, yellow blades, weighed 265 mg, 90%, mp 164-165°.

The crystals contain aromatic impurities which derive from the deblocking procedure. These are usually removed by trituration with ether; however the ether solubility of 2 prevented this step and an analytically pure sample was not obtained.

IV.5. Preparation of nucleotides

IV.5.1. 4-Amino-1-ribofuranosylbenzimidazole-5' phosphate 5

4-Nitro-1-ribofuranosylbenzimidazole 1 (590 mg, 2 mmoles) was suspended in 30 ml of *m*-cresol in a 50-ml round-bottomed flask and the mixture was cooled to 0° in an ice bath. Pyrophosphoryl chloride (1 ml, 7.2 mmoles) was added and the mixture stirred for 4 hours with the temperature kept below 5°. During this time the solid dissolved.

The yellow solution was poured into 150 ml of ice

and water and stirred until the ice melted. The mixture was extracted with ether (2 X 40 ml) to remove m-cresol. The aqueous layer was separated and adjusted to pH 2 with 1.25 N sodium hydroxide solution.

The aqueous solution was applied to a column of water washed powdered charcoal (8 g, 2.5 cm i.d.) by means of suction. The column was washed with water until the effluent was neutral. The nucleotide was eluted with ethanol-ammonium hydroxide-water (50/2/48, v/v/v). Fractions containing product were determined by measuring the absorbance at 317 nm. The appropriate fractions were pooled and evaporated to dryness under reduced pressure.

4-Nitrobenzimidazole nucleoside-5' phosphate, ammonium salt, was isolated as a yellow glass (620 mg, 76.2%).

The nitrobenzimidazole nucleotide (531 mg, 1.30 mmoles) was dissolved in 10 ml of distilled water. The solution was added to a suspension of 346 mg of 5% Pd/C in 100 ml of ice-cold methanol in a 250-ml Parr bottle. The mixture was placed on a Parr hydrogenation apparatus and shaken with hydrogen at 50 psig for 30 minutes.

The catalyst was removed by filtration and washed with additional ice-cold methanol. The filtrate and washings were combined and concentrated to about 10 ml under reduced pressure. The solution was diluted to 30 ml with

distilled water and transfered to a 50-ml beaker. After adjusting the pH to 3.75 with 2 M ammonium formate, the beaker was covered and stored at 4° for 48 hours. 4-Amino-benzimidazole nucleoside-5' phosphate 5 precipitated as a pinkish gel. The gel was isolated by filtration and dried under high vacuum for 48 hours. After drying, the amorphous product weighed 315 mg, 70% based on nitro-benzimidazole nucleotide. TLC on PEI-cellulose in 1 M lithium chloride showed a single spot, Rf 0.67.

Subsequently, 4-amino-1-ribofuranosylbenzimidazole-5' phosphate 5 was prepared by concentrating the aqueous solution resulting from the direct phosphorylation to 10 ml and reducing the nitrobenzimidazole nucleoside-5' phosphate without prior isolation. In this manner, nucleotide 5 was produced in 60% yield from nucleoside 1.

IV.5.2. 4-Amino-1-ribofuranosylbenzimidazole-5' diphosphate 7

IV.5.2.1. P^1-Diphenyl-P^2-(1-ribofuranosyl-4-aminobenzimidazole-5'-)pyrophosphate 27

4-Amino-1-ribofuranosylbenzimidazole-5' phosphate 5 (17.3 mg, 50 μmoles) in a 25-ml pear-shaped flask was dissolved in 5 ml of 50% ethanol with the addition of excess tributylamine. The solution was evaporated to dryness under reduced pressure and the residue evaporated

twice with absolute ethanol and twice with dimethylform-amide (under high vacuum).

To the tributylammonium salt of 5 was added 0.5 ml of dry DMF, 15 μl of diphenylphosphoryl chloride, and 30 μl of tributylamine. The flask was sealed, shaken to mix the contents, and let stand at room temperature for 2.75 hours. The solvent was removed under high vacuum. The product was washed with dry ether by decantation (3 X 5 ml) and dried by evaporation with two 3-ml portions of dry DMF. P^1-Diphenyl-P^2-(1-ribofuranosyl-4-aminobenzimidazole-5')pyro-phosphate 27 was used immediately without further purifica-tion in the preparation of di- or triphosphate.

