SOME ENZYMES INVOLVED IN THE BIOSYNTHESIS OF THE NUCLEOTIDES OF PURINES, PYRIMIDINES AND BENZIMIDAZOLE IN WHEAT EMBRYOS

A Thesis
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for the Degree of
Doctor of Philosophy

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Manju Kapoor
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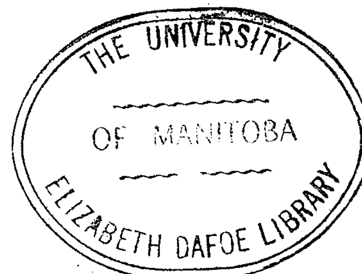

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ABBREVIATIONS

The following abbreviations have been used in the text: ATP, ADP and AMP for adenosine, tri-, di-, and monophosphate; NAD and red NAD, oxidized and reduced forms of nicotinamide adenine dinucleotide (DPN and DPNH); NADP, nicotinamide adenine dinucleotide phosphate (TPN); FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; PP-ribose-P and PRPP, 5-phosphoribosyl-1-pyrophosphate; R5P, ribose-5-phosphate; CP, carbamyl phosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribotide; FGAR, formylglycinamide ribotide; FGAM, formylglycinamidine ribotide; AICAR, 5-amino-4-imidazole carboxamide ribotide; O-5-P and OMP, orotidine monophosphate; DHO, dihydroorotate; UMP and UTP, mono-, and triphosphates of uridine; deoxy-UMP, monophosphate of deoxyuridine; CTP, cytidine triphosphate; TMP, thymidine monophosphate; BR, benzimidazole ribonucleoside; BMN, benzimidazole mononucleotide; BAD, benzimidazole adenine dinucleotide; TLC, thin layer chromatography; IAA, indoleacetic acid.

ABSTRACT

Although the pathways of biosynthesis of purine and pyrimidine nucleotides have been investigated in great detail in animal tissues and microorganisms, the corresponding reactions in plants have received little attention, until very recently. Therefore, in a study of the initial reactions of purine biosynthesis an attempt was made to determine the extent of similarity exhibited in these steps to those occurring in animal and bacterial systems. A comparison of the activity of various amide group donors in the formation of glycinamide ribonucleotide revealed that in wheat embryos asparagine was the most efficient of the donors as opposed to the situation in bacteria and animals.

Orotidine monophosphate pyrophosphorylase, an enzyme catalyzing the conversion of orotic acid to orotidine monophosphate, was studied in detail with the object of elucidating a similar reaction suspected to be undergone by benzimidazole. Since orotic acid reacts with phosphoribosyl pyrophosphate, it was argued that if benzimidazole was also involved in a similar reaction and also if the enzymes catalyzing the two reactions were present in the same preparation, orotic acid and benzimidazole would compete for PP-ribose-P. Based on this assumption an assay was devised for the conversion of benzimidazole to its mononucleo-

tide (BMN) and the enzyme catalyzing this reaction was referred to as BMN pyrophosphorylase, also it was assumed that pyrophosphate was a product of the reaction. BMN was isolated and analyzed chemically.

Another reaction of benzimidazole, the formation of benzimidazole adenine dinucleotide (BAD), catalyzed by an enzyme preparation obtained from wheat embryos was demonstrated. A major part of the enzyme was found to be confined to a particulate fraction. Almost immediately after being formed, the dinucleotide was broken down resulting in benzimidazole ribonucleoside and ADP. The possible significance of these reactions, in light of the recent work, has been discussed.

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I. GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

The text of this manuscript includes investigations on the initial steps of purine biosynthesis, orotic acid metabolism and metabolism of benzimidazole. A study of two of the reactions of benzimidazole, i.e. the formation of benzimidazole dinucleotide and that of benzimidazole adenine dinucleotide was the major subject of interest in this investigation. The enzymes of orotic acid metabolism were studied with the chief purpose of devising an assay system for the formation of benzimidazole mononucleotide. The experiments on the initial steps of the biosynthesis of purine nucleotides were performed simply as a sideline and a matter of curiosity.

II. THE INITIAL STEPS OF PURINE BIOSYNTHESIS

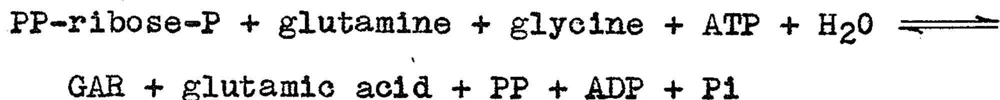
INTRODUCTION

In biological systems purine nucleotides are synthesized from small precursor molecules. With the application of radioisotope methodology and elaboration of procedures for the isolation and characterization of individual enzyme systems the pathway of purine biosynthesis has been established during the last decade or so. The precursors of individual atoms of the purine ring were determined by feeding compounds labelled with isotopes of carbon and nitrogen (Chargaff and Davidson, 1960). The origin of the carbon and nitrogen atoms of the purine ring is represented in Figure 1. The carboxyl carbons and the N atoms of glycine contribute to the carbons 4 and 5 and nitrogen 7 of the purine molecule. Carbon dioxide is the precursor of the carbon 6 and formate of carbons 2 and 8. Further investigations led to the clarification of the question of the immediate precursor of the nitrogen atoms (Buchanan *et al.*, 1957), showing thereby that aspartic acid contributed to N at position 1 and the amide group of glutamine to N atoms at positions 9 and 3.

The early studies contributing to the formulation of the scheme are those of Goldthwait *et al.* (1954), reporting the isolation of glycinamide ribotide (GAR). They substantiated the previous indication that ribonucleotides participated in the initial stages of the pathway.

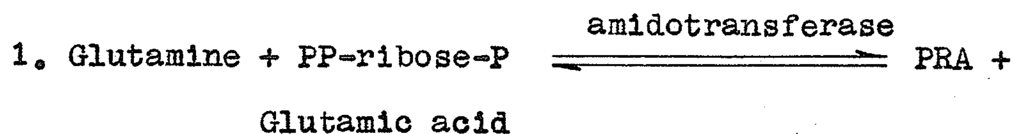
Using an acetone powder extract of pigeon liver they further demonstrated glycine, glutamine, ATP, and ribose-5-P to be necessary for the synthesis of GAR. In the following year Hartman et al. (1955) showed PP-ribose-P to be a more immediate precursor of GAR than ribose-5-P.

Purification and fractionation of a soluble protein extract from avian liver (Levenberg et al., 1955) led to the conclusion that two separate enzyme preparations were required for GAR formation from glutamine, glycine and ATP, and PP-ribose-P, the reaction sequence being represented as;



PP-ribose-P + glutamine + glycine + ATP + H_2O $\rightleftharpoons$
GAR + glutamic acid + PP + ADP + Pi

This complex reaction is now known to proceed in two steps (Goldthwait et al., 1955). In other words, in animal tissues the first reaction of the purine biosynthetic pathway is the amidation of PP-ribose-P to form PRA. This highly unstable compound reacts immediately with glycine and ATP to give rise to GAR. The two reactions are catalyzed by PP-ribose-P amidotransferase and GAR kinosynthase respectively (Goldthwait et al., 1956; Hartman and Buchanan, 1958):



1. Glutamine + PP-ribose-P $\xrightleftharpoons{\text{amidotransferase}}$ PRA +
Glutamic acid

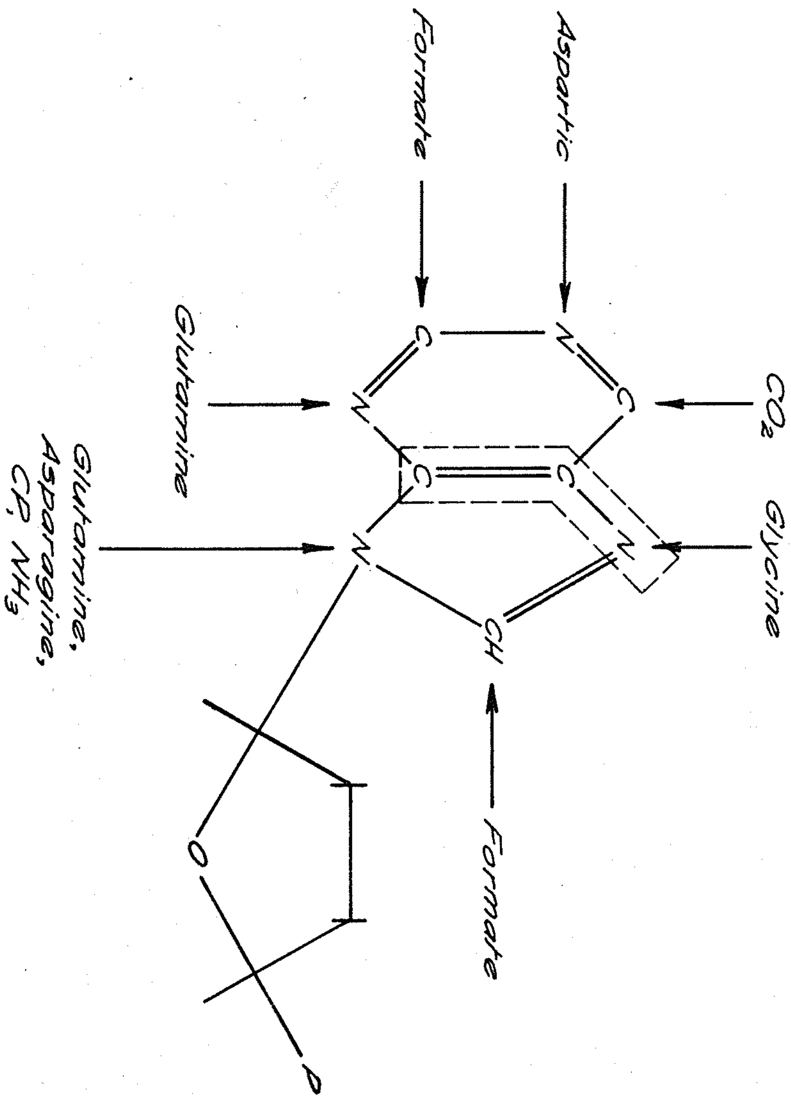

Aspartic
Formate
CO_2
Glycine
Glutamine
Glutamine,
Asparagine,
CP, NH_3
Formate

Figure 1. The precursors of carbon and nitrogen atoms of the purine ring.

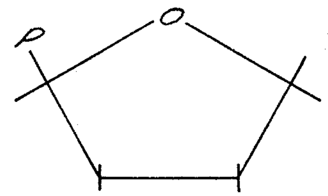
2. PRA + glycine + ATP $\xrightleftharpoons{\text{GAR kinosynthase}}$ GAR

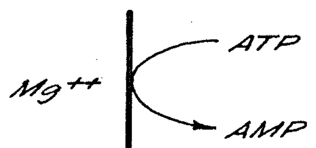
(Figure 2)

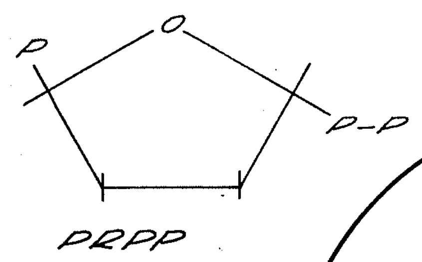
Hartman et al. (1956) isolated and purified GAR and FGA[R] formed in the presence of glycine and ATP by enzymes of pigeon liver by using azaserine. The latter causes an accumulation of GAR and FGAR by competitively inhibiting the enzyme catalyzing the conversion of FGAR to FGAM. In their system glutamine was found to be the only effective donor of the amide group in the first reaction. Alternate potential donors like asparagine, ammonium ion, aspartic and glutamic acids failed to react. However, the situation is somewhat different in bacterial systems. Nierlich and Magasanik (1961) reported the formation of GAR from glycine and ATP in the presence of chicken liver extract or a crude extract from *Aerobacter aerogenes* both with PP-ribose-P and glutamine and also from R5P and ammonia. The same is true of extracts from *Escherichia coli*. In these systems ammonia was demonstrated to be a better donor of amide group in the bacterial preparations used, although glutamine was also active.

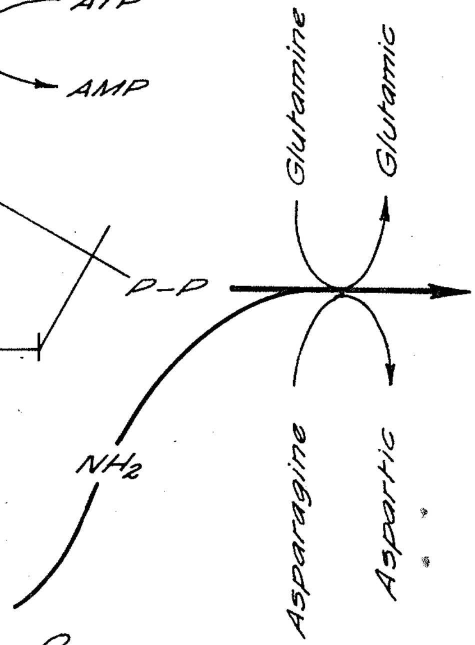
The pathways operating in the animal and bacterial systems are evidently different. A perusal of the existing literature showed no evidence as to which of these donor/acceptor systems participates in plant materials.

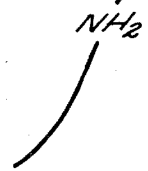
Figure 2. The first steps of purine biosynthesis.



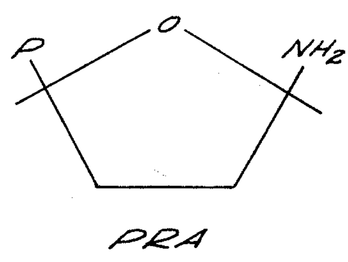






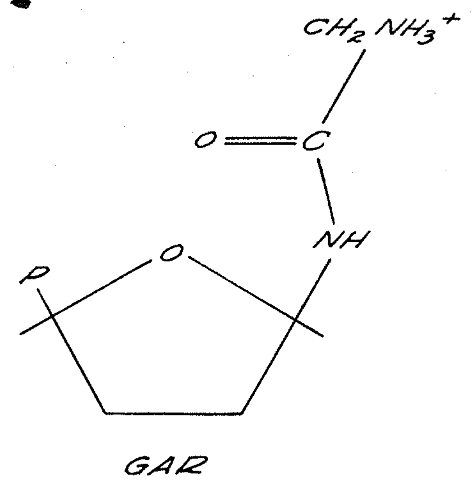


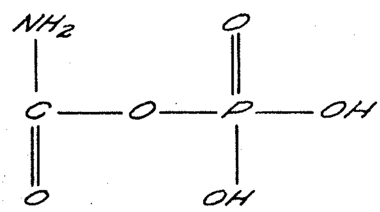
NH_3 + Ribose
+ ATP



Glycine ATP

ADP P





The present study was an attempt to elucidate the first steps of purine biosynthesis in a representative of the plant kingdom. A comparison of the activity of four amide group donors, namely, glutamine, asparagine, carbamyl phosphate, and ammonium ion was made using enzyme preparations obtained from commercial wheat germ and viable wheat embryos.

EXPERIMENTAL

MATERIALS

Commercial wheat germ for the enzyme preparations was purchased from Maple Leaf Milling Company, Winnipeg, and was stored in a cold room at 4°C. Wheat embryos were isolated from the wheat variety Selkirk.

Glutamine, asparagine, carbamyl phosphate, ATP and PP-ribose-P were purchased commercially (Sigma Chemical Co.). Glycine-1, and 2-C^{14} was obtained from Calbiochem.

METHODS

ISOLATION OF WHEAT EMBRYOS

Embryos were isolated from seeds of wheat variety Selkirk by a modification of the procedure of Johnston and Stern (1957). Two hundred and fifty grams of wheat seeds previously stored at -12°C were ground in a waring blendor for 20 seconds. The homogenized materials were screened twice through 10, 14 and 28 mesh sieves (Tyler scale). The portion collecting between 14 and 28 mesh was separated from the bran with a seed blower. The remaining portion was then floated on a mixture of cyclohexane and carbon tetrachloride (2:5: v/v). The starchy

endosperm fragments settled at the bottom leaving the whole embryos and parts on the surface. The embryos were picked up from the surface, air dried and stored at 4°C. The embryos thus isolated and separated by organic solvent mixture retained almost complete viability, showing a high germination percentage.

PREPARATION OF ENZYMES

Enzyme I: - PP-ribose-P amidotransferase was prepared by a slight modification of the method of Goldthwait and Greenberg (1955). Ten grams of acetone powder of commercial wheat germ was extracted with 50 ml of phosphate buffer (0.05M), pH 7.4, at 0°C for 40 minutes. The mixture was centrifuged, the supernatant diluted to twice the volume and 1 M potassium acetate buffer was added gradually to lower the pH to 5.5. The precipitate was then removed by centrifugation, and the pH of the supernatant lowered again to 5.0 with 1 N acetic acid. The solution was dialysed against 0.05 M K_2HPO_4 for 4 hours. To 45 ml of the supernatant 11.2 ml of precooled (-40°C) absolute ethanol was added and the temperature lowered to -50°C. The precipitated protein was separated by centrifugation at 20,000 x g and dissolved in Tris - HCl buffer (0.02 M), pH 8.0.

Enzyme II: - Five grams of wheat embryos were homogenized in a mortar with 50 ml of 0.05 M phosphate - 0.1 M cysteine buffer, pH 7.4. The homogenate was passed through four layers of cheesecloth and centrifuged at 10,000 x g for 10 minutes. The pH of the supernatant was adjusted to 7.4 with ammonium hydroxide and enough ammonium sulphate was added to make it up to 0.2 saturation. To the supernatant obtained after centrifugation at 20,000 x g for 10 minutes more ammonium sulphate was added to make up to 0.45 saturation. The protein precipitating between 20-45% saturation of ammonium sulphate was dissolved in 50 ml water and refractionated from ammonium sulphate twice. The precipitate obtained after the third fractionation was dialysed against distilled water overnight. The protein was then dissolved in Tris (0.02 M), pH 8.0.

Enzyme III:- Fifty grams of commercial wheat germ was ground with 250 ml of phosphate buffer (0.05 M) at pH 7.0 in a mortar and centrifuged at 10,000 x g for 10 minutes. To the supernatant 0.2 volume of precooled 90% ethanol was added and the precipitate discarded. To the supernatant an additional 0.25 volume of 90% ethanol was added and the precipitate collected and dissolved in 5 ml of Tris (HCl 0.02 M), pH 8.0.

Enzyme IV:- Five grams of viable wheat embryos were homogenized with 50 ml of phosphate buffer (0.05 M), pH 7.4, and centrifuged at 10,000 x g for 10 minutes. The pH of the supernatant was lowered to 5.5 with 1N acetic acid and the precipitate was discarded. Ammonium sulphate precipitation was then carried out and the protein fraction precipitated at a saturation of 0.33 - 0.65 was collected after centrifugation at 20,000 x g and dissolved in tris HCl buffer (0.02 M), pH 8.0

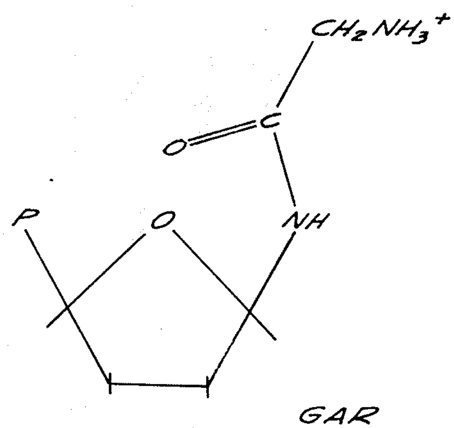
PROTEIN DETERMINATION

Protein determinations were done by the micro-kjedahl procedure throughout the study, assuming 16 percent nitrogen.

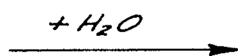
PAPER ELECTROPHORESIS

Paper electrophoresis was conducted in a Spinco Durrum cell at 25 milliamperes, in formate buffer (0.05 M), pH 3.5, in the cold room.

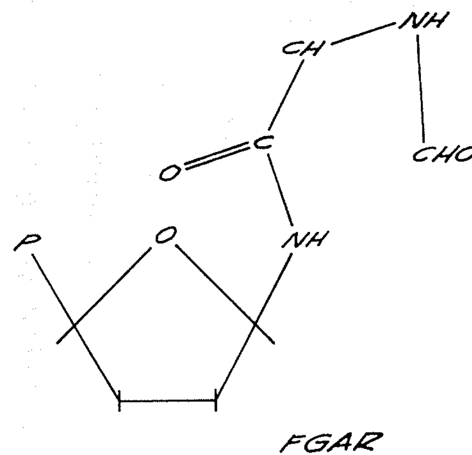
Figure 3. Determination of glycinamide ribonucleotide.



CH2NH3+
O
C
P
O
NH
GAR



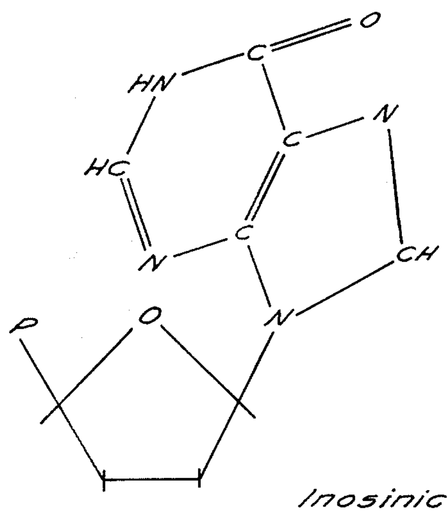
+ H2O



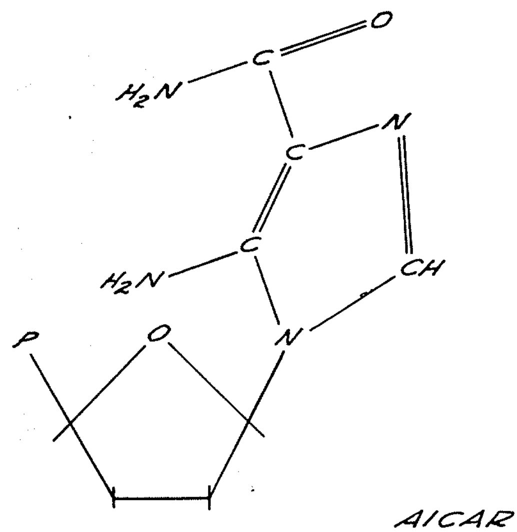
CH
NH
CHO
O
C
P
O
NH
FGAR

+

+



O
C
HN
N
C
HC
C
N
CH
N
P
O
Inosinic



O
C
H2N
N
C
C
H2N
CH
N
P
O
AICAR

RESULTS AND DISCUSSION

The reaction was conducted in three stages. The first two stages consisted in the formation of PRA which is subsequently converted to GAR. The third stage involves the procedure for estimation of GAR formed in the first two and is not directly participating in the reaction. In principle the reaction mixtures containing the amide donor and acceptor systems in appropriate amounts were incubated and the efficiency of each combination was assessed by determination of the quantity of GAR formed. The acceptors, PP-ribose-P and ribose-5-P were permitted to react separately with each of the four donors, i.e., asparagine, glutamine, carbamyl phosphate and ammonia.

FORMATION OF GAR

The first stage of reaction was initiated by incubating the mixture for 50 minutes at 37°C, the components were, Tris-HCl buffer, pH 8.5, 60µ moles; $MgCl_2$ 4µ moles; glutathione 1µ mole; enzyme I and II 0.2 ml each (protein 17 and 21 mg/ml respectively); and one of the amide donors, asparagine, glutamine, carbamyl phosphate or ammonium chloride 4µ moles; in a total volume of 0.6 ml. The reaction was started by adding 2.68µ moles of PP-

ribose-P to the system at zero time or alternately 4 μmoles of ribose-5-P and 2 μmoles of ATP.