IV.5.2.2. 4-Amino-1-ribofuranosylbenzimidazole-5' di-phosphate 7

To pyrophosphate 27 (from 50 μmoles of nucleotide 5) was added 0.100 mmole of tributylammonium phosphate in 0.250 ml of dry pyridine. The flask was sealed and shaken to mix the contents. The mixture was kept at room temper-ature for four hours, and the solvent was removed under high vacuum. The residue was dissolved in 5 ml of water and extracted with two 5-ml portions of ether. The aqueous layer was made basic with ammonia and again extracted with two 5-ml portions of ether. The aqueous layer was separ-ated and evaporated to dryness under high vacuum.

The nucleotide residue was dissolved in 10 ml of 0.02 M ammonium bicarbonate and applied to a column of DEAE-cellulose (2.5 X 20 cm) equilibrated with 0.02 M ammonium bicarbonate. The column was washed with the equilibrating solvent until the absorbance of the effluent at 254 nm fell to zero. The product was eluted with a linear gradient of 350 ml of 0.3 M ammonium bicarbonate into 350 ml of 0.02 M ammonium bicarbonate. The effluent was monitored continuously for absorbance at 254 nm; 5-ml fractions were collected. The exact location of 4-aminobenzimidazole species was determined by measuring the absorbance of each fraction at 263 nm. Fractions 116-132 were pooled and evaporated to dryness under high vacuum. The dry material, which contained ammonium bicarbonate, was heated in a vacuum desiccator for 12 hours. 4-Amino-1-ribofuranosylbenzimidazole-5' diphosphate 7 was isolated as a tan powder (13.4 mg, 28.2 μmoles); Rf on PEI-cellulose in 1 M lithium chloride, 0.43.

IV.5.3. 4-Amino-1-ribofuranosylbenzimidazole-5' triphosphate 9

P^1-Diphenyl-P^2-nucleosidepyrophosphate 27 (from 50 μmoles of nucleotide 5) was dissolved in 0.50 ml of a solution of tetrakis(tributylammonium)pyrophosphate in DMF. The solution was kept at room temperature for 48

hours. The DMF was removed under high vacuum. The solid residue was dissolved in 10 ml of water and extracted with 5 ml of ether. The aqueous layer was made basic with ammonia and extracted twice with 5 ml of ether. The aqeous layer was separated and evaporated to dryness under high vacuum.

The residue was dissolved in a minimum of 0.02 M ammonium bicarbonate and applied to a column of DEAE-cellulose equilibrated in the same solvent. The column was washed with 0.02 M ammonium bicarbonate until the absorbance at 254 nm fell to zero. Nucleotides were eluted with a linear gradient of ammonium bicarbonate (0.02 M to 0.4 M, 350 ml each). The effluent was monitored continuously at 254 nm; 5-ml fractions were collected. Exact peak locations were determined by measuring the absorbance of each tube at 274 nm. Tubes 99-111, containing 4-amino-1-ribofuranosylbenzimidazole-5' triphosphate 9, were pooled and evaporated to dryness under high vacuum. The tan solid remaining was heated in a desiccator to remove ammonium bicarbonate. The yield of nucleoside-5' triphosphate 9 was estimated from the absorbance at 274 nm to be 16.9 μmoles; Rf on PEI-cellulose in 1 M lithium chloride, 0.21.

IV.5.4. 4-Nitro-1-ribofuranosylindole-5' phosphate 6

4-Nitro-1-ribofuranosylindole 2 (29.4 mg, 100 μmoles)

was added to a flask containing 1 mg of zinc chloride, 0.5 ml of ethyl orthoformate and 2 ml of dry acetonitrile. The flask was sealed, shaken and let stand at room temperature for 4 hours. Four drops of concentrated ammonium hydroxide were added and the solvent removed under high vacuum. The gummy yellow residue was washed by decantation with petroleum ether (2 X 2 ml). The crude 2',3'-ethoxyformylidine-nitroindole nucleoside 25 was dissolved in chloroform and filtered to remove zinc salts.