At the end of the first stage the pH of the reaction mixture was lowered to 7.0 by the addition of 100 μmoles of KH_2PO_4. The second stage incubation was then continued after adding 4.0 μmoles of glycine and 2 μmoles of ATP and 0.2 ml of enzyme preparation III (protein 40 mg/ml) for another 45 minutes. In another series of experiments the components of the first and the second were incubated simultaneously and the system was referred to as the 'complete system'.

DETERMINATION OF GAR

Aliquots from stage 2 and the 'complete' system were withdrawn and the amount of GAR present was estimated by a slight modification of the procedure of Hartman and Buchanan (1958). GAR is assayed by reacting it with IMP which acts as one-carbon donor to GAR and is itself converted to 5-amino4-imidazole carboxamide ribotide (Figure 3). The enzyme preparation catalyzing the reaction is a crude mixture of inosinicase, carboxamide ribotide transformylase, cyclohydrolase and glycinamide ribonucleotide kinosynthase. Since AICAR is an aminoimidazole it is capable of undergoing diazotization and coupling with

N-1-naphthylenediamine dihydrochloride and yields a pinkish purple product which can be measured spectrophotometrically (Bratton and Marshall, 1939).

After the first two steps the following were added to the reaction mixture: EDTA 30 μmoles; sodium inosinicate 2.0 μmoles and 0.2 ml of enzyme IV (protein 35 mg/ml). After incubation for 45 minutes at 37°C the mixture was deproteinized with 0.1 ml of 30 percent trichloroacetic acid and the precipitate removed. The solution was then treated with acetic anhydride for 20 minutes. Thus the aromatic amines present are acetylated leaving behind the aminoimidazole compounds unacetylated. Then 0.3 ml of 1 N H_2SO_4; 1 percent $NaNO_2$ 0.1 ml and after 5 minutes 0.1 ml of 0.5 percent of ammonium sulphate and 0.1 ml of 0.1 percent solution of N-1-naphthalenediamine dihydrochloride were added. The coloured complex which resulted was measured 540 mμ . An absorbancy of 0.204 represents empirically 0.01 μmole of GAR. The molecular extinction coefficient for 5-amino imidazole carboxamide ribotide is 26,400.

Results of the two experiments with ribose-5-P and PP-ribose-P as acceptors and various amide donors are presented in Table I.

TABLE I. Effect of various amide donors on formation of GAR.

Amide donor	µmoles of GAR formed with ribose-5-P (A)	ribose-5-P (B)	PP-ribose-P (A)	PP-ribose-P (B)
Glutamine	2.00	0.00	7.20	4.40
Asparagine	10.10	1.40	21.00	17.00
NH_4Cl	12.30	9.90	2.45	0.98
Carbamyl phosphate	0.00	0.00	13.20	7.00

From these results it is evident that:-

a. The yield of GAR is greater if the systems are preincubated for PRA formation, i.e. a three-stage (A) instead of a two-stage (B) incubation. This is more pronounced in the case of asparagine.

b. Asparagine is the most efficient of the donors tested in combination with PP-ribose-P.

c. Ammonium chloride is the next in order of yield of GAR. It is reactive largely with ribose-5-P as the acceptor. The amount of GAR formed with PP-ribose-P is almost negligible, and that too if there is a preincubation for PRA.

d. Only a small amount of GAR is formed with glutamine/PP-ribose-P combination.

e. Carbamyl phosphate donated the amide only to

PP-ribose-P and not at all to ribose-5-P.

It follows from b. and c. that asparagine and carbamyl-P participate directly in the first step of purine biosynthesis and not through conversion to glutamine and/or ammonia. It is reasonable to assume that each of the donors is associated with a specific enzyme and acceptor. The possibility of the contamination of the enzyme preparation by PP-ribose-P synthesizing enzyme cannot however, be overlooked. This would explain the slight reactivity of asparagine and glutamine with ribose-5-P.

The formation of GAR was also confirmed by paper chromatography and paper electrophoresis. Figure 4 represents scan from radioelectropherograms of the four reaction mixtures incubated with glycine-2-C^{14}. A, B, C, and D represent samples from reaction mixtures with glutamine, asparagine, CP and NH_4Cl as amide donors. R is the glycine reference. In all the four cases peaks 1 and 2 represent the two isomeric forms of GAR (Levenberg et al., 1956). In this particular experiment the peaks were almost the same height in all the systems, and the fact that asparagine was a better donor is not evident here.

The present knowledge of intermediates and enzymic

Figure 4. Formation of GAR from glutamine, asparagine, CP and Ammonium chloride.

R is a scan of reference glycine-2-C^{14}

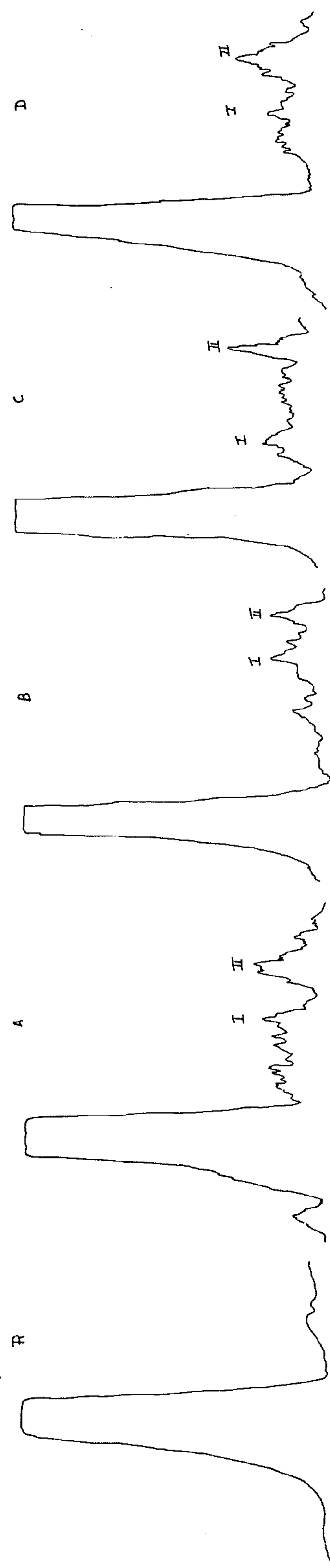
A represents scans from radioelectropherogram of a reaction mixture containing glycine-2-C^{14} and glutamine as the amide group donor

B the same as A with asparagine as the amide donor

C contains NH_4Cl as the amide donor

D contains CP as the amide donor

I and II are the two isomeric forms of GAR


R
A
I
II
B
I
II
C
I
II
D
I
II

reactions of the purine biosynthesis pathway is based, mainly if not entirely on investigations on animal and microbial systems (Carter, 1956; Buchanan and Hartman, 1959; Reichard, 1959; Chargaff and Davidson, 1961). The fact that till recently very little was known concerning the purine biosynthesis in plant has been emphasized (Wang and Waygood, 1962). The reviewers in this field have tended to present a generalized, unified and composite picture applicable to a wide variety of biological systems. Mechanisms of purine and pyrimidine biosynthesis and of intermediate steps in plants have been inferred largely on the basis of an overall similarity to the system described in mammalian and bacterial systems (Moat and Friedman, 1960). Even though showing a similarity to animal and microbial metabolism in a broad sense, plants, especially higher plants, do have distinctive features of intermediary metabolism and also differences of a lower order as illustrated by the donor/acceptor interrelationships in initial steps of purine biosynthesis in wheat embryos. It is too early to draw a general conclusion with respect to all the plants or even to plants of one particular group. Plant tissues do accumulate significant quantities of asparagine in contrast to bacteria,hence the opportunity to utilize the source

available. Besides, there is very little reason for glutamine and not asparagine to be able to donate amide group in this reaction. The close similarity in the structure of the two is an argument against any such theory.

III. OROTIC ACID METABOLISM

INTRODUCTION

The sequence of biosynthetic reactions involved in pyrimidine metabolism has been established on the basis of information gained from the work with micro-organisms (Reichard, 1959; Crosbie, 1961). The implication of orotic acid in the synthesis of pyrimidine nucleotides in bacteria and animal tissues emerged as a result of several investigations (Wright *et al.*, 1951; Hurlbert and Potter, 1952) and the fact that orotic acid could replace the pyrimidine requirement of several bacteria led to the assignment of a central role to orotic acid in the pyrimidine biosynthetic pathway.

Reichard and Lagerkvist (1953) studied the incorporation of N^{15} labelled ammonium chloride, C^{13}-bicarbonate, L-aspartate N^{15}; L-aspartate-1,4-C^{13}; L-aspartate-2,3,-C^{14} and L-carbamyl aspartate-N^{15} into orotic acid in rat liver slices. These results, in addition to the conclusions of the previous workers confirmed aspartic acid, carbamyl aspartate, and orotate to be the precursors of UMP in bacteria and animal tissues. The first step in the formation of the pyrimidine ring is the synthesis of carbamyl aspartate. This compound was demonstrated to originate from carbamyl phosphate which is synthesized

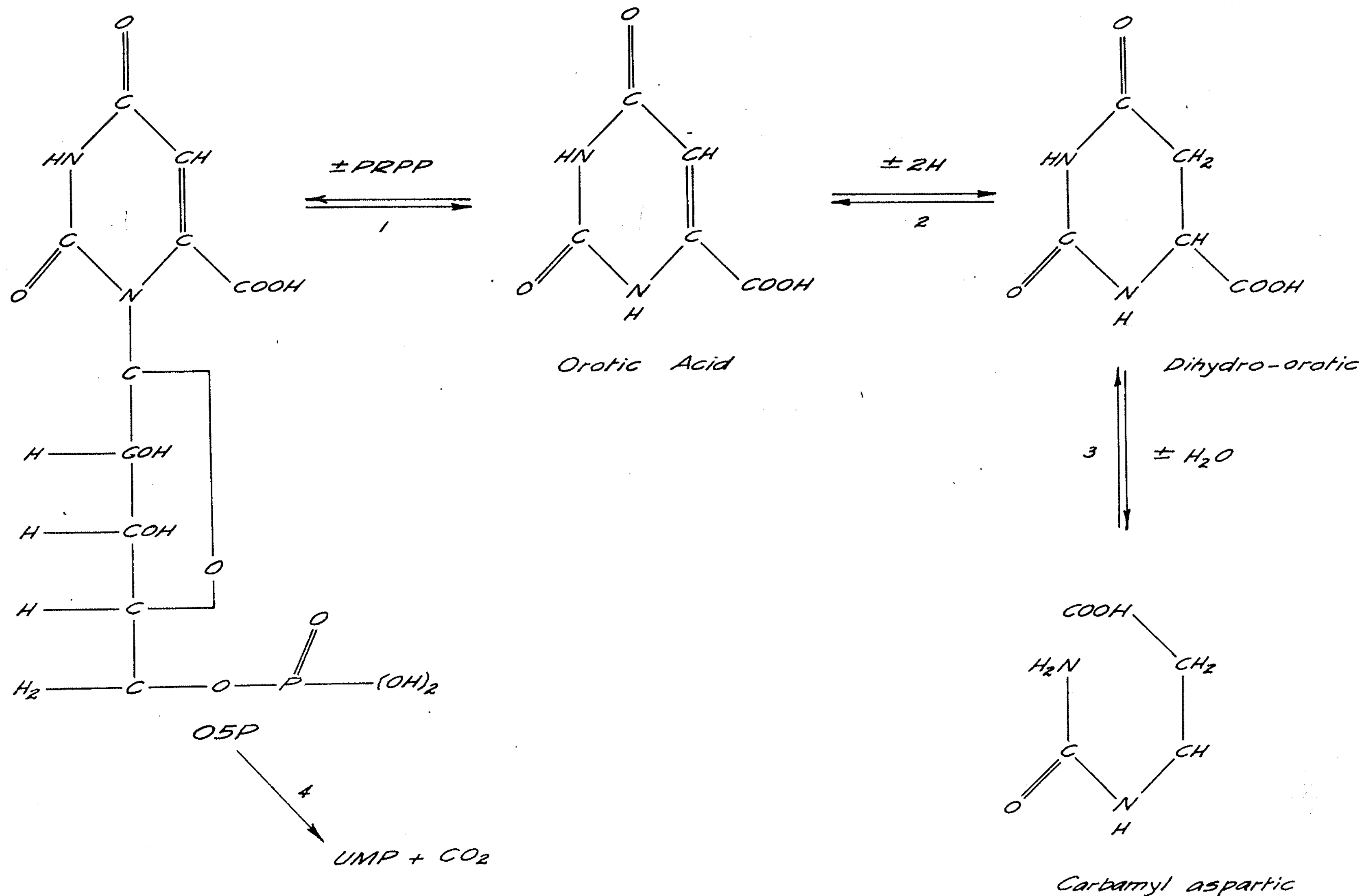
from small precursor molecules or alternately with an active form of carbamyl compound (Cohen and Grisola, 1948) participating in a condensation reaction with aspartate in the presence of aspartate carbamyl transferase (Reichard and Hanschoff, 1956). The enzyme catalyzing the ring closure in the next step to give dehydroorotate was discovered by Lieberman and Kornberg in Zymobacterium oroticum obtained from soil in enrichment culture media, in the presence of exogenous supply of orotic acid (Lieberman and Kornberg, 1955).

From the same organism Lieberman and Kornberg (1953) isolated another enzyme, dihydroorotic dehydrogenase which catalyzes the reduction of orotic to dehydroorotic acid in the presence of red NAD (Figure 4).

Friedman and Vennesland (1958) purified dihydroorotic dehydrogenase and showed the enzyme to be a flavoprotein. The presence of a sulphyhydryl group compound such as cysteine was found necessary for enzyme activity. The bacterial enzyme was demonstrated to catalyze the reduction of orotate and also of fluoroorotate by red NAD, the oxidation of dihydro-orotic acid by NAD or acetylpyridine - NAD and the oxidation of reduced dihydroorotate and reduced NAD by oxygen and methylene blue. The enzyme was subsequently crystallized and shown to contain FAD

Figure 5. Metabolism of orotic acid.

1. represents orotidine-5-phosphate pyrophosphorylase
2. dihydroorotic dehydrogenase
3. dihydroorotase



METABOLISM OF OROTIC ACID

and FMN in equal amounts along with iron as the metal component in the enzyme molecule (Friedman and Vennesland, 1960). Purification of NADP specific dihydro-orotic dehydrogenase from aerobic bacteria has recently been reported from the same laboratory (Udaka and Vennesland, 1962).

The orotic acid thus formed both in animals and bacterial tissue is converted to UMP through a ribotidation step followed by decarboxylation. The remaining pyrimidine nucleotides arise from UMP by interconversions; CTP arises by amination of UTP and TMP through deoxy-UMP.

OMP pyrophosphorylase catalyzes the conversion of orotic acid into OMP by condensation with 5-phospho-ribosyl-1-pyrophosphate (Liebermann et al., 1955). By a further decarboxylation of OMP in the presence of orotidine-5-phosphate decarboxylase, UMP is formed.

Blair, Stone and Potter (1960) have presented evidence with studies on a multienzyme system to demonstrate that OMP is an intermediate in the formation of UMP form orotic acid in rat liver also.

The present report embodies an investigation into the orotic acid metabolism of wheat embryos. The general pattern of metabolism and the intermediates formed was studied by following the fate of orotic-6-C^{14} in homogenates

and purified protein extracts of wheat embryos. A study of the enzymes participating in some reactions of orotic acid, namely, dihydroorotic dehydrogenase and OMP pyrophosphorylase was also conducted. The last named enzyme system was of special interest here because of reasons which will be obvious later.

EXPERIMENTAL

MATERIALS

The wheat embryos were isolated according to procedure outlined in part 1.

The following chemicals were purchased commercially: orotic acid and dihydroorotic acid (Nutritional Biochemical Corporation), NAD, red NAD, and PP-ribose-P (Sigma Chemical Co.). Orotic-6-C^{14} was purchased from California Corporation for Biochemical Research. All compounds were used without further purification.

Orotic acid and derivatives were detected on paper chromatograms by means of an Ultra violet lamp purchased from Edmund Scientific Co., N.J. Quantitative detection of compounds eluted from paper as well as of reference samples was accomplished by Beckman-DU recording spectrophotometer.

Radioactivity was determined by using a Nuclear-Chicago semiautomatic counter Model 186 fitted with a windowless gas flow counter tube or a Nuclear-Chicago actigraph Model C-100B having a windowless counter tube (continuous gas flow).

Kodak no-screen x-ray film was used for recording radioactive areas on paper chromatogram.

METHODS

General - Orotic acid and derivatives were chromatographed both in reaction mixtures and for reference purposes using Butanol; Ethanol; formic acid; water (5:3:2:1) as the solvent in a descending system (Fink et al., 1959). The individual compounds were detected on paper by spraying with Ehrlich reagent.

DIHYDROOROTIC DEHYDROGENASE

Preparation of enzyme

Ten grams of viable wheat embryos were homogenized in a mortar with 100 ml of K_2HPO_4 (0.05 M), cysteine (0.01 M) buffer, pH 7.4. The extract was then passed through four layers of cheesecloth and residue discarded. The supernatant was centrifuged at 20,000 x g for 10 minutes. To the supernatant, solid ammonium sulphate was added gradually to raise the saturation to 0.33 and the protein precipitate was removed by centrifugation. The supernatant was now made 0.65 saturated with regard to ammonium sulphate and the precipitated protein was dissolved in 10 ml of phosphate buffer (0.02 M), pH 6.5.

Assay of DHO-Dehydrogenase

Lieberman and Kornberg (1953) devised an assay system utilizing the measurements of the decrease in UV absorption at 280 mμ accompanying the reaction of orotate

to dihydroorotate. In the present study an assay system based on the one used by Friedman and Vennesland (1960) was employed.

The assay system contained the following:

Sodium phosphate buffer, pH 6.5	200 µmoles
$MgCl_2$	6 µmoles
Cysteine hydrochloride	20 µmoles
Sodium orotate (recrystallized)	0.6 µmoles
Red NAD (3×10^{-3} M)	0.1 ml

and 0.1 ml of the enzyme containing approximately 0.5 - 1.0 mg protein in a total volume of 3.0 ml. The reaction was carried out in silica cuvettes of 1 cm light path. The reaction was started by the addition of orotate to the cuvettes and the reaction rate was measured by following the decrease in optical density at 280 mµ accompanying the disappearance of orotate.

Cysteine solution was freshly prepared by dissolving cysteine-HCl in water and neutralizing it with NaOH. Sodium orotate was prepared from orotic acid, recrystallized from water.

Results

Figure 5 shows a typical progress curve for the reaction. The reaction rate is linear approximately for the first one minute after which it decreases steadily

till an equilibrium is attained.

Effect of substrate and enzyme concentration

The rate of the reaction was studied as a function of orotate concentration. Figure 6 shows that concentrations above 6.5×10^{-5} are inhibitory to the enzyme. Friedman and Vennesland (1958) have reported K_m values of 1.3×10^{-4} for both orotate and 5-fluoroorotate. Their values for orotate are in good agreement with that obtained by Lieberman and Kornberg (1953) (1.1×10^{-4} M). In the present study the K_m was found to be 2.5×10^{-5} M for orotate. In the systems of Lieberman and Kornberg (1953) and Friedman and Vennesland (1958) there was no inhibition of the enzyme at higher concentrations of the substrate.

The rate of reaction is linear with the enzyme concentration (Figure 7).

pH optimum

The pH optimum of the reaction as shown in Figure 8 is in agreement with that reported by Friedman and Vennesland (1958). The curve shows a broad optimum with a peak at 6.0.

Figure 6. Progress curve of dihydroorotic dehydrogenase.

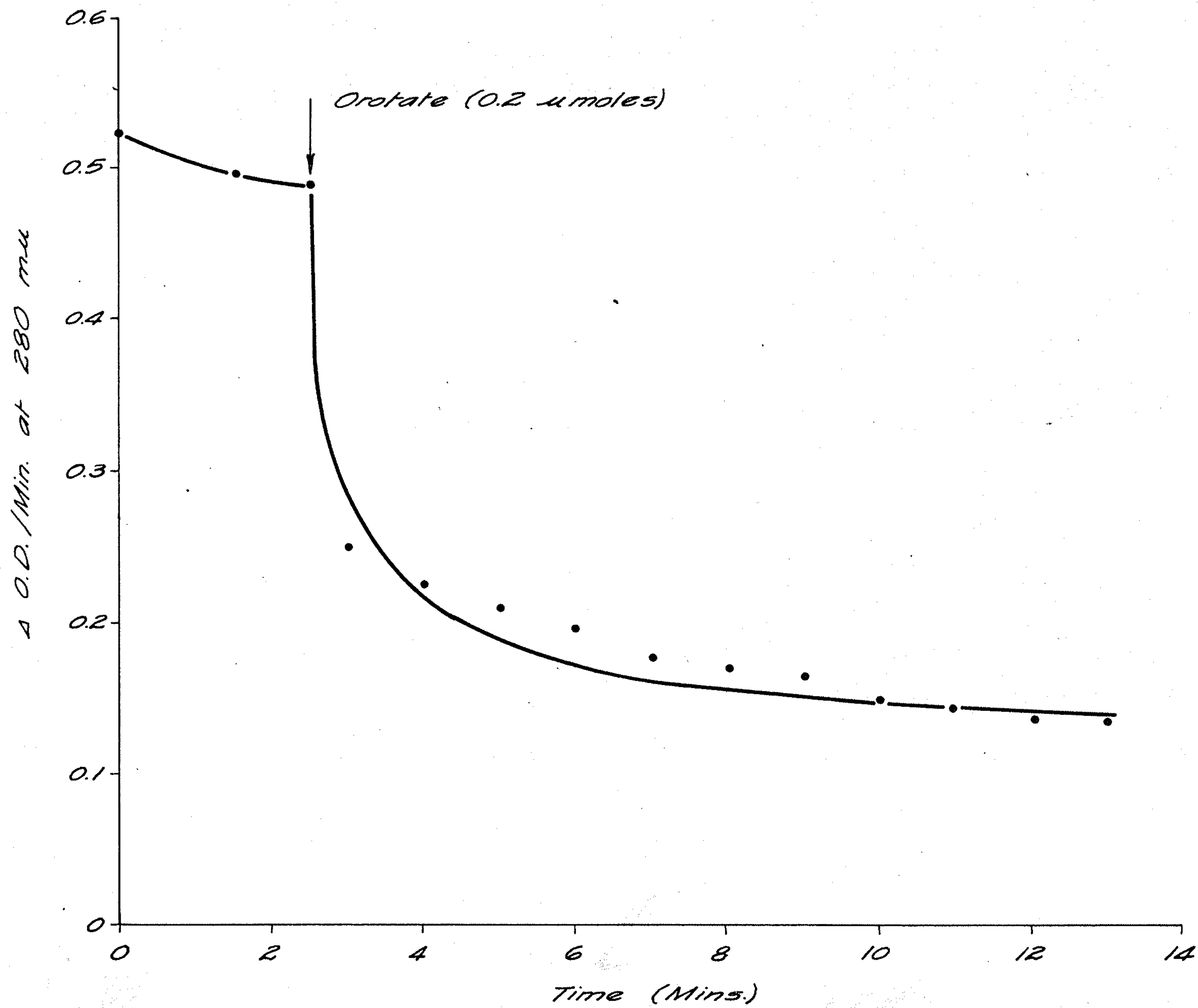

Orotate (0.2 μmoles)
Δ O.D./Min. at 280 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
Time (Mins.)

Figure 7. The effect of substrate concentration on the rate of dihydroorotic dehydrogenase reaction.