The chloroform solution was transfered to a 25-ml pear-shaped flask and evaporated to dryness under reduced pressure. To the flask was added 0.40 ml of a 1 M solution of cyanoethyl phosphate in dry pyridine and 84.4 mg of mesitylene sulfonyl chloride. The flask was sealed, shaken and kept at room temperature for 16 hours.

The pyridine solution was cooled in an ice bath. One milliliter of 5% ammonium hydroxide was added and the solution stirred for 30 minutes at 0° to decompose unreacted mesitylene sulfonyl chloride. The solvent was removed under high vacuum and the residue dissolved in 15 ml of ethanol. The ethanol solution was transfered to a 50-ml round-bottomed flask and an equal volume of concentrated ammonium hydroxide was added. The ethanol-ammonia mixture was stirred for 2 hours in a water bath at 65°. The solvent

was removed under high vacuum and the residue dissolved in 10 ml of 80% acetic acid. The acetic acid solution was kept at room temperature for 4 hours. The acetic acid was removed under high vacuum. The last traces of acetic acid were removed by dissolving the residue in absolute ethanol and evaporating to dryness three times. 4-Nitroindole nucleoside-5' phosphate _6_ was separated from polyacrylonitrile by extracting the residue with 5% ammonium hydroxide. The ammonia solution was filtered to remove polymer and evaporated to dryness under high vacuum.

The residue was dissolved in 15 ml of 0.02 _M_ ammonium bicarbonate and applied to a column of DEAE-cellulose equilibrated with the same solvent. The column was washed with 0.02 _M_ ammonium bicarbonate until the absorbance at 254 nm fell to zero. The effluent was monitored continuously at 254 nm; 5-ml fractions were collected. Products were eluted with a linear gradient of ammonium bicarbonate (0.02 _M_ to 0.3 _M_, 350 ml each). The exact location of product peaks was determined by measuring the absorbance of each tube at 368 nm. Fractions 140-153 were pooled and evaporated to dryness under high vacuum. Ammonium bicarbonate was removed by heating the residue in a desiccator under high vacuum. The yield of 4-nitro-1-ribofuranosylindole-5' phosphate was estimated from the absorbance at

368 nm to be 17.9 μmoles.

IV.5.5. 4-Nitro-1-ribofuranosylindole-5' diphosphate 8

IV.5.5.1. P^1-Diphenyl-P^2-(1-ribofuranosyl-4-nitroindole-5')-pyrophosphate 26

4-Nitro-1-ribofuranosylindole-5' phosphate 6, ammonium salt, (20 mg, 50 μmoles) was dissolved in 50% ethanol with the addition of tributylamine. The solution was evaporated to dryness under high vacuum. Traces of ethanol and water were removed by two codistillations with dry DMF. To the nucleotide tributylammonium salt was added 0.5 ml of dry DMF, 15 μl of diphenylphosphoryl chloride and 30 μl of tributylamine. The flask was sealed, shaken to mix the contents and kept at room temperature for 2.75 hours. The solvent was removed under high vacuum. The residue was washed by decantation with two 3-ml portions of ether-petroleum ether and dried by two co-evaporations with 2 ml of dry DMF. The crude product, P^1-diphenyl-P^2-(1-ribofuranosyl-4-nitroindole-5')pyrophosphate 26 was used immediately without futher purification.

IV.5.5.2. 4-Nitro-1-ribofuranosylindole-5' diphosphate 8

Pyrophosphate 26 was dissolved in 0.5 ml of 0.4 M tributylammonium phosphate in dry pyridine. The flask was sealed, shaken and left at room temperature for 4 hours. The pyridine was removed under high vacuum. The solid

residue was dissolved in 10 ml of water and extracted once with 5 ml of ether. The aqueous layer was made basic with ammonia and extracted twice with 5-ml portions of ether. The aqueous layer was separated and evaporated to dryness under high vacuum.