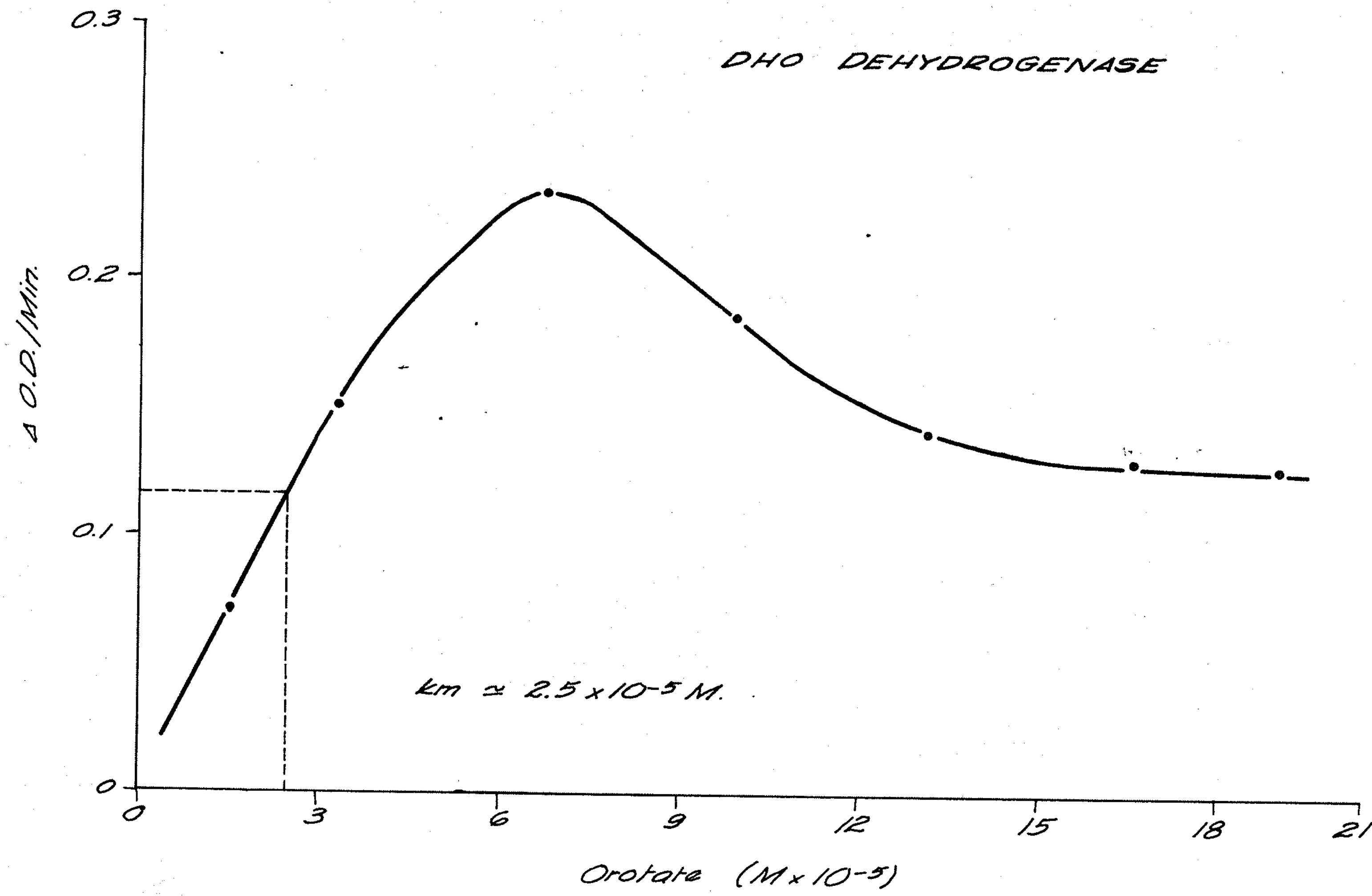

DHO DEHYDROGENASE
Δ O.D./Min.
0.3
0.2
0.1
0
km ≃ 2.5 x 10-5 M.
0
3
6
9
12
15
18
21
Orotate (M x 10-5)

Figure 8. The effect of enzyme concentration.

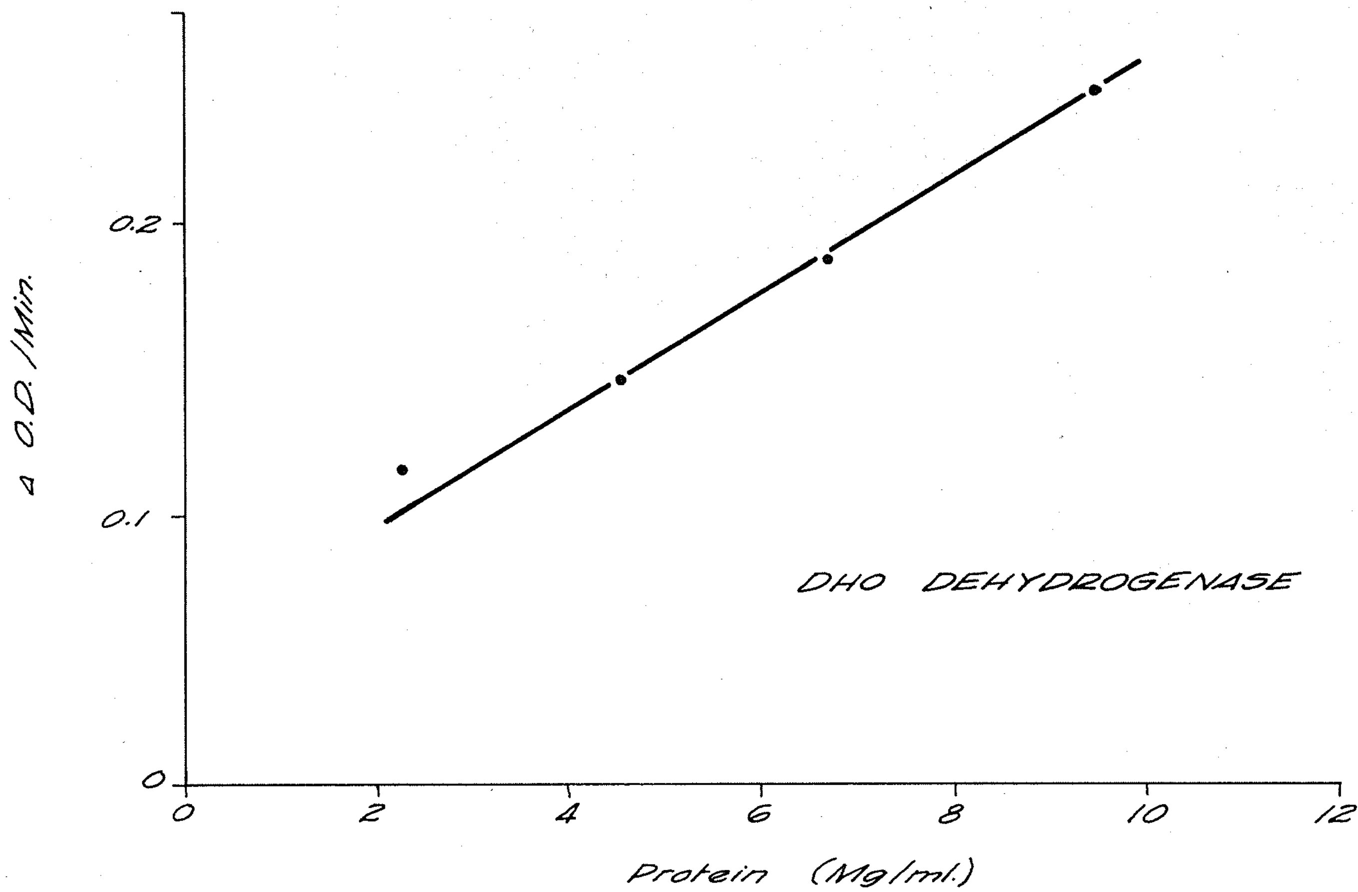
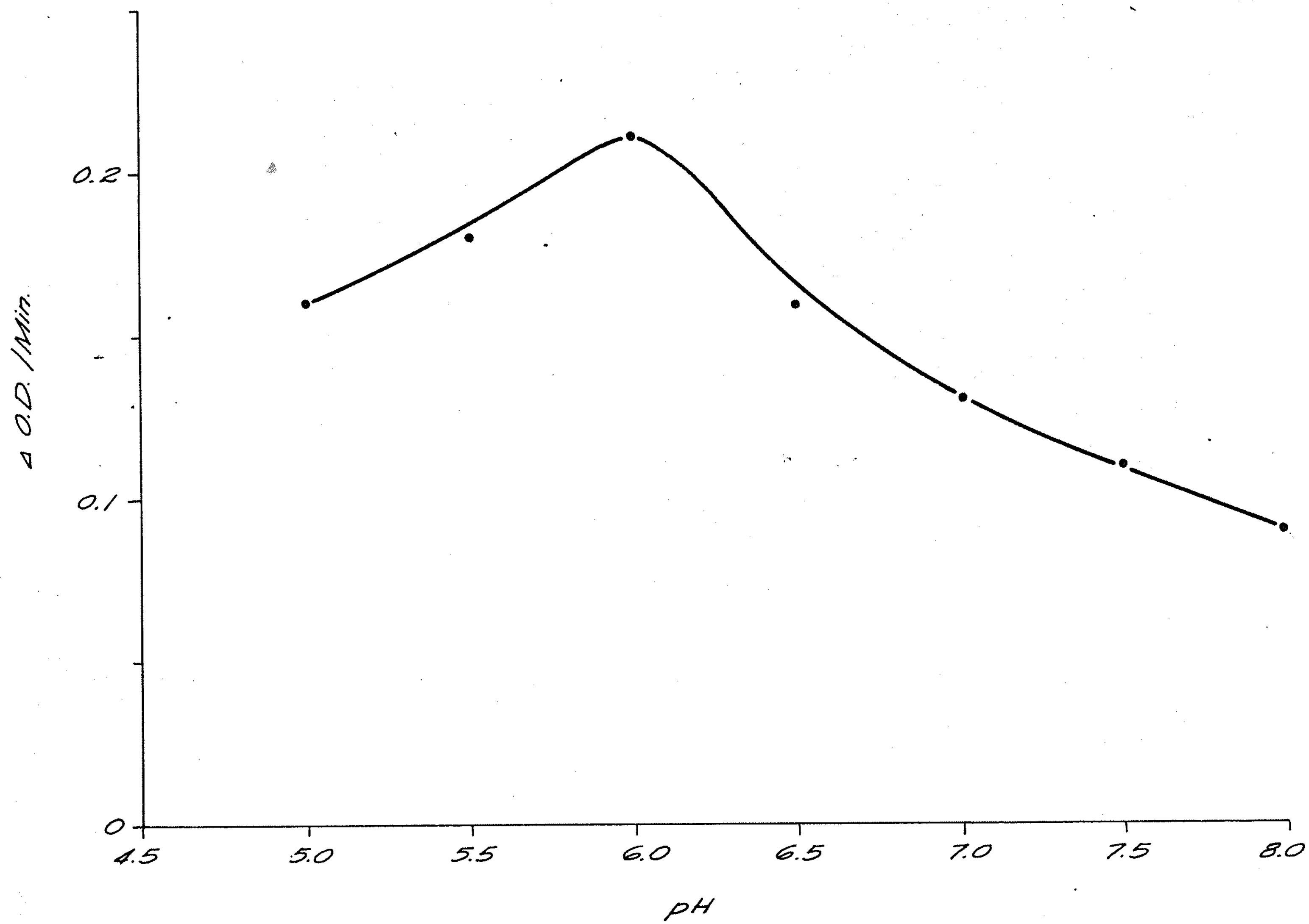

Δ O.D. /Min.
0.2
0.1
0
0
2
4
6
8
10
12
Protein (Mg/ml.)
DHO DEHYDROGENASE

Figure 9. The pH optimum of dihydroorotic dehydrogenase.


0.2
0.1
0
Δ O.D. /Min.
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

ORITIDINE-5-PHOSPHATE PYROPHOSPHORYLASE

Enzyme Assay

The assay of the enzyme is based on the decrease in optical density 295 mμ accompanying the disappearance of orotic acid from the medium. The system is a modification of that used by Lieberman, Kornberg and Simms (1955).

The test system contained:-

Tris-HCl buffer, pH 8.5	60 μmoles
$MgCl_2$	6 μmoles
Sodium orotate	0.3 μmoles
PP-ribose-P	0.34 μmoles

and 0.2 ml of enzyme preparation containing approximately 0.5 mg of protein in a total volume of 3.0 ml. The reaction was measured in a Zeiss Spectrophotometer in silica cuvettes of 1 cm light path. The reference cuvette lacked orotic acid.

A unit of enzyme is defined as the amount causing a decrease of 0.01 in optical density in one minute. Under the conditions of the assay the decrease in optical density was proportional to the enzyme concentration. The reaction rate was linear for approximately one minute.

Isolation and Purification of the Enzyme

Five grams of viable wheat embryos were homogenized

with Tris-HCl buffer (0.02 M), pH 7.4 containing 1×10^{4}M β-mercaptoethanol. The homogenate was passed through four layers of cheesecloth and then centrifuged at 20,000 x g for 10 minutes. The residue was rejected and the pH of the supernatant lowered to 5.5 with 1 N acetic acid. The protein precipitating at the acid pH was removed by centrifugation (acid precipitation fractionation).

Ammonium sulphate precipitation: To the supernatant from the acid precipitation fraction solid ammonium sulphate was added till the saturation was 0.33. The precipitate was removed by centrifugation and the supernatant again was made 0.65 saturated with regard to ammonium sulphate. The fraction precipitating between 0.33 and 0.65 saturation of ammonium sulphate was collected and 5-10 ml of tris-HCl buffer (0.02 M), pH 8.0 containing 1×10^{-4} M of β-mercaptoethanol was poured over it gently and it was allowed to stand for two hours at 0°C, during which time the precipitate dissolved completely in the buffer. Any disturbance in the form of stirring or other attempts at dissolving the protein led to a marked loss in the activity of the enzyme.

Norit-A treatment: The enzyme at the previous

stage contained approximately 28 mg of protein per ml. Fifty mg of norit-A were added to 5 ml of the solution and the norit was removed from the enzyme by centrifugation after keeping for 10 minutes at $0^{o}C$. Norit treatment did not result in a loss in activity but the removal of a considerable proportion of UV absorbing material proved to be of value in the subsequent purification.

<u>Gel treatment</u>: The enzyme from the previous stage was diluted to contain about 3 mg of protein per ml. To the diluted enzyme solution alumina C was added in the proportion of 0.2 ml of gel/ml of the enzyme (12% solids; Sigma Chemical Co.). The gel containing solution was stirred for fifteen minutes and then centrifuged. At this stage there was no change in the specific activity as compared to the previous step. More of gel was then added and the process repeated. There was only a slight decrease in protein but a large increase in the specific activity.

<u>DEAE cellulose treatment</u>: To the supernatant from the gel treated material 50 mg of DEAE-cellulose was added for every 5 ml of the enzyme solution, and this was stirred for 30 minutes in the cold. After this time

the cellulose had adsorbed almost all the active enzyme and only a negligible amount still remained in the supernatant. The cellulose was separated from the supernatant by centrifugation and the enzyme was eluted from it with Tris-HCl buffer (pH 8.0) at 0.05 M, 0.1 M and 0.2 M concentrations successively. The eluants were combined and lyophillized in lots of 5 ml each. For subsequent studies one lot derived from 5 ml was dissolved in 2.0 ml of buffer and used in the reaction. Table II shows the details of the purification procedure. It is evident that a 35-fold purification was achieved. The enzyme retained its activity for 3-4 days in the lyophillized state.

Norit-A treatment resulted in the removal of UV-absorbing substances, mainly nucleic acid fraction contaminating the preparation. Gel treatment aided in separation of some proteinaceous substance interfering in the reaction. The DEAE-cellulose step involved a further purification.

Properties of the Enzyme

In Figure 10 typical progress curve of the reaction is represented. The rate is linear for approximately one minute after which the rate decreases

progressively. Norit-A treated enzyme shows a similar progress curve though in some instances the rate was a trifle faster.

TABLE II. Purification of OMP pyrophosphorylase.

Fraction	Volume (ml)	Mg protein/ ml	Units/ ml enzyme	s.a.	Total Units
$(NH_4)_2SO_4$ 0.33 - 0.65 sat.	15	12.3	137.5	11.2	2055
Norit-treated enzyme	14	11.0	135.0	12.3	1890
Alumina C treated (supernatant)	44	1.81	40.0	22.2	1760
Supernatant after DEAE-Cellulose treatment	40	0.96	4	4.16	160
Eluate (combined)	32	0.1	40.0	400	1280

<u>Substrate concentration</u>: The rate of OMP pyrophosphorylase was studied as a function of the substrate concentration. As seen in Figure 11 concentrations of orotate higher than 1×10^{-3} M were inhibitory. The enzyme studied by Lieberman, Kornberg and Simms (1955) in yeast is not inhibited by the higher concentrations of the substrate. The K_m for the wheat embryos enzyme

Figure 10. The progress curve of OMP pyrophosphorylase.

PRPP was added at 2.5 minutes.

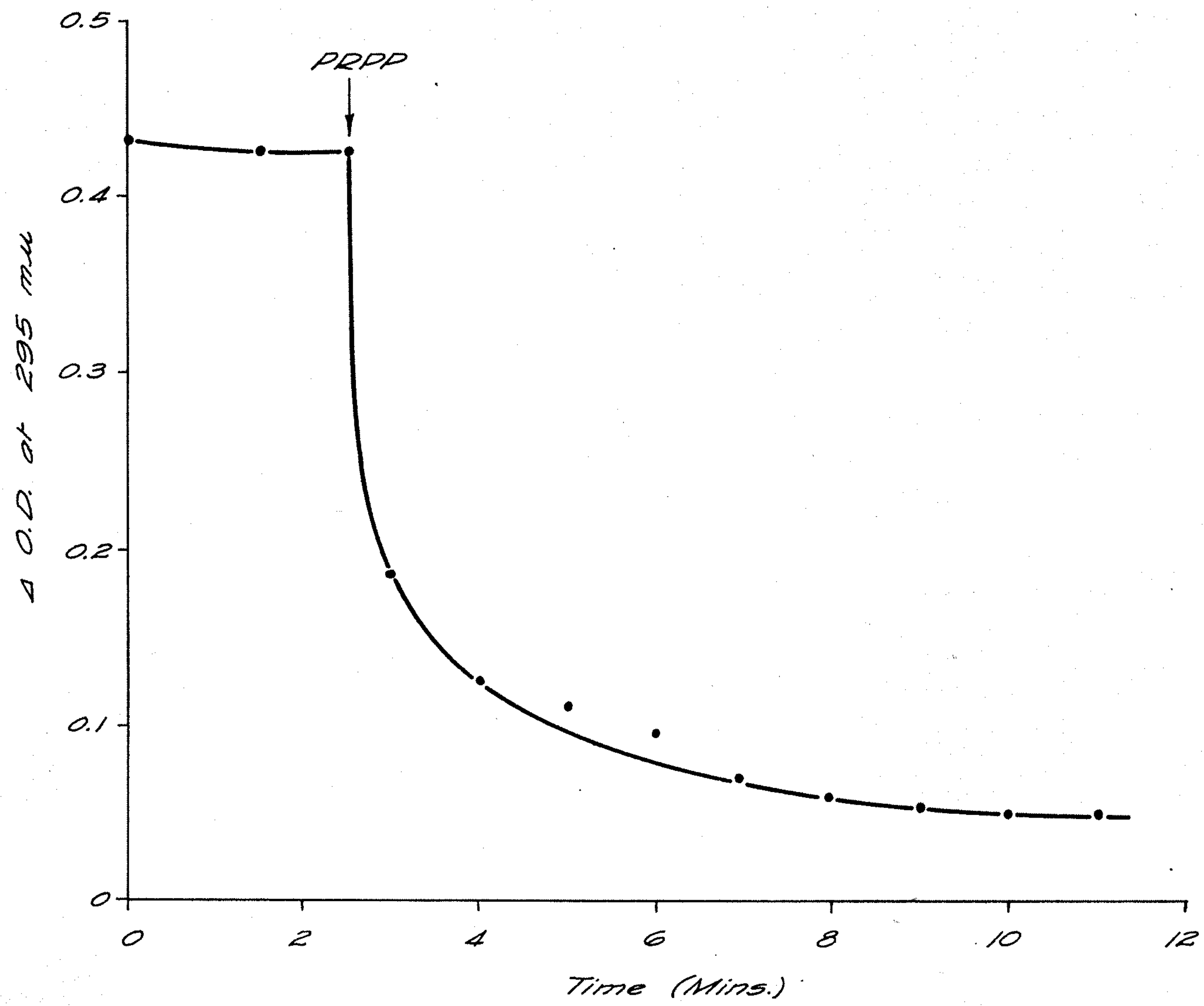
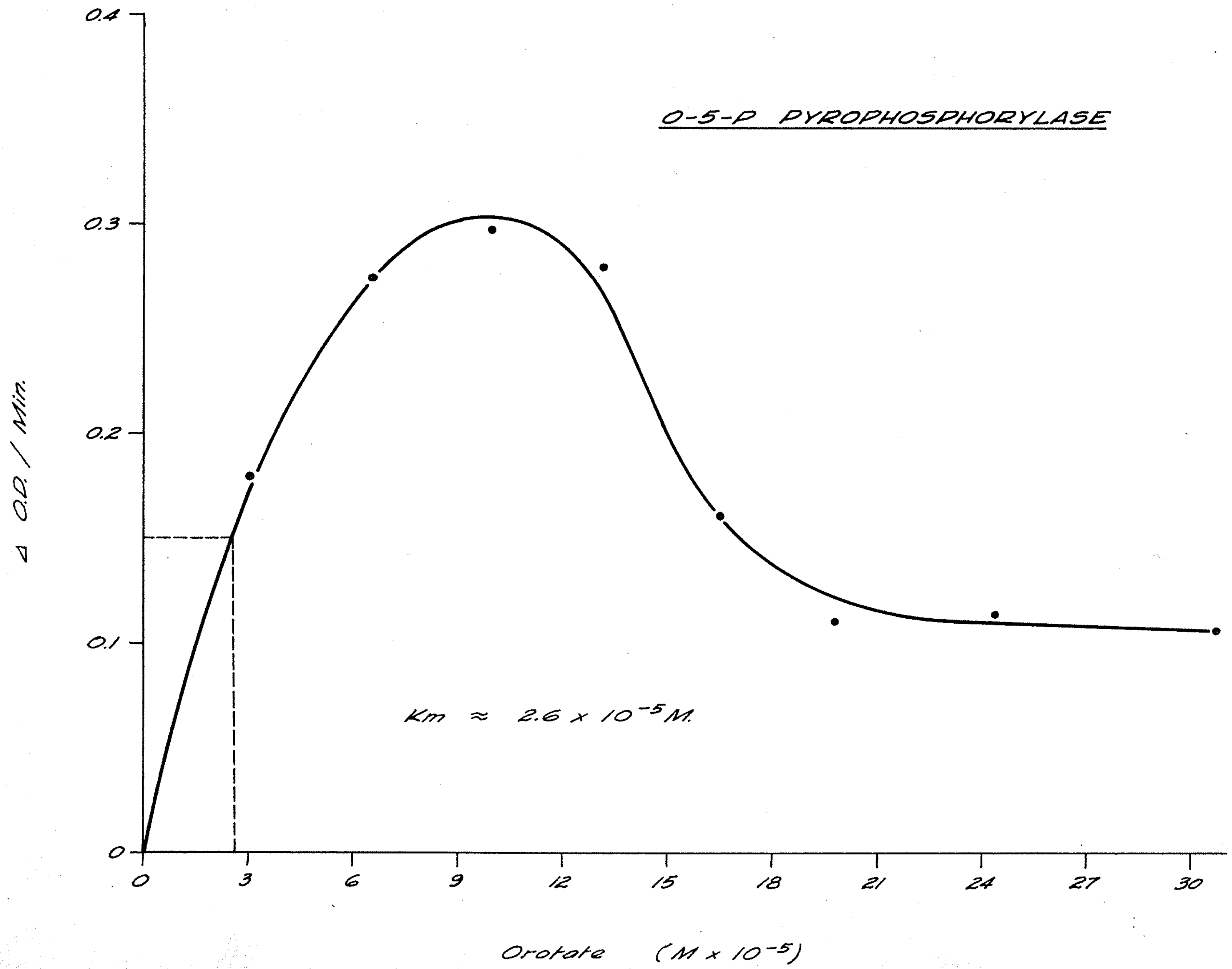
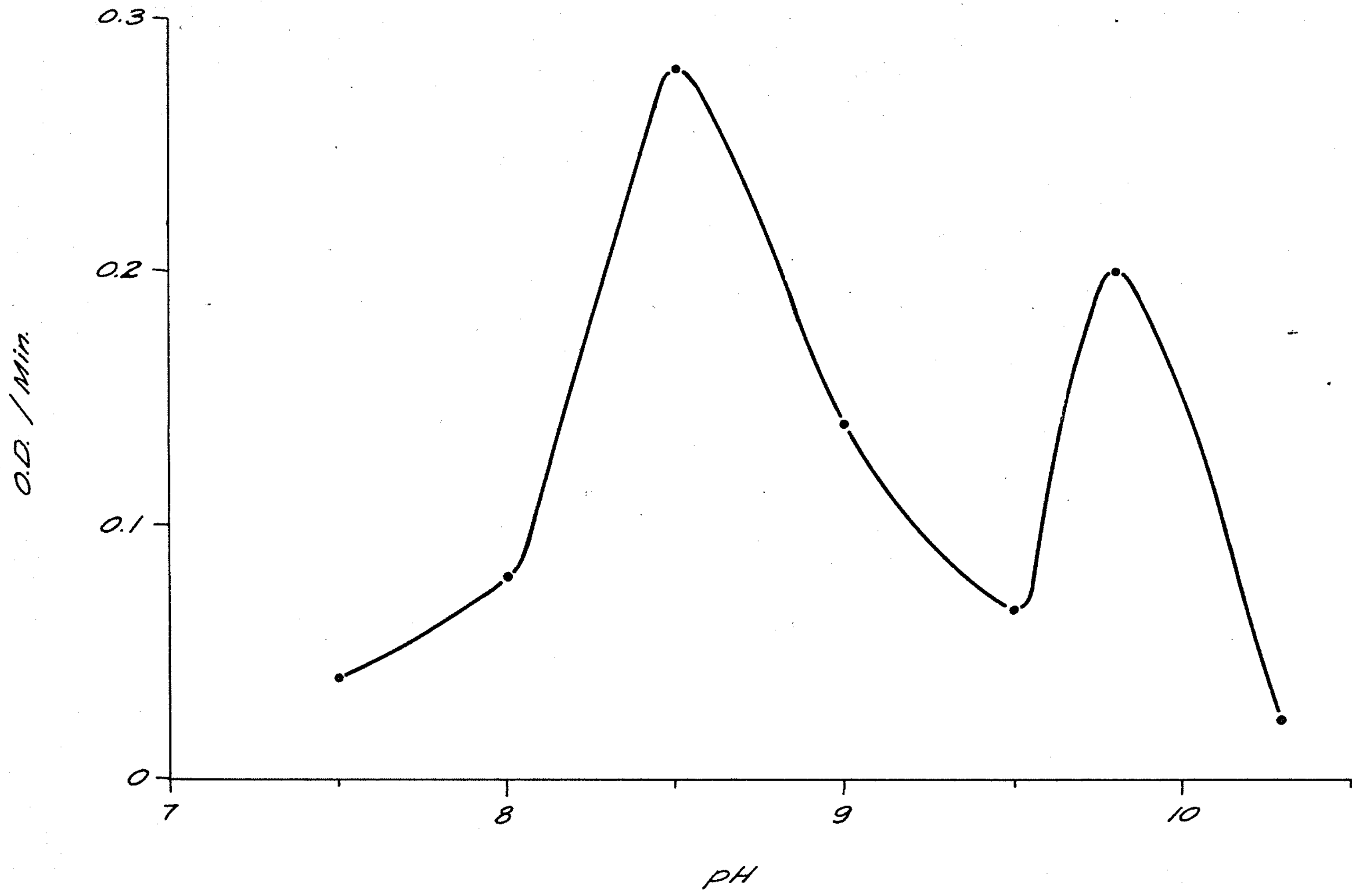

0.5
0.4
0.3
0.2
0.1
0
Δ O.D. at 295 mμ
PRPP
0
2
4
6
8
10
12
Time (Mins.)

Figure 11. Effect of substrate concentration on the rate of OMP pyrophosphorylase reaction.
$K_m = 2.6 \times 10^{-5}$ M.



O-5-P PYROPHOSPHORYLASE
Δ O.D. / Min.
0.4
0.3
0.2
0.1
0
0
3
6
9
12
15
18
21
24
27
30
Km ≈ 2.6 x 10⁻⁵ M.
Orotate (M x 10⁻⁵)

Figure 12. The pH optima of OMP pyrophosphorylase. Two pH optima at 8.5 and 9.7 are seen.


0.3
0.2
0.1
0
O.D. / Min.
7
8
9
10
pH

was 2.6×10^{-5} M as compared to that of yeast enzyme, 2.5×10^{-5} M reported by Lieberman, et al. (1955).

pH optimum: OMP pyrophosphorylase isolated from wheat embryos has a pH optimum at 8.5 (Figure 12). The reaction rate decreases rapidly at pH 9.0 and 9.5 but a second peak is sometimes seen to appear at 9.8. This second peak has been observed in two experiments and is difficult to interpret.

Effect of metal ions: $MgCl_2$ appears to be essential for enzyme activity, although a slow reaction does occur in its absence also. Optimum concentration of $MgCl_2$ is 2×10^{-3} M (Figure 13) above this level the rate of the reaction falls off considerably. The reaction was also performed in the absence of Mg and in the presence of chlorides of Mn, Cu, and Ni instead at the same concentration. As seen in Figure 14, the enzyme is only slightly inhibited in the presence of Cu and Mn whereas Ni reduces the reaction rate to about 50 percent of the normal. In some experiments Cu, Mn and Mg appeared to be equally effective.

Effect of inhibitors: Sodium azide, arsenate, fluoride, α, α'-dipyridyl (1×10^{-5} M) and p-chloromer-

curibenzoate (5×10^{-4} M) were used in the reaction mixture and the rate followed. The conversion of orotate to OMP is reduced to 42.4 per cent in the presence of α, α'-dipyridyl at 1×10^{-3} M. Inhibition with sodium azide, arsenate and fluoride at 1×10^{-3} M is 71, 67, and 67 per cent respectively, (Figure 14). The initial reaction rate, however, is not affected very much but the equilibrium is shifted by the addition of inhibitors. Inhibition by α, α'-dipyridyl, a chelating agent is suggestive of the involvement of a metallic group.

It is interesting that PCMB causes an acceleration rather than an inhibition at a concentration of 5×10^{-4} M. The reaction rate is increased by about 23 per cent in the presence of this inhibitor. This increase, if true, could possibly be due to inhibition of a side reaction which would normally utilize PP-ribose-P or perhaps some enzyme to responsible for its breakdown. In such a situation PCMB would provide a protection mechanism for the phosphoribosyl donor of the reaction.

Figure 13. The effect of $MgCl_2$ concentration.

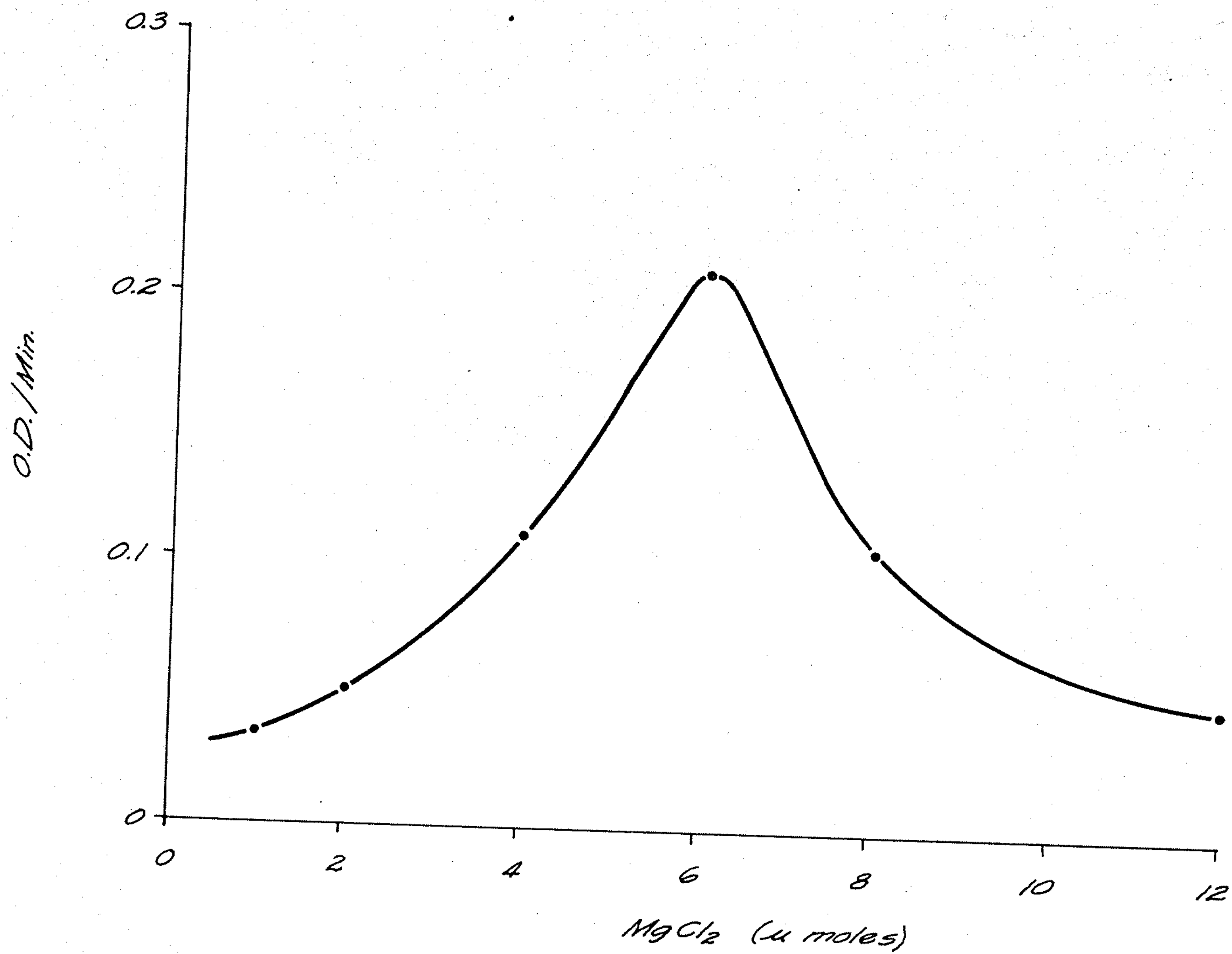

0.3
0.2
0.1
0
O.D./Min.
0
2
4
6
8
10
12
MgCl2 (μ moles)

Figure 14. The effect of metal ions.

Cu, Mg and Mn were equally effective.

Ni was inhibitory.

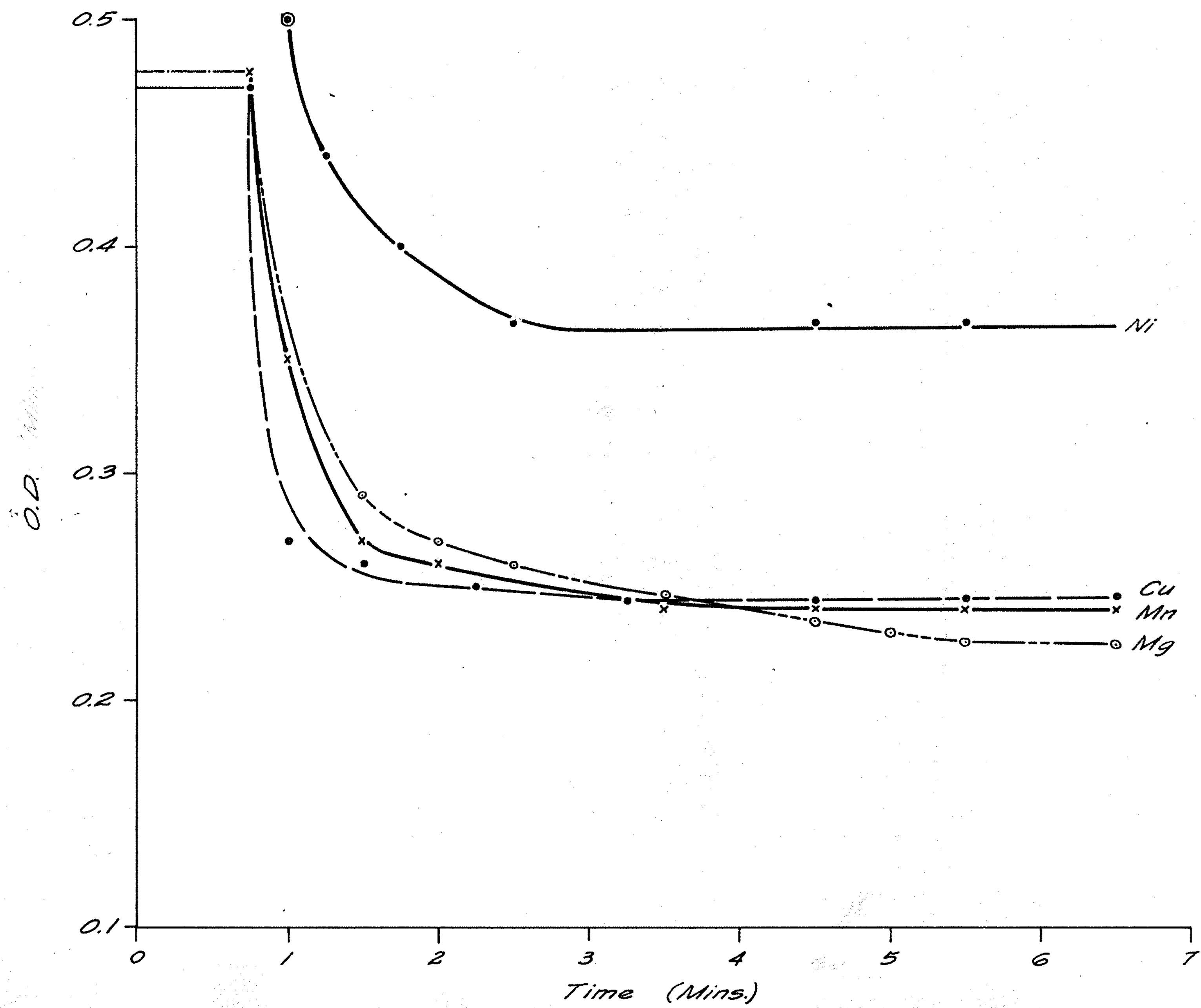
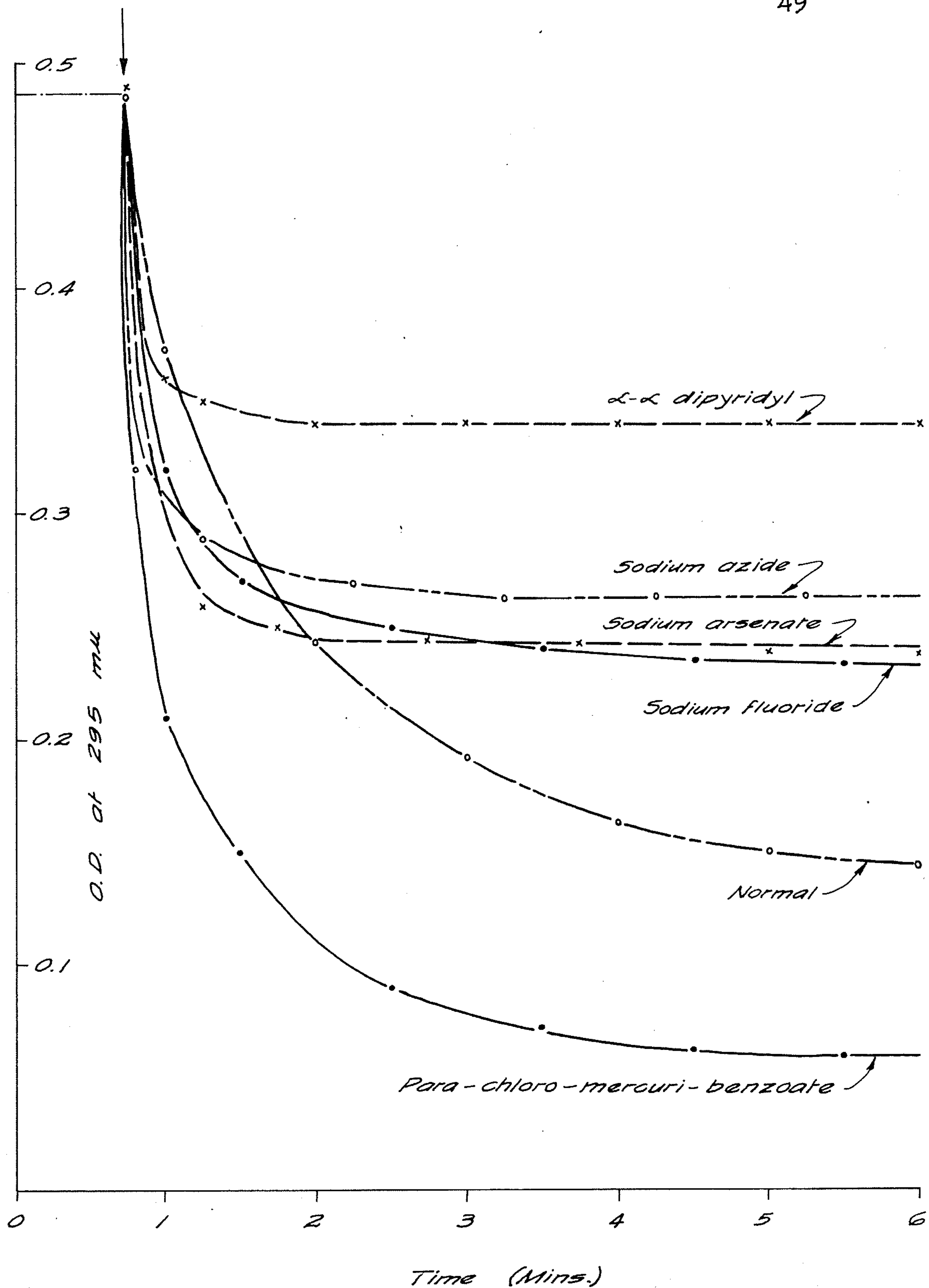

0.5
0.4
0.3
0.2
0.1
O.D.
0
1
2
3
4
5
6
7
Time (Mins.)
Ni
Cu
Mn
Mg

Figure 15. Effect of inhibitors.


0.5
0.4
0.3
0.2
0.1
O.D. at 295 mμ
α-α dipyridyl
Sodium azide
Sodium arsenate
Sodium fluoride
Normal
Para-chloro-mercuri-benzoate
0
1
2
3
4
5
6
Time (Mins.)

III. BENZIMIDAZOLE METABOLISM - BMN AND BAD FORMATION

A. BENZIMIDAZOLE, SOME BIOLOGICAL IMPLICATIONS

The processes of cell division, enlargement, tissue maturation and cellular senescence are mutually complementary. Growth and differentiation of tissues and organs both in plants and animals, are controlled by a complex interplay of physiological and biochemical factors and are invariably succeeded by the onset of senescence. Any one of these phases could be experimentally prolonged or omitted entirely. At the time of transition from a mature tissue to a gradually senescing stage a "switch" occurs in the metabolic pattern. During growth there is a preponderance of biosynthetic reactions as opposed to degradative ones. In biological systems a subtle balance operates between the synthetic and degradative processes and whether an organism will enter into a phase of growth, quiescence or senescence is determined by the existing metabolic balance manifested at the tissue, cellular and perhaps molecular level,

Although the subject of growth and differentiation has provoked a considerable amount of research, the problem of senescence and aging has received little attention. The metabolism of aging tissues and cells is, nevertheless, gradually being investigated into by an

increasing number of individuals and research schools, especially with regard to higher plants (Varner, 1961). Excised plant parts, more frequently leaves, have provided excellent model systems for a study of senescence at the metabolic level. It must be understood, however, that monocotyledons and dicotyledons differ in metabolism and growth processes in a striking and yet incompletely explored manner. The studies conducted on these processes in plants are limited to one or two species only and extreme caution should be exercised before making any generalizations.

It has repeatedly been demonstrated that in a mature leaf the phase of senescence can be initiated artificially by excising it from the plant. For this very reason detached leaves provide an extremely suitable model system for the study of senescence. In such a state, three chief chemical changes are noticeable:

(a) The protein levels undergo a rapid decline with a concurrent decrease in (b) chlorophyll content and consequently (c) the life span of the leaf is reduced (Mitchael, 1935; Chibnall and Wiltshire, 1954) in proportion resulting in eventual death. If a detached leaf is floated on water an exactly identical pattern

of behaviour is encountered. This metabolic sequence is interrupted if the leaf roots at the cut end. In the event of rooting of the petiole or any other part of the leaf, a biochemical and metabolic rejuvenation is evident and the leaf reverts back to a state of normal photosynthetic activity and protein synthesis (Gregory and Samantrai, 1950).

Studies of Vickery et al. (1946) and those of Mothes and Engelbrecht (1956) have demonstrated that the decline in protein content of leaves is not caused by a breakdown of carbohydrate metabolism or an inavailability of nutrients. Detached leaves, on the contrary retain the capacity to incorporate labelled nitrogen and carbon into proteins (Chibnall and Wiltshire, 1954; Racusen and Aronoff, 1954), but they appear to lose the capacity to synthesize certain amino acids, consequently shifting the ratio of synthesis to breakdown in favour of the latter. Since in the event of rooting, the leaf can synthesize all the amino acids and incorporate them into proteins, a root factor (a substance or substances synthesized by the root and transported elsewhere in the plant) has been invoked to explain the phenomenon.

The factors involved in senescence and the accompanying deterioration of metabolic machinery are not

understood very well. During the last decade several attempts have been made to retard the onset of senescence in leaves and several chemicals have been tested for their effect. Growth regulators, chiefly, IAA and derivatives sometimes retarded protein degradation in *Xanthium* leaves but the effect was rather erratic (Richmond and Lang, 1957). Kinetin (6-furfuryl-aminopurine), however, was demonstrated by the same authors to inhibit protein degradation consistently. Fully expanded leaves of *Xanthium pennsylvanicum* were excised and the petiole put in water or dilute kinetin solutions. The leaves floated on water lost protein and chlorophyll but those floated on kinetin retained the green color and maintained a steady protein level for a considerable length of time.

In the same year Person *et al.* (1957) conducted extensive experiments on excised wheat leaves and studied the effect of purine and pyrimidine derivatives. They demonstrated the dramatic effect of benzimidazole (a growth antagonist, Gillespie *et al.*, 1954) in the retention of green color and its capacity to support the growth of leaf and also stem rust on the leaf up to a period of one month. In a comparative study of effect of floating leaves on water and benzimidazole solutions they came to the conclusion that i) whereas in water

floated leaves an increase in respiratory rate was seen, the benzimidazole treated leaves remained normal; ii) the water floated leaves revealed a much higher level of free amino acid and benzimidazole treated ones showed no increase in size of free amino acid pool over the controls; iii) on transferring the water floated leaves to benzimidazole solutions a synthesis of proteins occurred.

The effects of benzimidazole on the detached leaves enumerated above are more or less parallel to those of kinetin reported for *Xanthium* leaves. In addition benzimidazole also maintains the property of resistance to rust in detached leaves of a wheat variety, Khapli (Samborski *et al*., 1958). Khapli leaves are normally resistant to race 15-B of stem rust but on detachment and floating on water they succumb to infection. By benzimidazole treatment, on the other hand, the leaves exhibit the property of resistance just as in the normal attached leaves.