The solid was dissolved in 10 ml of 0.02 M ammonium bicarbonate and applied to a column of DEAE-cellulose equilibrated in the same solvent. The column was washed with 0.02 M ammonium bicarbonate until the absorbance at 254 nm fell to zero. The product was eluted with a linear gradient of ammonium bicarbonate (0.02 M to 0.6 M, 350 ml each). The absorbance of the effluent was monitored continuously at 254 nm; 5-ml fractions were collected. The exact location of peaks was determined by measuring the absorbance of each tube at 368 nm and plotting absorbance against tube number. Fractions 99-109 were pooled and evaporated to dryness under high vacuum. Ammonium bicarbonate was removed by heating the solid in a desiccator under high vacuum. The yield of 4-nitro-1-ribofuranosylindole-5' diphosphate 8, estimated from the absorbance at 368 nm, was 19.0 μmoles.

IV.5.6. Attempted preparation of 4-nitro-1-ribofuranosylindole-5' triphosphate 10

To P^1-diphenyl-P^2-(1-ribofuranosyl-4-nitroindole-5')-

pyrophosphate 25 (from 50 μmoles of nucleotide 6) was added 1.0 ml of 0.2 M tetrakis(tributylammonium)pyrophosphate in dry DMF. The mixture was shaken and left at room temperature for 48 hours. The solvent was removed under high vacuum and the residue dissolved in 10 ml of water. Thw aqueous solution was extracted once with 5 ml of ether, made basic with ammonia, and extracted with ether (3 X 5 ml). The aqueous solution was evaporated to dryness under high vacuum.

The nucleotide mixture was dissolved in a minimum of 0.02 M ammonium bicarbonate and chromatographed on DEAE-cellulose (bicarbonate) in the same manner as the previous nucleotide 8. The products were eluted with a linear gradient of ammonium bicarbonate (0.02 M to 0.6 M, 350 ml each). The plot of absorbance at 368 nm versus tube number showed that the nucleotides were not completely resolved (Figure 20, page 48). Fractions 92-126 were pooled and evaporated to dryness under high vacuum. After removal of excess ammonium bicarbonate by sublimation, the nucleotide mixture was dissolved in 0.1 M ammonium bicarbonate and applied to a column of DEAE-cellulose (1.5 X 20 cm) equilibrated in the same solvent. The column was eluted with a linear gradient of ammonium bicarbonate (0.1 M to 0.5 M, 350 ml each); 5-ml fractions were collected.

The plot of absorbance at 368 nm versus tube number showed a small broad peak spread over tubes 40-80 (Figure 21, page 49). The loss of optical density at 368 nm compared to the original mixture as well as the failure to resolve the components caused the abandonment of the process.

IV.6. <u>Enzyme reactions</u>

IV.6.1. <u>Snake venom 5'-nucleotidase</u>

Nucleoside-5' monophosphate (3-6 mg) was dissolved in 1.0 ml of tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer, pH 8.0, 0.04 M in magnesium chloride. To this solution was added 1.0 mg of lyophilized *Crotalus adamanteus* venom (Sigma Chemical). The mixture was shaken and incubated at room temperature. The reaction was monitored using thin layer chromatography. An aliquot (10 μl) of the nucleotide solution prior to the addition of venom was applied to a cellulose TLC plate. After a suitable interval, another aliquot (10 μl) was applied to the same plate and the plate developed in an appropriate solvent.

IV.6.1.1. <u>Benzimidazole nucleoside-5' phosphate 5</u>

4-Amino-1-ribofuranosyl benzimidazole-5' phosphate 5 after a 2 hour incubation with venom was completely cleaved to the nucleoside. TLC (butanol-acetone-acetic acid-5% ammonia-water, 7/5/3/3/2, by volume) showed only one spot, Rf 0.80, identical to a sample of 4-amino-1-ribofuranosyl-

benzimidazole which had been prepared for the purpose of comparison. The nucleotide in the same solvent had an Rf of 0.68.

IV.6.1.2. Nitroindole nucleoside-5' phosphate 6

4-Nitro-1-ribofuranosylindole-5' phosphate 6 showed no apparent cleavage of phosphate after 24 hours (TLC; cellulose in 1 M ammonium bicarbonate). Addition of an equivalent solution of 6 to a Tris buffer solution of AMP 12 and venom resulted in a significant inhibition of the cleavage of phosphate from adenosine after 2 hours. In the absence of 6, AMP was completely cleaved to adenosine and inorganic phosphate in two hours.