EFFECT OF BENZIMIDAZOLE ON METABOLISM

PROTEIN

The studies of Samborski *et al*.(1957) on the effect of benzimidazole on metabolism of detached leaves led to the conclusion that in water floated leaves there

is a progressive increase in the level of soluble nitrogen and a decrease in the insoluble nitrogen but on benzimidazole treated leaves there was practically no change in these two fractions. The progressive decrease in insoluble N in water floated leaves was interrupted on transferring them to benzimidazole solution and a new equilibrium was established. It is quite clear here that benzimidazole is directly or indirectly causing the "switch" from the metabolic trend exhibited by the water floated leaves to the normal ones. Thus benzimidazole is capable of not only retarding protein breakdown but also of reversing the degradation already initiated.

CARBOHYDRATE

An accumulation of water-soluble carbohydrates, especially glucose and fructose and sucrose in water floated but not in benzimidazole treated leaves was also reported by Samborski *et al.* (1958). It is not unreasonable to assume that this is indicative of nonutilization of sugar for polysaccharide synthesis in water floated leaves. Polysaccharide biosynthesis is also maintained by benzimidazole treatment.

CHLOROPHYLL

Benzimidazole not only stimulates chlorophyll biosynthesis but also protects it from destruction (Wang and Waygood, 1959). When excised etiolated leaves were subjected to illumination and treated with benzimidazole, the chlorophyll concentration increased rapidly during the first two days. The benzimidazole treated leaves, however, synthesized far more chlorophyll than the controls. On transferring the leaves to dark, benzimidazole treatment again shielded chlorophyll from destruction.

The effect of benzimidazole on the incorporation of glycine-2-C^{14}, succinic-2, 3-C^{14}, glutamate-C^{14} and urea-C^{14} was investigated by Wang et al. (1961) again using the excised wheat leaves. It was concluded from their experiments that benzimidazole caused an enhancement in the rate of incorporation of glycine and succinate into chlorophyll whereas there was no effect on the incorporation of glutamate and urea. In water-floated leaves a gradual, progressive impairment of the capacity to incorporate glycine was observed but benzimidazole treatment maintained the normal level of incorporation for as long as four days.

INHIBITION AND PROMOTION OF THE BENZIMIDAZOLE EFFECT

The expression of the benzimidazole effect appears to be effected by the presence of other totally unrelated compounds in the test system. Effect of 60 ppm of benzimidazole on rust resistance could be counteracted by providing 1 per cent glucose (Samborski et al., 1959). In addition to this the same concentration of glucose could cause an increase in level of soluble Nitrogen. Addition of one per cent glucose made the benzimidazole treated leaves mimic the water floated sample. The benzimidazole effect could, nevertheless, be restored by applying a higher benzimidazole concentration.

5,6-dimethylbenzimidazole appears to be a potent inhibitor of benzimidazole. Due to a close similarity in chemical structure it could be suspected of being involved in competitive inhibition of some reaction or a complex formation.

A somewhat complicated situation with regard to inhibition of benzimidazole effect and its reversal by various metal ions was elicited by Wang (1959). These studies indicated that 1 ppm of cobalt ion could completely reverse the glucose interference in the benzimidazole. Ni^{++}, Zn^{++}, Mo^{++} and Cu^{++} were also tested for

effects if any on inhibition and reversal of the benzimidazole effect and also to ascertain if the Co^{++} requirement was specific. Ni^{++} was found to be effective consistently in reversing both glucose and 2,6-dimethyl benzimidazole inhibition. The effect of Ni may, however, be completely unrelated to that of benzimidazole, because it is known to prevent rust infection of leaves quite independently of any other factor (Wang *et al.*, 1958). A simplified version of the situation is presented in Table III.

In spite of the fact that Co^{++} could effectively remove the glucose and dimethylbenzimidazole inhibition, it proved to be toxic at concentrations of 6 ppm and higher. It is interesting to note here that ribose relieved the toxicity caused by Cobalt if used in the presence of benzimidazole. By itself ribose was indifferent toward rust development.

A large number of other compounds were also tested by Wang *et al.* (1961) in combination with benzimidazole to study their interaction. Here a distinction must be made between the effect of these compounds on benzimidazole effect with regard to the rust reaction and that in chlorophyll development. Adenine, 2-chlorobenzothiazole, benzotriazole, Xanthine, 8-chloroxanthine, 8-azaxanthine

and azaguanine were antagonistic towards benzimidazole in its effect on rust development but indifferent towards its effect on chlorophyll development. Evidently rust reaction and chlorophyll biosynthesis are influenced through separate mechanisms.

TABLE III. Inhibition and reversal of the benzimidazole effect.

System	Treatment	Situation
Detached leaves		S
	Water	S
	Benzimidazole	N
	Benz. Glucose	S
	Benz. dimethylbenz.	S
	Benz. Glucose Co	N
	Benz. Dimethylbenz. Co	N
	Benz. Glucose Ni	N
	Benz. dimethylbenz. Ni	N

S stands for senescence and N for the normal state. Dimethylbenz. is used for 2,6-dimethylbenzimidazole.

BENZIMIDAZOLE IN ION UPTAKE

Apart from modifying the host-parasite relationship in the rust development and influencing chlorophyll

and protein metabolism, benzimidazole is known to influence a variety of other apparently physical processes, too; i.e., uptake of ions by the plant roots. Hillman (1955) demonstrated that benzimidazole treatment led to a decreased activity of Copper containing enzymes in Lemna minor plants. A binding of the Copper ions by benzimidazole was suggested by the observation that the amount of Copper remained unchanged in the plant.

Klingensmith (1961) employing detached Hordeum vulgare roots demonstrated that benzothiazole, chlorobenzimidazole and benzotriazole repressed the uptake of potassium ion. Benzimidazole, on the contrary, stimulated the uptake of potassium and sodium by 50 per cent when used at a concentration of 1×10^{-3} M. The enhancement of the uptake of potassium ion has, now been found to be due to the buffering action of benzimidazole (Parr and Norman, 1962). In the course of the potassium uptake from a solution of K_2SO_4 the pH of the solution progressively decreased resulting in a lower rate of absorption. This decrease in pH was checked in solutions containing benzimidazole and also benzimidazole pretreated roots. The ability of benzimidazole to act as an effective buffer is due to its amphoteric nature; the imino hydrogen of the imidazole ring being responsible

for the acidic properties and the pyridine nitrogen for basic properties.

STRUCTURAL REQUIREMENTS FOR THE BENZIMIDAZOLE EFFECT

Studies of Wang *et al.* (1961) suggest that both the imidazole and benzine ring are essential for the complete manifestation of the benzimidazole effect. The introduction of a methyl, amino or other group into the benzimidazole molecule results in a change in activity.

NATURAL OCCURRENCE OF BENZIMIDAZOLE AND DERIVATIVES

It has been pointed out repeatedly that benzimidazole appears to substitute for a root factor, perhaps required continuously by the plant. Although it has not been shown yet that benzimidazole is a natural factor in plants, benzimidazole and related compounds occur in animal, bacterial, and plant systems as a prominent portion of the vitamins and coenzymes of the group B_{12}.

Vitamin B_{12}, a molecule unique among naturally occurring compounds in the B_{12} series, in being a Cobalt-coordination complex (Rikes, *et al.* 1948a) was characterized chemically, microbiologically and clinically and isolated in 1948 (Rikes *et al.*, 1948b). Because of the known physiological relationship of the compound to

pernicious anemia it was suspected to contain a cobalt complex of a polypyrrole, resembling a porphyrin. In addition to cobalt, "cobalamin" molecule yielded 5,6-dimethylbenzimidazole on acid hydrolysis. Shortly thereafter, 1-α-D-ribofuranosyl-5-6-dimethylbenzimidazole was identified as a vital part of the "cobalamin" molecule (Kaczka et al., 1952). Another class of compounds referred to as pseudovitamin B_{12} have adenine, instead of 5,6-dimethylbenzimidazole, built in their molecule.

Vitamin B_{12} is known to be synthesized by or necessary for the growth and metabolism of bacteria, brown, red and blue-green algae (Holm-Hansen et al., 1954) but not by green algae (Robbins et al., 1953). The possibility of the occurrence of vitamin B_{12} in green plants has been debated at length (Darken, 1953). Robbins and coworkers (1952) detected traces of vitamin B_{12} in plant roots by the Euglena test but these were attributed to microbial contamination. The suspicion that green plants do, indeed, contain B_{12}-like factors would be substantiated by a positive requirement for cobalt. The root nodules of leguminous plants are known to excrete vitamin B_{12} (Burton and Lockhead, 1951) but this was more likely to be a product of the symbiotic

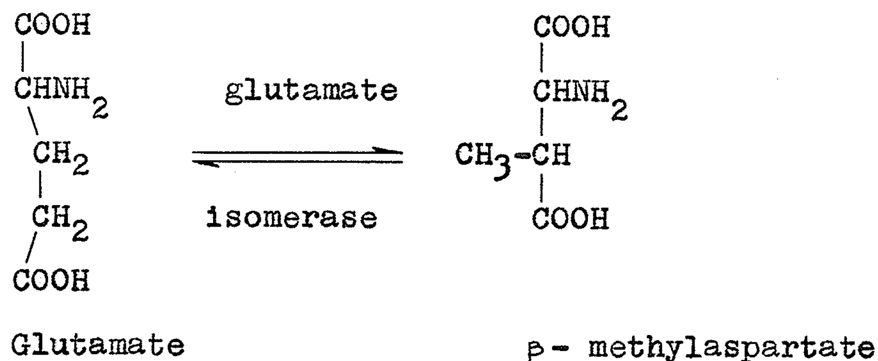
bacteria. In the absence of a positive requirement for cobalt by higher plants the only available information on the effect of vitamin B_{12} on seed plants is the data of Reinert and White (1956) on tumor tissue of *Picea glauca*. Recently, Fries (1962) has succeeded in isolating vitamin B_{12} from pea plants, maintained in aseptic culture conditions, by charcoal adsorption. He employed a microbiological assay using a B_{12}-less mutant of *E.coli* (strain 113-3). Vitamin B_{12}-like factors were also detected in wheat and lupins by the same technique.

Coenzymes of B_{12} group were recognized during studies on glutamate fermentation by *Closteridium tetanomorphum* (Barker *et al*., 1959). Coenzymes containing 5,6-dimethylbenzimidazole or alternately variously substituted benzimidazole or purine in its place, are analogous to vitamins of the group B_{12}. Properties of the coenzymes B_{12} are similar to those of vitamin B_{12} but they differ sharply in spectral characters and also chemically by having an extra adenine and a sugar moiety probably in the form of a nucleoside (Weissbach *et al*., 1960) which are readily dissociated by exposure to light. Coenzymes also contain divalent cobalt (Bernhauer *et al*., 1961).

The B_{12} coenzymes catalyze three reactions. The

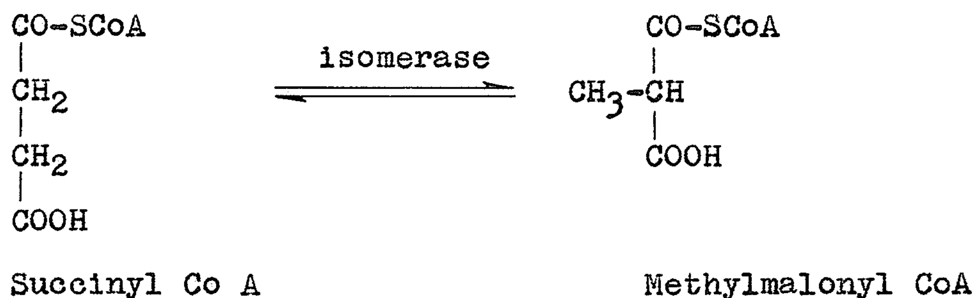
first is the glutamate isomerase reaction observed, in *Clostridium tetanomorphum*, (Barker *et al.*, 1960a) and *Rhizobium meliloti* (Kliewer and Evans, 1962). The enzyme required for the conversion of glutamate to β-methyl asperate was purified from *C. tetanomorphum* (Barker *et al.*, 1960a). The same group of workers crystallized benzimidazole cobalamide and 5,6-dimethylbenzimidazole cobalamide from *Propionibacterium shermanii* (Barker *et al.*, 1960b).

The reaction is as follows:



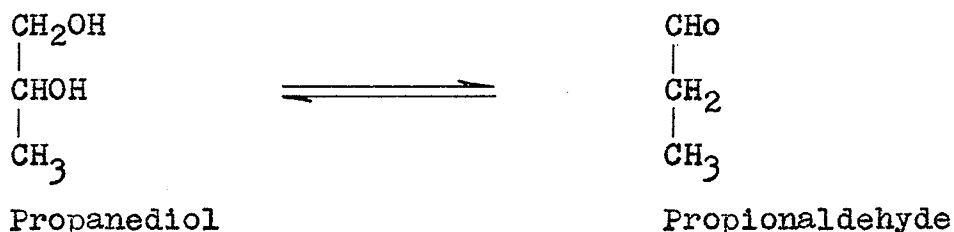
β-methyl aspartate is subsequently converted to mesaconate by deamination.

The second reaction, the succinyl-methylmalonyl CoA isomerase occurs both in animal and bacterial systems (Stadtman, *et al.*, 1960; De Hertogh and Evans, 1962):-



The isomerase enzyme of P. shermanii can be easily separated from the coenzyme by charcoal treatment and the system can be reactivated by adding back the benzimidazole containing analogues.

A third B_{12} coenzyme reaction catalysed by extracts of Aerobacter aerogenes was demonstrated by Abels and Lee (1961). In this reaction a glycol is converted to the corresponding 2-dioxyaldehyde. The enzyme system studied here converts 1, 2-propanediol to propionaldehyde.



These reactions have not, so far, been investigated in plant tissues but it would be illuminating to know exactly how the conversion of glutamate to mesaconate proceeds in higher plants.

With regard to the biosynthesis of vitamin B_{12} two controversial view points exist. Dellweg et al. (1956) supported the view that in E. coli 5,6-dimethylbenzimidazole is incorporated into B_{12}, as a free base. Bernhauer et al. (1959) also came to similar conclusions from work with P. shermanii. Recently Friedman and Harris (1962) have presented evidence to the effect that ribonucleosides of benzimidazole and 5,6-dimethylbenzimidazole mediate the biosynthesis pathway rather than the corresponding free bases.

Another molecule of biological interest, namely, firefly luciferin, contains a 6-hydroxybenzothiazole compounds, closely related to benzimidazole (White et al., 1961).

Very little is known about the reactions benzimidazole could possibly be undergoing after being administered to a biological (especially on plant) systems. Enzymatically catalyzed reactions between benzimidazole and NAD have recently been discovered (Alivisatos et al., 1962) and their significance will be discussed later.

B. BENZIMIDAZOLE MONONUCLEOTIDE

INTRODUCTION

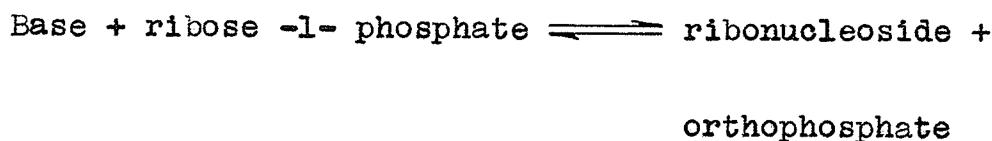
A survey of the literature as presented in the previous pages revealed that although considerable attention had been paid to the morphological and the descriptive aspect of the benzimidazole effect as in modified rust reaction, maintenance of protein and chlorophyll biosynthesis, the mechanism of its action was very poorly understood, if at all. Dr. D.T.T. Wang in our laboratory initiated experiments designed at locating the site and elucidation of the mode of action of benzimidazole. A reasonable approach was to investigate into chemical changes undergone by benzimidazole upon entry into a plant cell. The realization 'what does the plant do to benzimidazole', is a more vital question than, and deserving priority over 'what does benzimidazole do to the plant', brought forth a shift in the design of experiments.

The initial experiments were aimed at simply following the fate of benzimidazole-2-C^{14} fed to the excised leaves at the cut ends. On extraction and fractionation of the leaf extracts it appeared that all the radioactivity was confined to the so called "nucleic acid fraction" which also contained protein in the method being used. Subsequent paper chromatography of

the fraction in acidic solvents separated out two compounds on paper. One of these compounds was identified to be benzimidazole on the basis of Rf value and a comparison with genuine samples. The other, however, did not migrate from the point of application and showed properties characteristic of a nucleotide or -side. On hydrolysis, this compound yielded benzimidazole proving thereby that it was a derivative of benzimidazole. On administering radioactive ribose and benzimidazole the same compound was detected in very small quantities. These results indicated strongly the formation of a mononucleotide or a riboside of benzimidazole, when the latter came in contact with the plant enzymes.

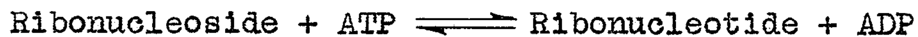
With this background, in the present investigation a search was made for a reaction or reactions which could possibly lead to the synthesis of benzimidazole mononucleotide from free benzimidazole. In the purine and pyrimidine biosynthetic pathways the utilization of preformed bases is accomplished through the formation of mononucleotides or ribosides in one of the following reactions:

1. <u>Ribonucleoside phosphorylase:</u>



2. Ribonucleotide-pyrophosphorylase:

3. Ribonucleoside phosphokinase:



It is clear that in a single step reaction 2 can achieve the product of combined effect of both reactions 1 and 3. Therefore, the second reaction was employed for the conversion of benzimidazole to BMN by an enzyme system isolated from wheat embryos. The formation of OMP from orotic acid also utilizes the same reaction (Lieberman *et al.*, 1955). The orotic-OMP system was employed for the measurement of benzimidazole-BMN reaction.

MATERIAL AND METHODS

Viable wheat embryos, isolated according to the procedure described in part I were used at a source of enzyme preparations throughout this study.

Benzimidazole (Eastman Kodak Co, Rochester, N.J.) PP-ribose-P, NAD, (DPN), NADP (TPN), acetyl pyridine-NAD (Ap-DPN) (Sigma) were commercially obtained. Benzimidazole-2-C^{14} was obtained from Calbiochem.

ASSAY OF BENZIMIDAZOLE MONONUCLEOTIDE

Since the formation of OMP from orotic acid proceeds by a pyrophosphorylase reaction involving PP-ribose-P it was assumed that the formation of BMN would also proceed by a similar reaction catalyzed by a different enzyme which may be present in the partially purified preparation of OMP pyrophosphorylase. If this assumption was correct it was argued that a given enzyme preparation would catalyze both OMP formation and BMN formation. Since both the reactions require PP-ribose-P there will be a competition between the two for this reactant. Therefore in a system containing both orotic acid and benzimidazole, the amount of OMP formed would be less than that formed in the absence of benzimidazole. In other words, the conversion of orotic acid to OMP would decrease in proportion to the amount of benzimidazole

converted to BMN, due to the inavailability of a portion of PP-ribose-P for OMP formation. The assay enzyme preparation used for BMN reaction was the same as that for OMP formation. The reaction mixture contained the following:

Tris-HCl buffer, pH 8.5	60 µmoles
$MgCl_2$	4 µmoles
Orotate, pH 7.0	0.3 µmoles
Benzimidazole	0.3 µmoles
PP-ribose-P	0.34 µmoles

and 0.1 ml of the enzyme preparation in a total volume of 2.8 ml. The reaction was initiated by adding PP-ribose-P to the reaction mixture and measuring the decrease in the absorption at 295 mµ (accompanying the disappearance of orotate) in the presence and absence of benzimidazole. The difference between the two equilibrium values was used as an index of the activity of BMN pyrophosphorylase.

Preparation of embryo homogenates

Five grams of wheat embryos was homogenized with 50 ml of 0.5 M K_2HPO_4 - 0.01 M cystein (pH 7.4). The coarse material was filtered through four layers of

cheesecloth and the filtrate centrifuged at 10,000 x g for 5 minutes. The residue was discarded and the supernatant at this stage, was used in experiments with the whole homogenate.

Incubation

To 20 ml batches of the whole homogenate in Erlenmeyer flasks benzimidazole-2-C^{14} (approximately 200,000 cpm) was added with and without orotate and PP-ribose-P and the mixture incubated at 30°C for 2 hours. The reaction was terminated by boiling the mixture for 5 minutes and adding 95% ethanol. The protein thus precipitated was centrifuged off, washed twice with water and the washings, and supernatant were combined. The pooled supernatant was dried under a jet of cold air, dissolved in a known volume of water, and its radioactivity recorded. The samples were subsequently subjected to chromatography and electrophoresis which will be described presently.

Bulk preparation

A bulk preparation for isolation and characterization of the reaction product incubated for 2 hours at 20°C contained the following components:

Tris-HCl buffer pH 8.5	2000 µmoles
$MgCl_2$	60 µmoles

Benzimidazole	25 μmoles
PP-ribose-P	25 μmoles
Benzimidazole-2-C^{14}	approximately 200,000 cpm as a marker

and 50 ml of wheat embryo protein precipitating between 0.33 - 0.65 saturation of ammonium sulphate (obtained from 5 gms of wheat embryos). After incubation the mixture was deproteinized with 3% perchloric acid which was removed by neutralization with 5 N KOH. The volume (approximately 150 ml) was reduced to 50 ml.

SEPARATION OF BENZIMIDAZOLE AND THE PRODUCT

Electrophoresis

The reaction mixture and the concentrated homogenate was subjected to electrophoresis in a Spinco Durrum cell in formate (0.05 M) buffer at pH 3.5. A 0.02 ml sample was applied on to the filter paper strips by means of a special applicator after the paper had equilibrated for one hour against the same buffer, the current was constant at 25 milliamperes. Each set was accompanied by a reference sample containing benzimidazole-2-C^{14}. The paper strips were scanned on a Nuclear Chicago automatic rate meter (actigraph; continuous gas flow).

Chromatography

Paper chromatography: The reaction mixtures and samples from the homogenates incubated with labelled benzimidazole were chromatographed on Whatman No.1 and 4 filter paper by application in the form of spots or streaks. The chromatograms were developed using the following solvent systems in an ascending manner for 18 hours:

1. N-butanol: acetic acid: H_2O (2:1:1, V/V/V).
2. Isopropanol: H_2O (70:30; V/V) in an atmosphere of Ammonia (0.35 ml conc. NH_4OH per liter of air).
3. Propanol: H_2O (70:30; V/V).
4. n-Butanol: acetone: acetic acid: 5% NH_3: H_2O (3.5:2.5:1.5:1.5:1).
5. Tertiary amyl alcohol: formic:H_2O (3:2:1; V/V/V).

Thin layer chromatography: Thin layer chromatography (TLC) was used as an alternative to paper chromatography. Glass plates 20 cm x 20 cm and 20 cm x 5 cm were used as a support for layer of absorbents. Silicic acid (Malinkrodt; chromatography grade), 200 mesh and Aluminium oxide with 600 mg of Calcium phosphate as a binder,

were employed as absorbents. The developing solvent systems used are listed below:

1. Butanol:acetone:acetic acid: NH_3:water (3.5:2.5:1.5:1.5:1).
2. Propanol:H_2O (7:3; V/V).
3. Methanol: chloroform (8:2; V/V).
4. Tertiary amyl alcohol: formic:H_2O (3:2:1; V/V/V).

Column chromatography: Columns of cationic and anionic exchange resins, Dowex-50 H^+ form and Dowex-1, AG, formate form (0.7 cm x 10 cm) were employed for separation of small samples of reaction mixtures.

DEAE-cellulose columns were prepared as follows:- ten grams of DEAE cellulose were equilibrated against Tris (0.02 M) buffer (pH 8.0) by excessive washing in the same buffer. The slurry was then poured into a column 1.1 cm x 20 cm. Five ml of the bulk preparation were then loaded on the column. The column was washed with ten bed volumes of the original buffer (Tris, 0.02 M, pH 8.0). The eluate was collected in ten ml fractions. The fractions were dried, dissolved in 1 ml water and their radioactivity was determined. They were also checked for the presence of benzimidazole or BMN by means of TLC.

DETECTION AND ELUTION

Benzimidazole and derivatives appear as fluorescent spots on thin layer chromatograms when viewed under UV. Fluorescence is not easily detected on paper, and also with some absorbent solvent systems, but it was very pronounced on silicic acid plates. The fluorescent zones observed under UV illumination were delineated with a pencil and the dry power in the absorbing region was scraped off by means of a flattened spatula and transferred to a funnel fitted with a sintered glass filter. The developing solvent was layered on top of the absorbent powder in the funnel and allowed to seep through the powder over a period of a few hours. Eluants from three successive elutions were combined, evaporated to dryness, dissolved in water and diluted as required after adjusting the pH to neutrality. 0.1 ml samples were withdrawn for radioactivity counts and obtaining the ultra violet absorption spectra. The samples were rechromatographed in the same manner and recorded on tracing paper and the Rf values were calculated.

The plate was photographed under illumination from two UV lamps placed on either side using a yellow filter and an exposure of 90 minutes.

ANALYSIS OF THE PRODUCT

Spectral

Ultra violet absorption spectra of the product separated by TLC were obtained by using a Zeiss or a Beckman DK automatic recording spectrophotometer.

Chemical

Known quantities (2 to 3 mg) of the product were hydrolysed in 6N HCl at 100°C for 6 hours. Both free and esterified phosphate were determined by the method of Waygood (1948) which is a modification of the procedure of Lowry and Lopez (1946).

Ribose was determined after hydrolysis by Mejbaum's orcinal procedure (1939). Indirect evidence for the presence of ribose was also obtained by the furfuryl spot test for carbohydrates (Feigl, 1960), as follows:-

One to two mg of the sample was placed in a crucible. One drop of syrupy phosphoric acid was added, and the crucible was covered with filter paper, moistened with aniline acetate solution (10 per cent solution of aniline in 10 per cent acetic acid). A watch glass was used to hold the filter paper in place. The crucible was then heated gently for 20 to 30 seconds. A pink color appeared on the filter paper showing the presence

of carbohydrate.

Molecular weight determinations

A wide variety of methods are available for micro determination of molecular weight (Millard, 1921) which are chiefly adaptations of methods used at a macro scale. These include ebullioscopic, cryoscopic and vaporimetric methods. Ebullioscopic methods take into account the elevation of boiling points of the solvent. Cryoscopic methods the depression of the freezing and melting points and the vaporimetric methods are based on vaporization of the substance in a closed system. Two of the most commonly used are the Rast method, (cryoscopic) and the Signer method (Steyermark, 1961). The latter is based on the principle of isothermal distillation. Due to the convenience and the short duration of the experimental period the Rast method was employed for the microdetermination of the product of benzimidazole - PP-ribose-P reaction. This method involves the principle that the melting and freezing point of a solvent is depressed in proportion to the molar fraction of the solute present. The solvent employed was camphor (BDH: microanalytical reagent, resublimed in vacuum). Roth (1958) has compiled a list of the solvents that can be used. Two important

properties of a solvent are that it should not react with the sample and the solute should not decompose at the melting temperature of the solvent. These two criteria impose limitations on the solvent to be used.

The procedure was as follows:-

A micro capillary was weighed, closed and packed with the solid solvent through the open end and weighed again. In a second tube a small amount of the sample was taken and the solvent inserted on top. The weight of the capillary tube sample and solvent was recorded. The two tubes were then sealed under vacuum and heated in an assembly capable of maintaining a constant temperature gradient (melting point apparatus; Scientific Glass Co.) to a temperature adequate to melt the contents completely. The temperature at which the last crystal disappeared was taken as the melting point. The bath was cooled till the contents of the capillary tubes resolidified and then reheated. The process was repeated until a reasonable agreement between consecutive values was obtained. The freezing points were recorded on the same samples by allowing the solution to cool gradually and noting down the "fogging" temperature. The molecular weight of the sample was calculated from the following equation (Millard, 1021):

$$M.W. = \frac{100 \times Kf \times g}{T \times G}$$

Kf represents the molar melting point depression

g = weight of the sample

G = weight of camphor

t = the depression of the freezing point of camphor.

The value Kf for camphor was obtained, by using benzimidazole as a standard, from the equation

$$Kf = \frac{118.13 \times G \times t}{1000 \times g}$$

(118.13 = molecular weight of benzimidazole).

Calculations for Kf:

Weight of benzimidazole = 0.9 mg

Weight of camphor = 9.0 mg.

Melting point of camphor = 173.2

Melting point of mixture = 141.0

t = 32.2

$$\text{Hence } Kf = \frac{118.13 \times 9 \times 32.2}{1000 \times 0.9}$$

$$= 38.0$$

EXPERIMENTAL RESULTS

Evidence for the formation of benzimidazole mononucleotide was obtained from various sources; firstly, competition with the OMP pyrophosphorylase reaction by benzimidazole for PP-ribose-P; secondly the conversion of orotate-6-C^{14} to labelled OMP by enzyme preparations from wheat embryos and finally the resolution of the reaction mixture into benzimidazole and the product by chromatography and chemical analysis of the product.

1. COMPETITION BETWEEN BENZIMIDAZOLE AND OROTATE FOR PP-RIBOSE-P IN THE PYROPHOSPHORYLASE REACTION

OMP pyrophosphorylase reaction was used for the assay of the enzyme catalyzing the reaction of benzimidazole and PP-ribose-P since it is assumed that PP is an end product.

A comparison of the progress of OMP pyrophosphorylase reaction with orotate and benzimidazole (O + B) and orotate alone (Figure 16) showed that only 50 per cent of the orotate was metabolised in the presence of benzimidazole. Since kinetin is known to effect protein and chlorophyll synthesis in a manner closely resembling the benzimidazole effect, it was also tested in the system. In the presence of kinetin also the conversion of orotate to OMP was decreased (O + K). This indicated

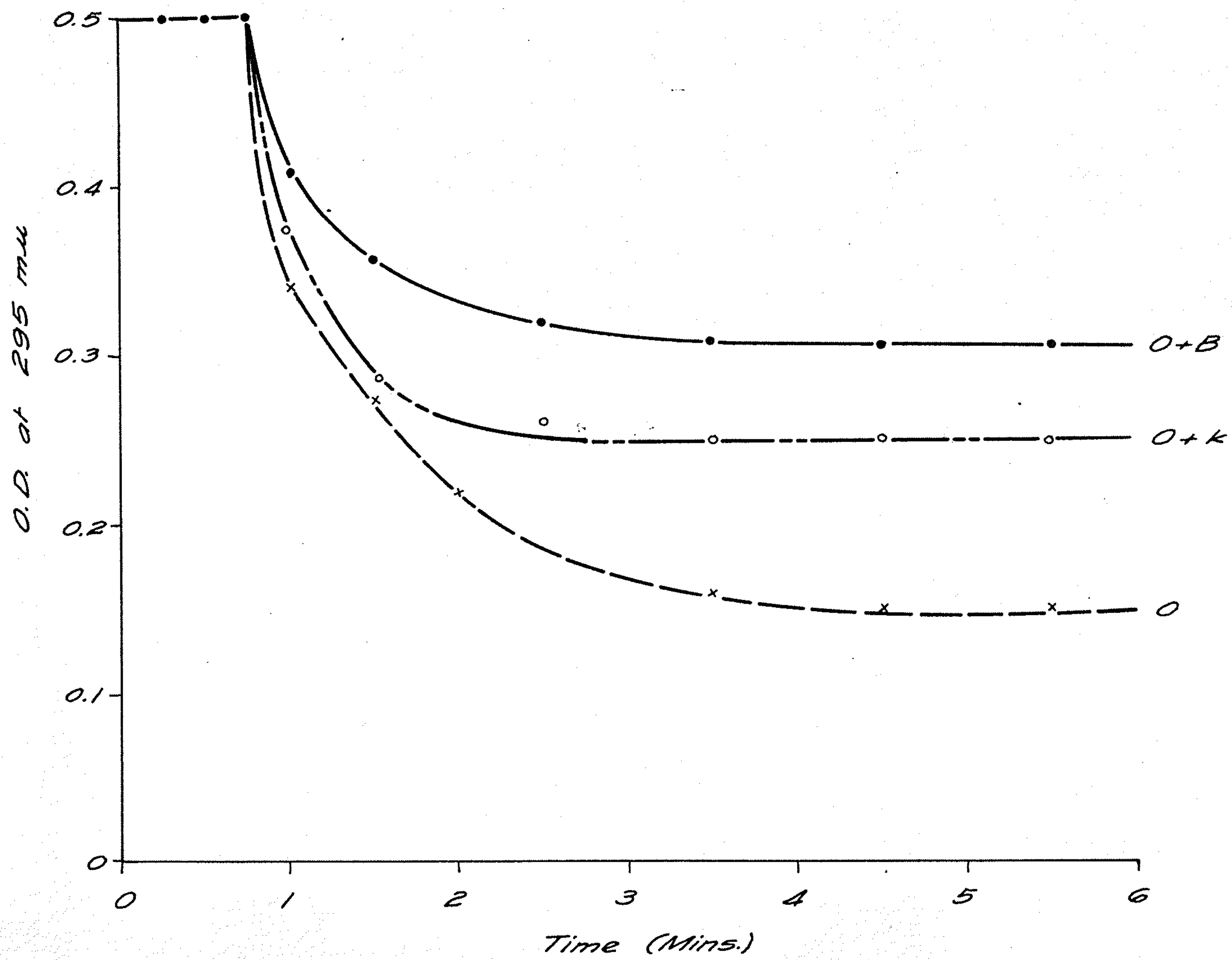
that kinetin also reacts with PP-ribose-P, which is quite possible considering that it has an intact purine ring. Using the same principle 2-methyl benzimidazole and 2-amino benzimidazole were also tested in the same system. These two derivatives of benzimidazole and kinetin are much less reactive (Table IV).

Figure 17 shows ultraviolet absorption spectra of kinetin and the product of the kinetin, PP-ribose-P reaction. Kinetin has an absorption peak of 260 mμ and a shoulder in the vicinity of 280 mμ . The product shows the maximum absorption at 266 mμ and another hump at 269 mμ . The absorption maxima of the kinetin and the product are not far removed from each other, but the product shows a lower extinction than kinetin. These spectra provide a useful assay for the reaction leading to formation of kinetin mononucleotide. It can be measured spectrophotometrically by following the decrease in optical density at 270 mμ associated with the disappearance of kinetin. At present, further studies were not carried out on the reaction of kinetin, although this is of interest, in view of the dramatic effect of kinetin on nuclear and cell division.

Figure 16. Effect of benzimidazole and kinetin on the OMP pyrophosphorylase reaction.

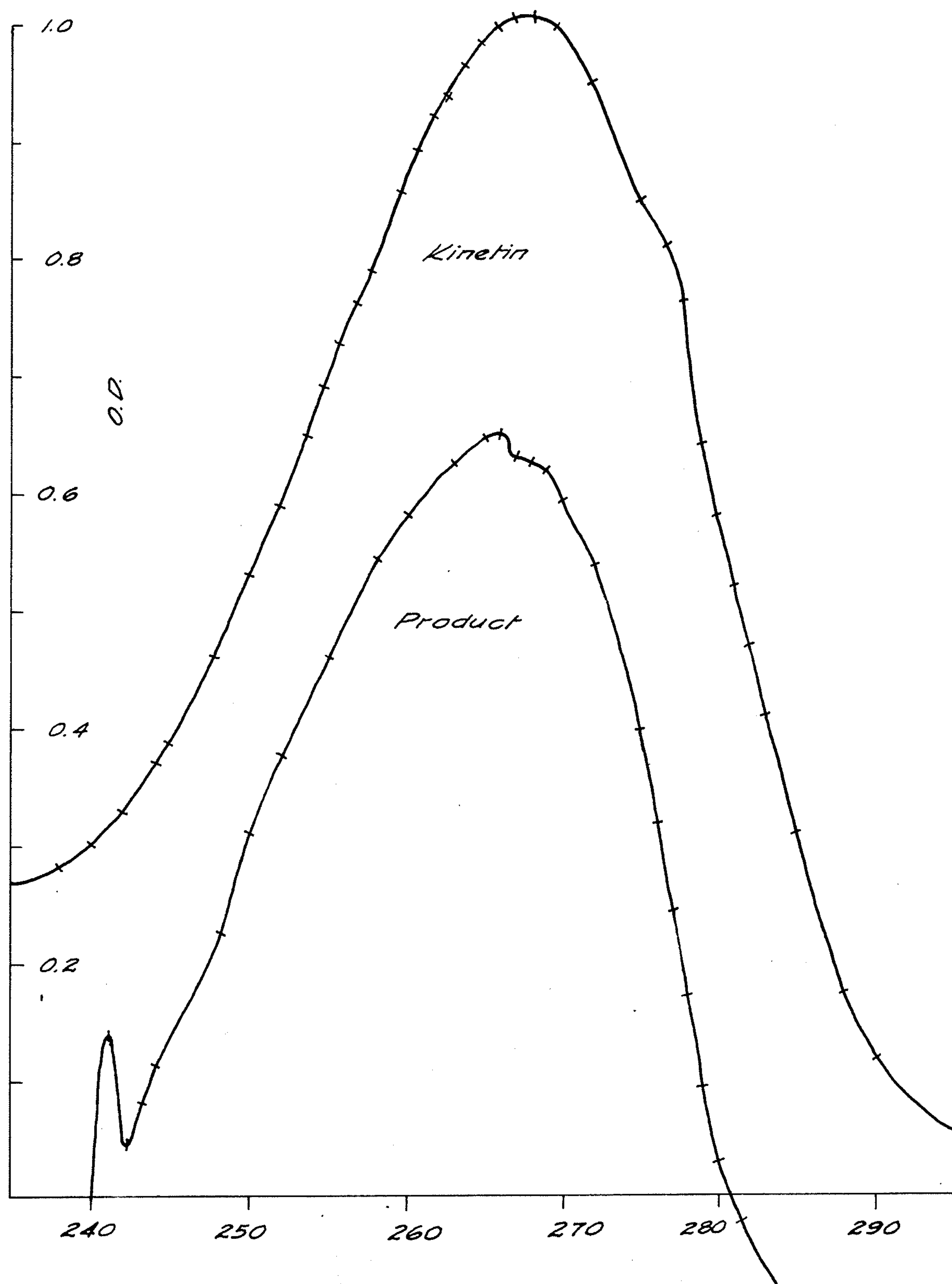
O stands for orotic acid,
B stands for benzimidazole and
K for kinetin.

Each curve was obtained from a reaction mixture in a cuvette containing 0.4 moles of orotic acid and the same amount of benzimidazole and kinetin wherever indicated.



0.5
0.4
0.3
0.2
0.1
0
O.D. at 295 mu
0
1
2
3
4
5
6
Time (Mins.)
O+B
O+K
O

Figure 17. Ultraviolet absorption spectrum of the product of reaction between kinetin and PP-ribose-P. Kinetin is shown to have the maximum absorption at 268 m and the product at 266 m .



1.0
0.8
0.6
0.4
0.2
O.D.
Kinetin
Product
240
250
260
270
280
290
mμ

TABLE IV. Effect of benzimidazole, its derivatives and kinetin on OMP formation.

System	Decrease in O.D. at 295 mμ	Decrease in the amount of OMP formed (percent)
No additions	0.40	-
Benzimidazole	0.15	62.5
2-aminobenzimidazole	0.25	37.5
2-methylbenzimidazole	0.30	25.00
Kinetin	0.20	50.00

A consideration of the assay for BMN pyrophosphorylase revealed a serious drawback in this method. Although competition for PP-ribose-P provided a convenient, and accurate method, its application was limited to preparations containing OMP pyrophosphorylase. For preparations devoid of this enzyme alternate procedures had to be employed. In the initial experiments the presence of BMN-forming enzyme in the protein preparations, appeared very variable. It was later evident that some factor, influencing the BMN pyrophosphorylase has been overlooked early. The duration of contact between ammonium sulphate and the preparation during fractionation procedures was found to be a very important factor in this. After the addition of ammonium

sulphate in the second step (0.33 - 0.65 saturation), if the precipitated protein was collected within one hour, there was practically no BMN pyrophosphorylase activity i.e., no inhibition[1] of OMP pyrophosphorylase reaction with benzimidazole. Increasing this time to 4 hours caused only a slight or no increase in OMP pyrophosphorylase activity, but a 10-15 percent inhibition by benzimidazole could now be detected. A 60-80 percent inhibition was detected when the solution was permitted to stir overnight and the precipitate collected the next morning (Table V). It seems logical to conclude that BMN pyrophosphorylase precipitates out gradually over a period of 12 to 13 hours. In all subsequent preparations ammonium sulfate precipitation (0.35 to 0.65 saturation) was allowed to proceed overnight.

In Table V relative values for OMP pyrophosphorylase and inhibition by benzimidazole are listed. These values are averages for three individual fractionations.

---

[1] There is a competition between OMP pyrophosphorylase and BMN pyrophosphorylase reactions. The term inhibition, has been used here and in the following pages, to indicate the decrease in OMP formation and not in the technical sense of the word.

TABLE V. Effect of duration of ammonium sulphate treatment.

Time	Relative activity for 5 OMP pyrophosphorylase (percent)	Inhibition by benzimidazole
1 hour	90 to 100	0 to 5
4 hours	100	10 to 15
overnight	100	60 to 80

On cold storage at -12°C, OMP pyrophosphorylase activity started to decline after two days. The relative values for the activity on the third, fourth, and fifth day of storage were 60, 50 and 20 percent. Almost no activity was detected after the fifth day (Table VI). Inhibition by benzimidazole on the other hand disappeared at a faster rate. Compared to a relative inhibition of 60-75 percent on the first day, by the third day only about 20 percent remained.

TABLE VI. Effect of duration of storage.

Age of preparation (Days in storage)	Relative activity of OMP pyrophosphorylase	Relative Inhibition by benzimidazole
1	100	60 - 75
2	95 - 90	50
3	60	20
4	50	-
5	20	-

These two experiments strongly suggest that OMP and BMN pyrophosphorylase are distinct proteins.

2. CONVERSION OF OROTIC-6-C^{14} TO OMP IN THE PRESENCE AND ABSENCE OF BENZIMIDAZOLE

This experiment was performed in order to measure the actual differences in the levels of OMP formed in the presence and absence of benzimidazole, in buffered wheat embryo homogenates. Two grams of viable wheat embryos were homogenized with 20 ml of 0.05 M K_2HPO_4 at pH 7.4. The extract was filtered through four layers of cheesecloth, residue discarded and the supernatant centrifuged at 15,000 x g for 10 minutes. The supernatant obtained at this stage was used as the "crude homogenate".

Five ml samples of the crude homogenate were incubated with the following:

A. orotic-6-C^{14}

B. orotic-6-C^{14} + PP-ribose-P, 1 µmole,

C. orotic-6-C^{14} + red NAD, 2 µmoles,

D. orotic-6-C^{14} + cold benzimidazole, 2 µmoles,

E. benzimidazole-2-C^{14}

F. benzimidazole-2-C^{14} + PP-ribose-P, 1 µmole,

for one hour at 30°C. At the end of incubation the mixtures were deproteinized, and evaporated to dryness. Each sample was dissolved in 1.0 ml of water and 0.5 ml

of each was chromatographed on paper in n-butanol: ethanol:formic acid: water (5:3:2:1) in a descending manner.

The radiochromatograms from this experiment revealed four zones containing radioactive material in samples A, B, C and D and only two in E and F. Zone 1 corresponds to the origin in all cases and the successive zones are numbered II, III, IV. In case of sample E and F since there are only two zones, numbers I and II, are used for the origin and second zone. Approximately 250,000 cpm of orotate were fed in the first four samples and 200,000 cpm of benzimidazole in E and F. Strips cut from radiochromatograms of samples B, C, and D are shown in Figure 18. Material from this zone was eluted in hot water and the counts per minute were recorded (Table VII).

On elution of these zones with hot water, their ultraviolet absorption spectra were obtained and these compounds were identified as OMP (I), UMP (II), orotic acid (III) and uracil (IV) on basis of their spectra. This was further confirmed by cochromatography of known samples of UMP, orotic acid and uracil.

The data presented in Table VI showed that the formation of OMP and UMP was enhanced in the presence of

added PP-ribose-P. Uracil appeared in A and B in considerable amount but in C and D it was detected only in traces. Sample D is the most interesting of all because in this sample "cold" benzimidazole was included in addition to orotic-2-C^{14} and PP-ribose-P. The conversion of orotic and to OMP was very poor, as anticipated, and consequently only a negligible quantity of UMP was encountered. The accumulation of orotic acid seen in sample D was anticipated on basis of inhibition by benzimidazole. As also seen in Figure 18 for some unexplainable reason in sample D, orotic acid failed to migrate to the position it did in A, B and C. The same also true for uracil.

TABLE VII. Radioactivity of samples before and after chromatography.

Sample	Total counts per fraction	Total counts per zone I (OMP)	II (UMP)	III (orotic)	IV (uracil)
A	248,600	25,570	2,300	200,180	11,594
B	201,800	29,300	25,500	166,450	7,500
C	226,000	19,350	14,230	148,390	790
D	242,000	1,640	580	246,300	468
E	184,700	4,500	207,400		
F	193,250	5,970	191,360		

The evidence obtained from sample D was, however, not fully substantiated in E and F. The substance that remained at the point of origin in E was in very small

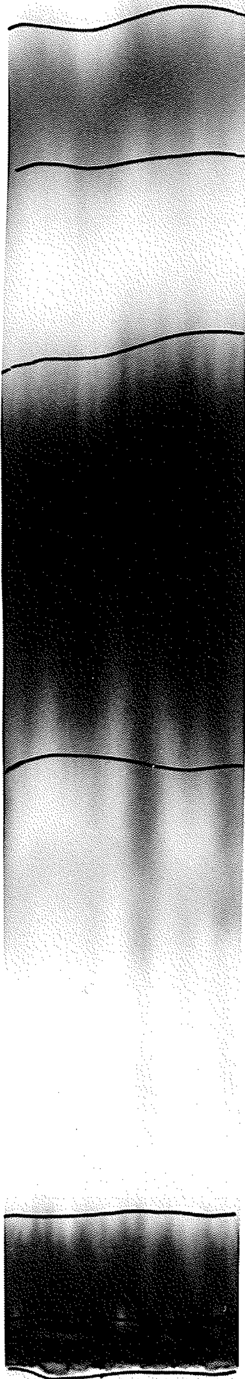
Figure 18. The conversion of orotic acid to OMP in the presence and absence of benzimidazole.
(Sectors cut from radioautograms are shown here).

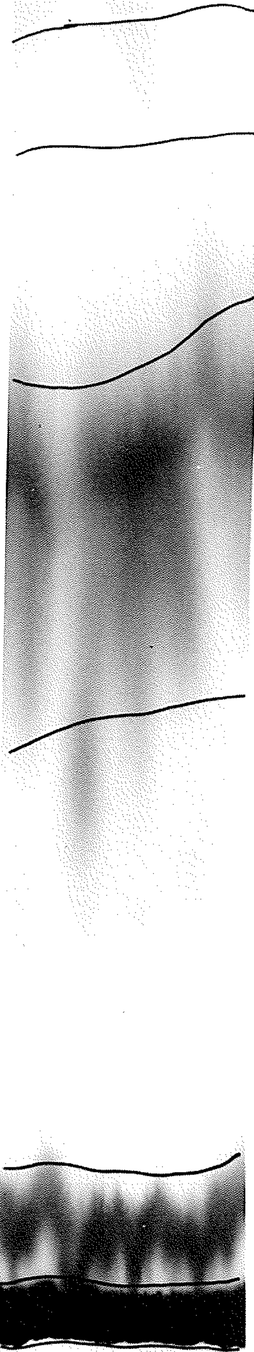
1, is a sector from radiochromatogram of a homogenate containing orotic-6-C^{14} + PP-ribose-P, 1 mole

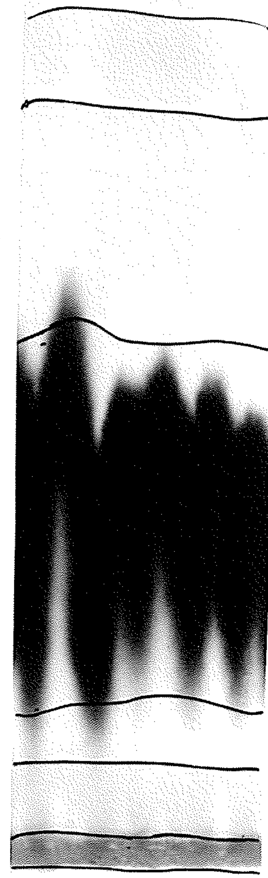
2, homogenate contains orotic-6-C^{14} + red NAD

3, homogenate contains orotic-6-C^{14} + PP-ribose-P + cold benzimidazole, 2 moles.