IV.6.1.3. Aminoindole nucleoside-5' phosphate

A sample of nitroindole nucleotide 6 in aqueous methanol was hydrogenated using 5% Pd/C as the catalyst. The catalyst was removed by filtration and the solvent evaporated. The aminoindole nucleotide was dissolved in Tris buffer, venom added and the mixture incubated at room temperature. TLC on PEI-cellulose in 1 M lithium chloride gave no clear indication of phosphate cleavage. Analysis of aliquots for inorganic phosphate with the Fiske-Subbarow reagent (Gunsalus, 1959) indicated some release of inorganic phosphate.

IV.6.2. Reaction with adenylate kinase

IV.6.2.1. TLC analysis

Nucleoside-5' diphosphate (2 mg) was dissolved in 1.0 ml of 0.1 M Tris hydrochloride buffer (pH 7.6), 0.1 M in potassium chloride and 0.01 M in magnesium chloride. To the solution was added 100 μg of adenylate kinase (obtained from Sigma Chemical as a suspension in ammonium sulfate). The solution was incubated at room temperature for 100 minutes.

Aliquots (10 μl) of each reaction mixture were spotted on PEI-cellulose plates together with the appropriate standards (monophosphate, diphosphate and triphosphate). The plates were developed in 1 M lithium chloride.

ADP 13 and adenylate kinase showed three spots after incubation, AMP 12 (Rf 0.64), ADP 13 (Rf 0.57) and ATP 14 (Rf 0.28).

Benzimidazole nucleoside-5' diphosphate 7 and adenylate kinase showed only one spot, 7, Rf 0.43.

A mixture of AMP 12 and benzimidazole nucleoside-5' triphosphate (1 mg each) under the same conditions showed four spots, AMP (Rf 0.64), ADP (Rf 0.57), 7 (Rf 0.43) and triphosphate 9 (Rf 0.21).

IV.6.2.2. Adenylate kinase kinetics

Kinetic information was obtained by coupling the adenylate kinase reaction to pyruvate kinase and lactate dehydrogenase and measuring the decrease in absorbance at 340 nm as NADH was oxidized. The buffer used was 50 mM Tris hydrochloride, pH 7.7, 25 mM in potassium chloride, 5 mM in magnesium chloride, 1 mM in phosphoenol pyruvate, 0.2 mM in NADH and 1 mM in AMP.

Solutions of nucleoside triphosphate were prepared using the stock buffer as solvent. Concentration of triphosphate was varied by dlution with stock buffer. Each reaction was initiated by the addition of pyruvate kinase-lactate dehydrogenase followed by adenylate kinase. Each assay solution (total volume 1 ml) contained 5 units of lactate dehydrogenase, 2.5 units of pyruvate kinase and 0.0235 unit of adenylate kinase. (1 unit of enzyme is defined as that quantity which will convert 1 μmole of substrate to product in one minute.)

Concentrations of ATP varied between 0.01 mM and 0.1 mM. Benzimidazole nucleotide 9 concentrations varied between 0.1 mM and 1.2 mM.

Initial rates of conversion were determined. Values of V_{max} and K_M were calculated by the double reciprocal method (Lineweaver and Burk, 1934) using the least-squares

method to obtain values for the slope and intercept.

IV.6.3. Pyruvate kinase kinetics

Pyruvate kinase was assayed by coupling to lactate dehydrogenase and measuring the decrease in absorbance at 340 nm as NADH was oxidized. Stock buffer contained 100 mM Tris hydrochloride, pH 7.7, 100 mM potassium chloride, 5 mM magnesium chloride, 2 mM phosphoenol pyruvate and 0.2 mM NADH. Nucleoside diphosphate solutions were prepared in stock buffer. The diphosphate concentration was varied by dilution with stock buffer. Assays were performed by placing 1.0 ml of the desired diphosphate concentration in a cuvette and adding lactate dehydrogenase (0.625 unit)-pyruvate kinase (0.313 unit) to initiate the reaction.

The concentration range for both ADP and benzimidazole diphosphate 7 was 0.1 mM to 1.2 mM.

Initial rates of reaction were determined. Values of V_{max} and K_m were calculated as in the previous section.

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