1 has been identified as OMP; II, as UMP; III as orotic acid; and IV as uracil.


IV
III
II
I
1


2


IV
III
II
I
3

quantities both in the presence and absence of added PP-ribose-P. This decrepency was attributed to the fact that the benzimidazole and BMN were not being separated effectively by the solvent used. Use of solvents 1 to 5 as listed in Methods also proved unsatisfactory.

3. ELECTROPHORETIC SEPARATION AND HYDROLYSIS OF BMN

Samples from reaction mixtures containing benzimidazole-2-C^{14} and PP-ribose-P were subjected to electrophoresis in formate buffer (0.05) pH 3.5 for 3 hours. In Figure 19 scans from selected portions of radioelectropherograms are redrawn. The peak at the point of application on extreme left is BMN and the other peak approximately an inch away from origin towards the cathode was identified to be benzimidazole.

It was argued that if the peak at origin was BMN it should yield benzimidazole on hydrolysis. The substance at the origin was then eluted in hot water and hydrolyzed in 6N HCl for 6 hours, concentrated and was re-electrophoresed. On the right hand side a portion of a scan from the electropherogram of the hydrolysed product (HP) is drawn. The product liberated on hydrolysis, did, indeed, move to the position corresponding to that occupied by benzimidazole. In the hydrolyzed

product there was nothing resembling the substance remaining at origin. Recovery of benzimidazole from the product again confirmed that it was a derivative of benzimidazole.

In some experimental mixtures three compounds were detected at the origin. The scans represented in Figure 20, show one peak at the position to which benzimidazole normally migrates and three peaks at the origin. These additional peaks are probably due to a partial hydrolyses of BMN and one of them may represent a ribosidic derivative. The group of peaks labelled "B" and "b" seen in the scan in the middle of Figure 20 were discerned on increasing the sensitivity of the ratemeter (actigraph) to a maximum. Both these peaks were in the benzimidazole region. One of them was benzimidazole and the other possibly a degradation product or an impurity present in the commercial sample. The scan on the extreme right is a benzimidazole reference. Altogether, four compounds were detected, three derived from the product of the reaction and one from benzimidazole itself.

4. ISOLATION OF BMN

The evidence obtained from experiments described in the preceding sections provided ample grounds for con-

Figure 19. The electrophoretic separation of benzimidazole and BMN in formate (0.05 M) buffer, pH 3.5, 25 ma for 1.5 hours.

B represents benzimidazole in the reaction mixture in diagram 1. BR is the product, BMN

In diagram 2, HP stands for the product of hydrolysis of BMN eluted form the previous electrophergram.

B, benzimidazole was the product of hydrolysis.

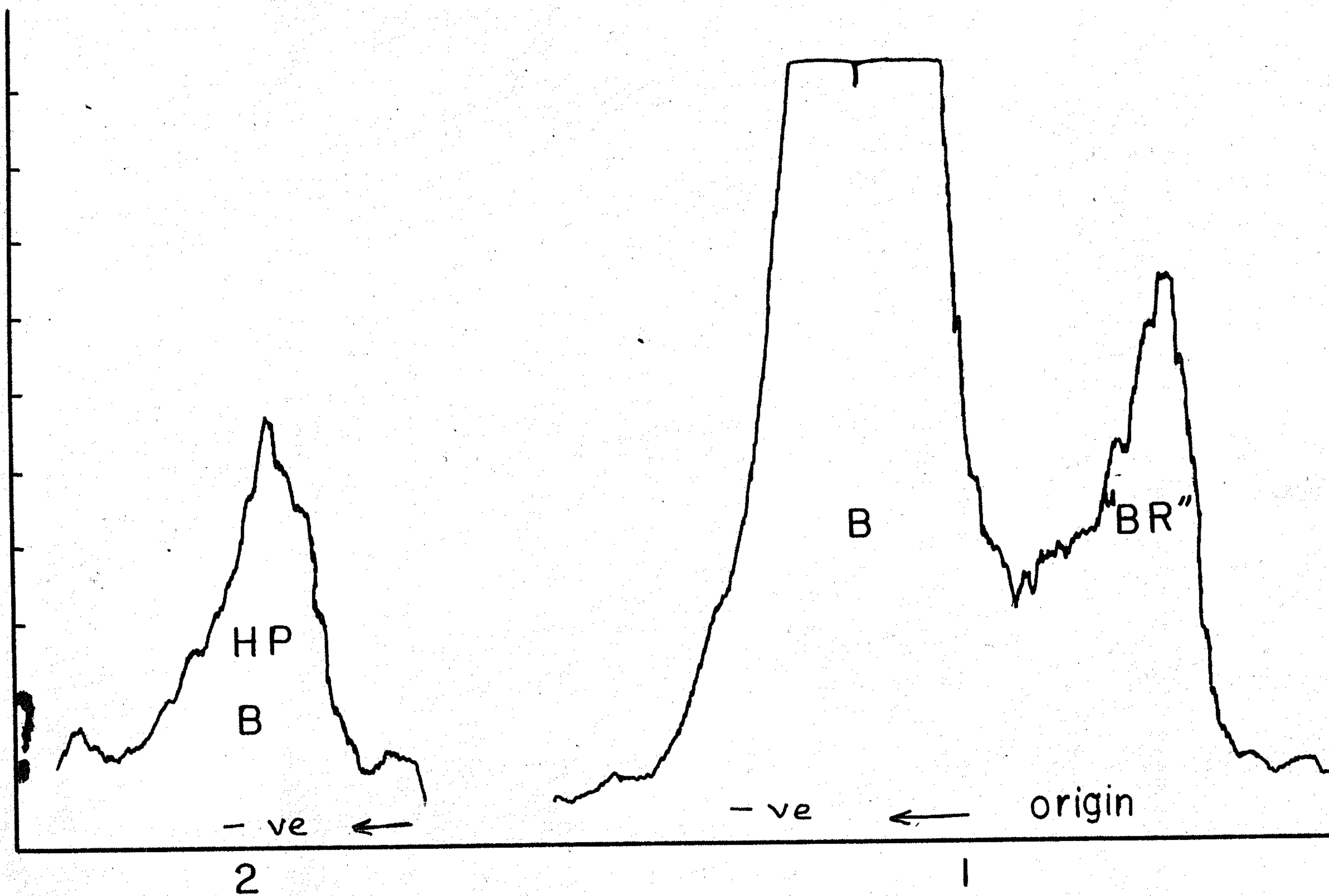
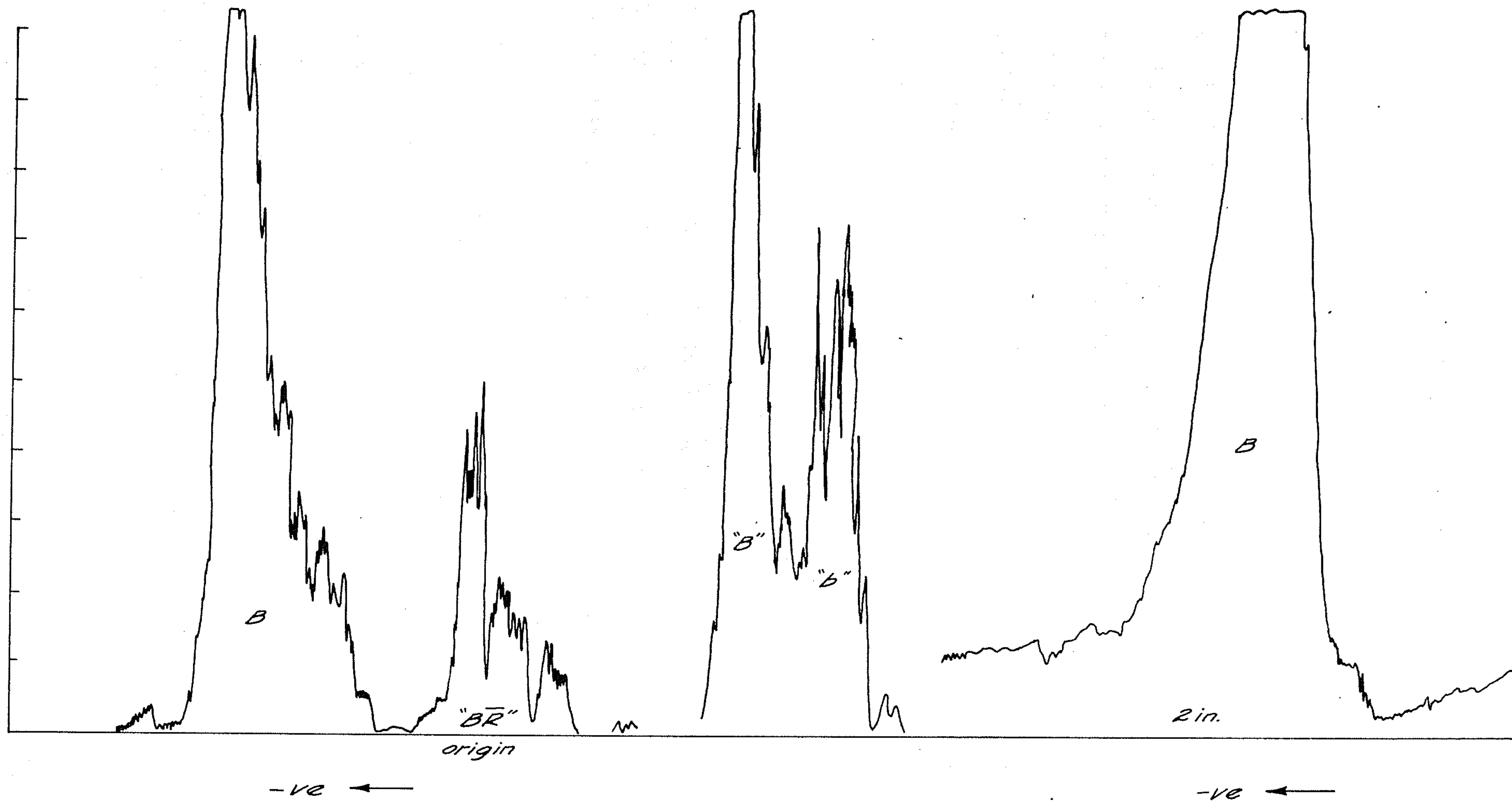

HP
B
– ve
2
B
"BR"
– ve
origin
1

Figure 20. The electrophoretic separation of benzimidazole and BMN continued.
The scan on the extreme right shows the benzimidazole (B) reference. (2 in. from the point of application).
The scan in the middle shows a further resolution of benzimidazole component isolated from a reaction mixture, into "B" and "b".

The scan on the extreme left shows an electropherogram of the reaction mixture, BR is BMN (resolved in this case into three peaks; and B is benzimidazole).


B
"BR̄"
origin
-ve ←
"B"
"b"
B
2 in.
-ve ←

sidering the reaction between benzimidazole and PP-ribose-P as established, and BMN pyrophosphate to be the enzyme responsible for the reaction as definite. But so far it had not appeared feasible to separate BMN from the mixture satisfactorily by the methods employed. Paper chromatography and electrophoresis did not yield BMN in a quantity sufficient for a chemical analysis.

The feasibility of application of thin layers chromatography (TLC) in separating purine and pyrimidine bases, and nucleotides was recently demonstrated (Randerath, 1961). The superiority of the technique over paper chromatography is illustrated by the fact that a mixture of four isomeric 2' and 3' purine nucleotides was resolved in 90 minutes on cellulose layers chromatogram compared to 24 to 48 hours with the paper (Randerath, 1962). The possibility of separating BMN and benzimidazole on TLC was hence explored.

<u>Thin layer chromatography</u>

Thin layer chromatography is a rapid technique adapted for microchromatography on a glass plate coated with a layer of adsorbent which corresponds to a paper chromatogram. Although first described as early as 1938 by Ismailov and Schraiber, it was used extensively by Kirchner, Miller and Keller (1951) in their work on sepa-

ration and resolution of essential oils. It was, however not until Stahl (1956) devised a practical equipment and standardized the technique, that it was available for use in a variety of fields of application.

The absorbant layer of a known uniform thickness is applied on to glass plates of a standard size, absorbents that can be used are Silica Gel, Aluminium Oxide, Kieselguhr G, acetylated and ion exchange celluloses, according to the requirements of the components to be separated. Before application a suitable binder, usually calcium sulphate is mixed with the absorbent to render the layer adhesive.

A series of glass plates (20 x 20 cm) are mounted on a plastic aligning plate, with a raised edge on one long and one short side (22 x 113 cm). At either end two short (5 x 10 cm) starting plates are kept. The standard applicator (suitable for application of a layer 250 to 2,000 μ thick) is now positioned on the first starting plate. Meanwhile the absorbant is made into a slurry with water and small quantities of adhesive. The slurry is poured into the applicator and the latter is drawn across the series of plates, manually. The whole series of operations, starting from the preparation of the slurry to the preparation of the absorbant layer

should not take more than two minutes, otherwise the binder begins to set. The coated plates are next allowed to dry at room temperature for 10 minutes followed by heating in an oven at 110° for 40 minutes, in a drying rack. This process is referred to as 'activation'. The activated plates are now ready for use after cooling. They can be stored in a desicator and if necessary reactivated before use.

Known quantities of the sample and the reference solution are applied on the plate with the aid of a micropippette, the position of the spots being determined by reference to a plastic template. The plates are then developed in glass tanks containing 1.0 to 2.0 cm of the appropriate solvent. The solvent is allowed to ascend for a distance of 10 to 15 cm. The detection of the substance on the chromatogram can be achieved by spraying with a reagent or examination under the UV or exposure to iodine or bromine vapors (for location of colored complexes formed due to the presence of double bond in the organic materials).

The selection of the solvent system for thin layer chromatography should be based upon the chemical nature of the substance or substances to be chromatographed. Stahl (1958) has proposed the following order

of solvents:

Hexane	Ether
Carbon disulfide	Ethyl acetate
Carbon tetrachloride	Methyl acetate
Ethylene trichloride	Acetone
Benzene	Propanol
Methylene chloride	Ethanol
Chloroform	Methanol

Solvents are employed in mixtures generally. For trial runs small (5 x 20 cm) glass plates are used with a variety of solvents till a satisfactory solvent system is obtained. Large plates are then used with a solvent thus selected. Two dimensional separations can also be achieved on using a different solvent for each direction.

The technique of thin layer chromatography has been widely employed in resolution of natural and synthetic mixtures as well as for preparative purposes (Ritter and Meyer, 1962). A successful separation of mixtures of amino acids (Mutschler and Rochelmeyer, 1959; Nurnberg, 1959) fractionation and resolution of lipid components and chemical groups (Mangold and Tuna, 1961) phospholipids (Vogel _et al_.,1962) sugars and alkaloids has been achieved. Recently Randerath (1961) explored the possibility of

applying the technique of chromatography on a thin absorbant layer to the separation of nucleic acid derivatives. Randerath (1962) used ECTEOLA cellulose plates for separation of purine and pyrimidine bases, ribosides and nucleotides and compared it with paper chromatography conducted under identical conditions using the same solvent systems.

The plates for thin layer chromatography were prepared as follows:

A slurry of silicic acid was prepared by mixing 30 gms with 60 ml of a solution of 1 per cent calcium sulphate. The suspension was poured into an applicator and spread over thoroughly clean glass plates (5 x 20 cm). The reaction mixture and benzimidazole were applied on the activated plates and the plates developed on the solvents listed in "Methods". Table VIII summarizes the properties of the solvent/adsorbent combinations used.

Of all the solvent/adsorbent systems tested MeCh/silicic acid was found to be the most desirable combination. The time required for the solvent to travel a distance of 10 cm was the shortest and MeCh was very easy to remove by evaporation. In addition MeCh is an inert solvent and hence would be unlikely to cause any hydrolysis or breakdown of any other kind in the substances chromatographed.

The Rf value of the product in MeCh was about half of that of benzimidazole (Table VIII) and a remarkable separation was achieved in a period of 25 minutes. This procedure, in the present work, proved to be far superior to paper chromatography.

A good separation was also obtained by the use of other solvent systems (Table IX) but due to the reasons mentioned earlier MeCh was used in all experiments.

In Figure 21, a UV photograph of a thin layer chromatogram of a sample from the bulk preparation, is shown. The reaction mixture had been applied to the plates coated with silicic acid, in the form of a streak. A benzimidazole spot was included for reference. The chromatogram was developed in MeCh for 20 minutes. An intense BMN zone compared to an extremely light benzimidazole one is seen. Although the benzimidazole zone did not show up very well on the chromatogram it is nevertheless, sharply separated from BMN. A high rate of conversion is apparent from the picture. The MeCh/silicic system was subsequently used as a convenient guide and a quick check for fractions collected from columns and after reaction mixtures.

<u>Column chromatography</u>

After standardizing the system for chromatographic

TABLE VIII. Rf values of benzimidazole and BMN in the solvents employed.

System	Composition		Rf value Benzimidazole	BMN
MeCh	Methanol	80	0.54	0.24
	Chloroform	20		
PW	Propanol	70	0.69	0.83
	Water	30		
AmF	Tert-amyl alcohol	30	0.65	0.44
	Formic acid	20		
	Water	10		
BuAAW	Butanol	35	0.57	0.73
	Acetone	25		
	Acetic acid	15		
	NH_3(5./.)	15		
	Water	10		

Figure 21. Ultraviolet photograph of a thin layer chromatogram of the reaction mixture, using a silicic acid/MeCh system.

The bright band (P) represents BMN, the faint band on top (B) is benzimidazole and the spot is a benzimidazole reference. O is origin.

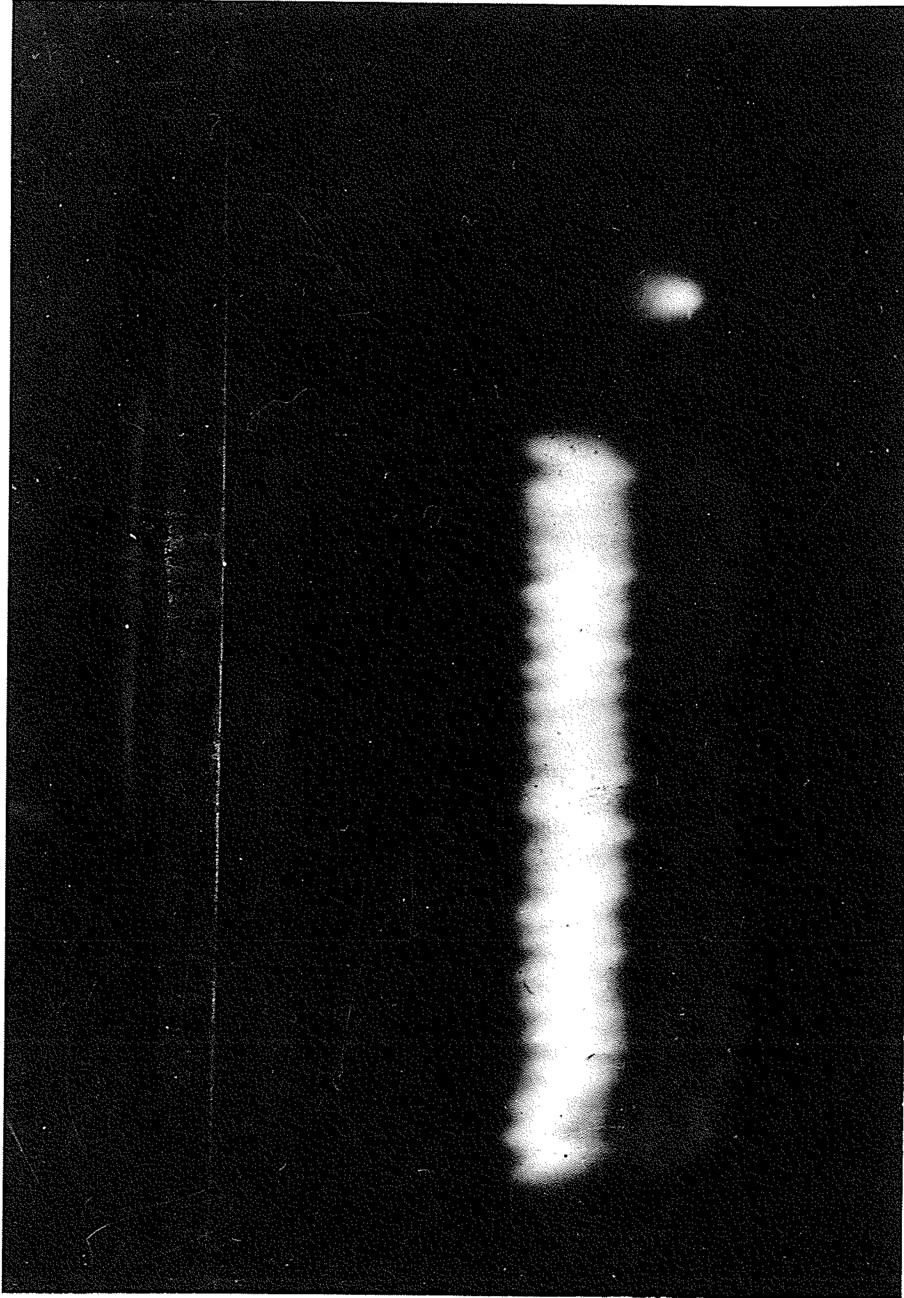

O
P
B

TABLE IX. The properties of solvent systems used for TLC

Solvent	Composition	Preparation	Time for 10 cm rise of solvent	Remarks
1. MeCh	Methanol 80 Chloroform 20	Contents shaken vigorously Good for 4 runs	25 min.	Separation good on silicic acid Plates dry up easily
2. PrCh	Propanol 80 Chloroform 20	Good, for 2 runs	45 min.	No separation at all
3. PrW	Propanol 70 Water 30	Good for 1 run only	1 hour (on aluminium oxide)	Separation fairly good
4. AmF	Tert-amyl alcohol 30 Formic acid 20 Water 10		1.30 hours	Separation good with aluminium oxide
5. BuAAW	n-butanol 35 acetone 25 acetic acid 25 W#3 (5./.) 15 Water 10	Prepared fresh before use	50 min.	Spots do not show up on aluminium oxide. Suitable only with silicic acid

separation and detection of benzimidazole and BMN, 1 ml aliquots were withdrawn from the bulk preparation and experiments were conducted to select a suitable column for a large scale separation. For this Dowex-50, H^+ and Dowex-1 AG, formate column were used. The trial run revealed a very poor separation. In each case either of the two substances tended to stick tenaciously to the column and the eluting buffer proved to be either too concentrated or too weak to effect a satisfactory separation. A suitable value for the ionic strength of the eluting buffer, just adequate for a resolution of the two components, could not be achieved.

The next in the series of ion exchanger used was DEAE-cellulose. One ml aliquots from the bulk preparation are loaded on to a DEAE-cellulose column which had previously been equilibrated against Tris-HCl buffer (0.02 M), pH 8.0. Five bed volumes of the same buffer were used for elution and six 10 ml fractions were collected and their radioactivity determined. Activity was concentrated in two fractions (Table X). The fractions were also checked on TLC.

The fractions containing the major part of radioactive material were distinctly separated by fraction number 3. Fractions 1 and 2 with the major portion of

counts were demonstrated to contain BMN and no benzimidazole (by TLC) whereas in 4,5 and 6 all the benzimidazole had appeared.

TABLE X. DEAE-cellulose chromatography of the bulk preparation.

Fraction number	Total cpm/ml	Compound (identified by TLC)
1 2	14,800	BMN
3	-	None
4	1,800	
5	600	Benzimidazole
6	950	

Although DEAE-cellulose column allowed a distinct and a very sharp separation of the two compounds the system had to be abandoned due to an uncomfortably long time required for drying the fractions completely.

Experience had shown that TLC consistently yielded excellent separation on silicic acid hence the feasibility of using a silicic acid column was explored on a small scale. A slurry of silicic acid was prepared in a mixture of methanol and chloroform (4:1), packed into a column of appropriate dimensions and allowed to settle down gradually. One ml of reaction mixture was loaded on the column and eluted with 5 bed volumes of

methanol:chloroform (4:1) followed by two bed volumes of chloroform. Five ml fractions were collected, evaporated to dryness and scanned by means of TLC. A successful separation was achieved, as anticipated.

The remainder of the bulk preparation was then subjected to chromatography on a larger (2.5 cm x 25 cm) column of silicic acid and the procedure used for the pilot run was repeated exactly. Forty 10 ml fractions were collected and scanned on TLC as usual. In Table XI a summary of the elution procedure is presented. Fractions 7 to 9 were found to contain a substance which on superficial observation appeared to migrate to the benzimidazole position on the thin layer plate, but on crystallization with methanol this substance (B_1) resolved into two components, a crystalline substance (P_3) and the supernatant. The symbol P_3 has been used arbitrarily to indicate that this compound was third to be detected and travelled beyond the benzimidazole position on the chromatogram; in fluorescence, however, it resembled benzimidazole. Fractions 9 to 11 could also be resolved further by crystallization into B_1 and B_2. B_2 behaved exactly like benzimidazole on TLC. The succeeding four fractions 12 to 16 contained all the major product, referred to as P_1 (assumed to be BMN from

the Rf value). In chromatographic behaviour P_1 resembled the product obtained on TLC before fractionating the reaction mixture on the column. P_1 was also crystallized by methanol, from an aqueous solution. In the last five fractions small quantities of a substance, with the same Rf value as P_1 but with a blue fluorescence, were detected.

The confusing array of compounds that appeared after fractionation on silicic acid column is explained by the occurrence of hydrolysis on the column and also during the processes of elution and drying. Elution was a comparatively slow process extending over a period of 30 hours. During this time probably a partial hydrolysis of the major product P_1 occurred resulting in a small amount of P_2 and P_3. P_3 could also have been present as an impurity in the benzimidazole used and might have escaped detection on the plate due to a lack of resolution. On the column a more efficient separation could have possibly led to the appearance of this compound.

Figure 22 is a tracing from a thin layer chromatogram wherein the situation, before and after the column fractionation, is outlined. This chromatogram was prepared prior to crystallization of the compounds.

Figure 22. The separation of BMN and benzimidazole by column chromatography on silicic acid.

This is a tracing from a thin layer chromatogram (silicic acid/MeCh).
B, benzimidazole reference
RM, reaction mixture before column chromatography
1, a sample from fractions 7 to 9
2, fractions 10 to 12
3, fractions 12 to 16
4, fractions 16 to 22.

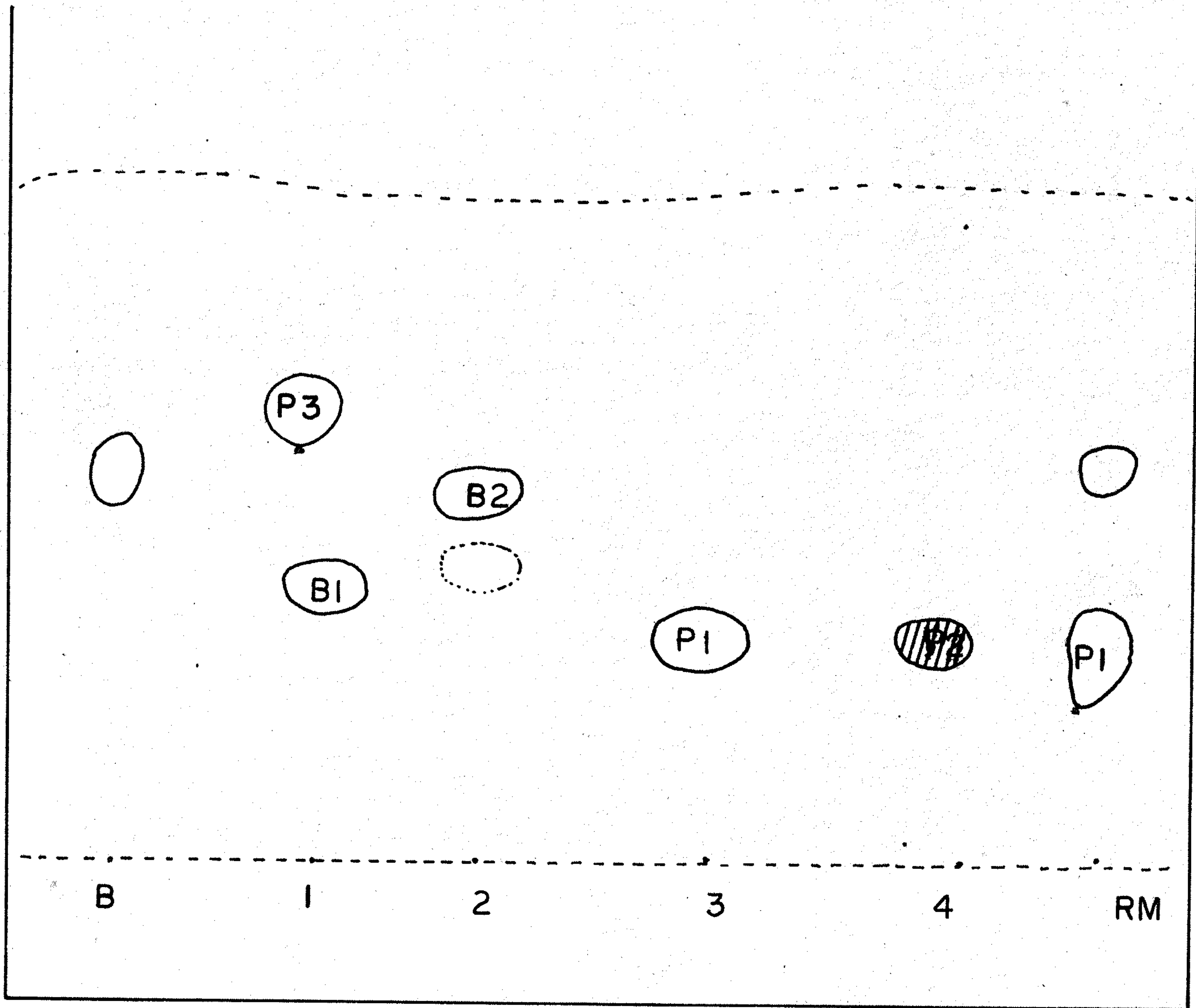

P3
B2
B1
P1
P2
P1
B
1
2
3
4
RM

TABLE XI. Analysis of fractions eluted from silicic acid column.

Fraction number	Compound (symbol)	Resolution on rechromatography into	Fluorescence (in UV)
1 to 6	-	--	
7,8 and 9	B_1	B_1 (crystals) B_3 (in supernatant)	violet blue
10 and 11	B_1	B_1 (small quantity) B_2 (crystals)	violet violet
12 to 16	P_1 (major product)	P_1 (crystals)	violet
17 to 22	P_2	P_2 (crystals)	violet

B is the benzimidazole reference spot. At positions 1, 2,3 and 4 samples from fractions 7 to 9, and 11, 12 and 16, and 17 to 22 were applied. RM is the reaction mixture before column fractionation.

5. IDENTIFICATION OF THE COMPOUNDS

For the purpose of identifying the compounds isolated from the column as described in the preceding section, some of their physical and chemical properties were studied. In Table XII the molecular weights of P_1, B_1, P_2 and B_2 are listed. Molecular weight

determinations were performed by the Rast method (1922) based on the freezing point depression of camphor. P_1 and B_1 were found to be the same compound (molecular weights 392.8 and 393.6 respectively). These values for molecular weights are accurate only within an error of 5 per cent.

Chemical analysis

The compounds P_1, B_1, P_2 and B_2 were analyzed for the presence of free and esterified phosphate and ribose. The samples were hydrolyzed in 6N HCl for 3 to 6 hours. The analytical data suggested that in P_1 and B_1 one molecule of esterified phosphate corresponds to one molecular of phosphate. Very small amounts of ribose were detected by the orcinol test (Majbaum, 1939) even after hydrolysis in 6N HCl. However, the presence of carbohydrate was comprised by a positive furfural test (Feigl, 1960). Since the only carbohydrate included in the reaction was ribose it seems reasonable to assume that the carbohydrate in question, indeed, was ribose. P_2 also contained ribose but no phosphate. No free phosphate was detected in any of the compounds, presumably it had been absorbed on the column.

The above analysis revealed that the product of

TABLE XII. Molecular weight determination: Rast Method.

Sample	Wt. of solute	Wt. of camphor	Mp of mixture	r	MW	Remarks
P_1	0.6	7.4	165.4	7.8	392.8	Clear solution
P_2	1.2	8.2	153.2	20.2	274.8	Slightly decomposed
B_1	1.4	8.0	156.3	16.9	393.6	Clear solution
B_2	0.8	6.8	154.6	18.6	240.5	Turned brown but remained clear

Melting point of camphor = 173.2°C
Molal freezing point constant = 38.0

Reliability of melting points ± 0.3°C

TABLE XIIa

Sample	Wt. of sample (mg)	ug P found	ug P calculated
P_1	1.2	43.5	40.8
P_2	1.5	-	-
B_1	1.1	40	37.4
B_2	1.1	-	-

Figure 23. The UV absorption spectrum of BMN at pH 2 (continuous line) and pH 11 (interrupted line).

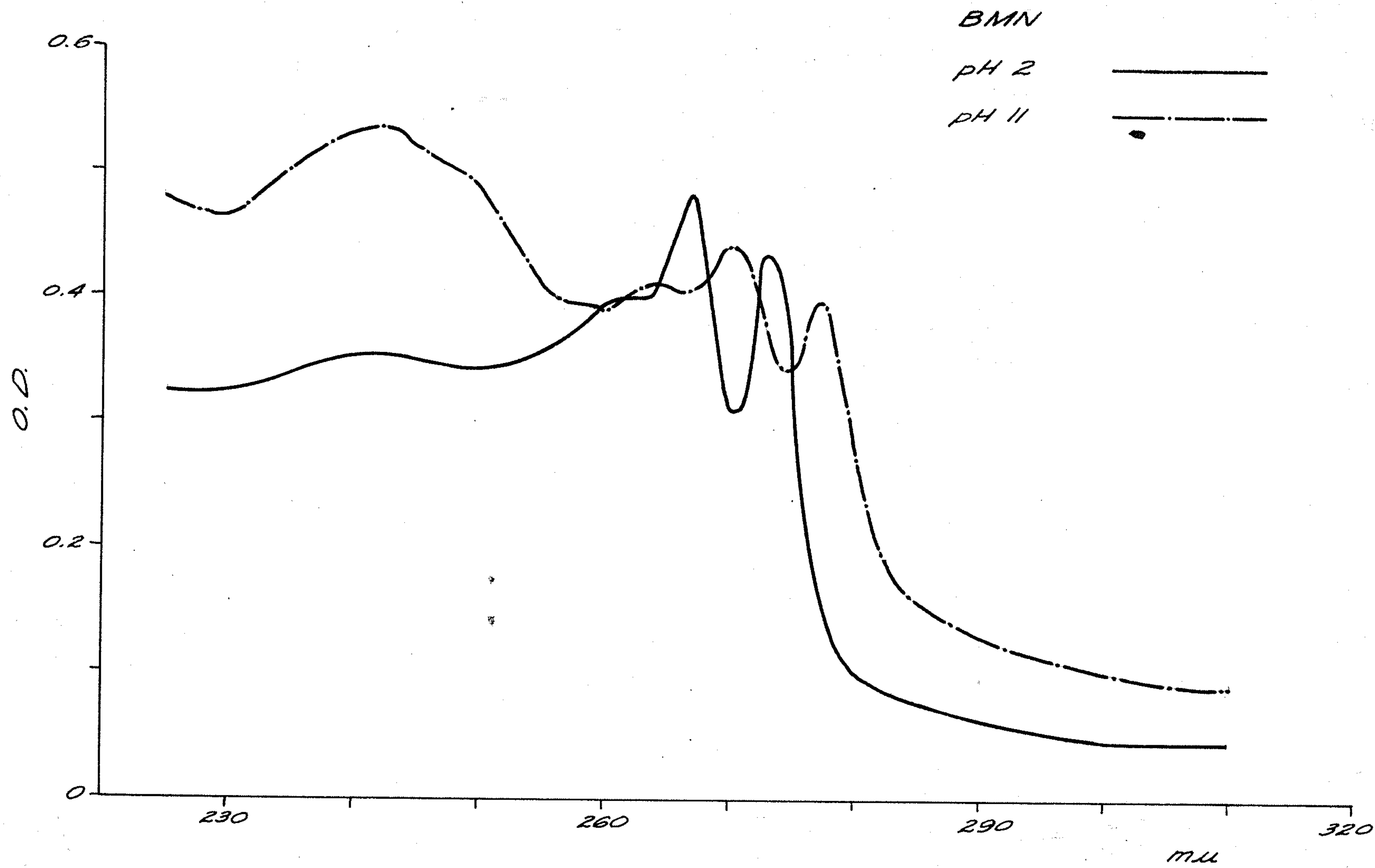
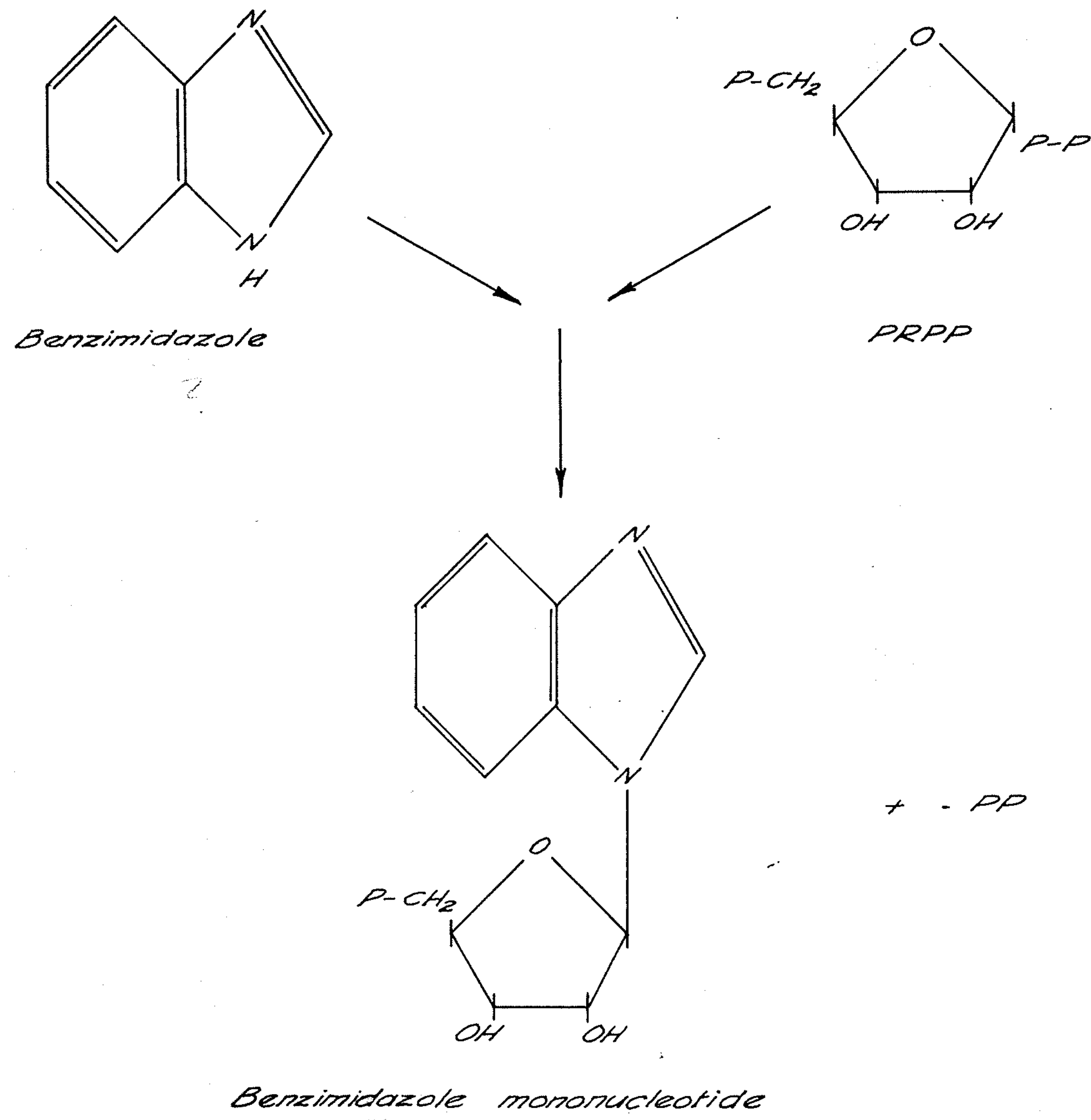

BMN
pH 2
pH 11
0.6
0.4
0.2
0
O.D.
230
260
290
320
mμ

Figure 24. A schematic representation of the reaction between benzimidazole and PP-ribose-P.


N
N
H
Benzimidazole
O
P-CH2
P-P
OH
OH
PRPP
N
N
O
P-CH2
OH
OH
+ - PP
Benzimidazole mononucleotide

the reaction between benzimidazole and PP-ribose-P yielding one molecule each of phosphate and ribose for every molecule of a partial hydrolysis on the silicic acid column giving a small but measurable quantity of a riboside.

The molecular weight of BMN in view of the analytical data should be approximately 366. The value actually obtained is slightly higher but still falls within the 5 per cent limit of error. The value of 274 obtained for P_2 approaches very closely that expected for benzimidazole riboside. Molecular weight of 240 for B_2 which is supposed to be benzimidazole is difficult to interpret. Benzimidazole and derivatives are known to undergo polymerization to produce bimolecular species through N-N linkage (Hoffmann, 1953). Though polymerization is a definite possibility, the suggestion is entirely speculative at this point.

The absorption spectrum of BMN (P_1) (Figure 23) was similar to, though not identical with that of free benzimidazole. At pH 11 three major absorption peaks appeared at 242.5 mμ, 271 mμ and 278 mμ, a shoulder at 249 and a minor peak at 263 mμ. At pH 2.0 in place of the peak around 240 mμ, a broad shoulder was seen, the

peaks in the longer wave length regions were also displaced to 267 mμ and 272.5 mμ accompanied by the appearance of an additional minor peak at 261 mμ. The relative absorbancies of the major peaks in the 260-280 mμ region were also different at acid and alkaline pH. The comparison of the absorption spectra with that of a sample of 1-β-D-ribofuranosyl benzimidazole synthesized by Davoll and Brown (1951) showed no significant differences. The ratios obtained in the present study were also in fair agreement with those reported for a ribofuranoside of benzimidazole by Friedman and Harris (1962) which had been isolated from media and extracts of *Propionilbacterium shermanii* (1.055, 1.24 and 1.04).

The spectral and analytical data provide ample support for the occurrence of a reaction between benzimidazole and PP-ribose-P resulting in the formation of BMN (Figure 24). The reaction is catalyzed by an enzyme, referred to as benzimidazole mononucleotide pyrophosphorylase by analogy with orotidine-5'-phosphate pyrophosphorylase.

C. BENZIMIDAZOLE ADENINE DINUCLEOTIDE

INTRODUCTION

Attempts to establish the essentiality and non-essentiality of the component groups of NAD (DPN is named NAD in this investigation according to the recommendation of the enzyme commission of the International Union of Biochemistry; Report of the commission on enzymes of the international Union of Biochemistry 1961; Dixon, 1960) have resulted in preparation of NAD analogues, enzymatically and chemically. In the enzymic method the exchange between the nicotinamide moiety of NAD and compounds structurally related to nicotinamide is catalized by DPNase (Zatman *et al.*, 1957; Kaplan *et al.*, 1956; and Kaplan and Stolzenbach, 1957). In the chemical method the adenine moiety of NAD was modified by a process of deamination (Kaplan *et al.*, 1952).

The use of NAD analogues in the study of the role of its functional groups, in elucidation of enzymic mechanisms (Kaplan *et al.*, 1956; Van Eys and Kaplan, 1957) and enzyme differentiation (Kaplan *et al.*, 1960) is a standard approach. As a result, a large number of NAD analogue have been prepared. Fawcett and Kaplan (1962) recently prepared some new nicotinamide dinucleotides by the replacement of 5^1-AMP moiety by

deoxy-adenylic, uridylic acid, thymidylic acid and ADP. The activity of these analogues was assessed by comparison with the natural coenzyme.

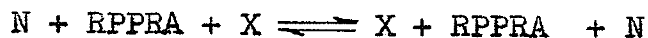
The displacement of the nicotinamide moiety by 5-amino-4-imidazole carboxamide (Alivisatos et al., 1955; 1956) histamine (Alivisatos et al., 1960) amidazole, histidine and amidazole acetic acid (Alivisatos et al., 1962a) have also been investigated.

The report by the same workers (Alivisatos et al., 1962b) of the occurrence of a reaction between NAD and certain benzimidazole derivatives was of great interest and significance in connection with the present work. Since benzimidazole is known to influence diverse metabolic pathways and thereby retard biochemical changes in a senescing leaf, it would be anticipated to participate in these reactions at the level of a common denominator. Such an influence would be exerted if benzimidazole were to replace a functional group in NAD. The latter being a universal reducing agent it reacts with a large number of unrelated compounds which branch off to unconnected metabolic sequences.

Alivisatos et al employed animal tissue NADase (beef spleen and pig brain) in all their investigations

and it was necessary to ascertain whether or not, an enzyme system capable of catalyzing the substitution of benzimidazole for nicotinamide moiety of NAD, could be isolated from plant material. Another point of interest was to investigate whether the NAD/NADase system of plants was similar to that in animal tissues.

Detailed studies have been conducted on the diphosphopyridine nucleotidase of animal origin (beef spleen and pig brain) and a number of NAD-analogues have been synthesized with the help of these systems. The beef spleen DPNase is a glycosidase catalyzing the reaction represented below:



N + RPPRA + X ⇌ X + RPPRA + N

(The enzyme is associated with a particulate fraction obtained after centrifugation of the tissue homogenate at 16000 x g (Kaplan, 1957). Alivisatos and Woolley (1956) succeeded in solubilizing the enzyme by treatment with solutions of DNA and isoamyl alcohol. They purified it considerably by treatment with alumina $C\gamma$ and Ammonium sulphate fractionation.

The spleen NADase is specifically inhibited by nicotinamide and the inhibition is of a competitive type. By use of C^{14}-labelled nicotinamide Zatman <u>et al</u>

(1953) have shown the inhibition to involve an exchange reaction between free and bound nicotinamide. Both pig brain and beef spleen NADases attack the oxidized forms of NAD and NADP. Nicotinamide mononucleotide and nicotinamide riboside are not hydrolyzed by them. Both enzymes hydrolyze acetylpyridine-NAD also.

Nason *et al* (1951) purified a NADase from *Neurospora* grown in a zinc deficient medium. The *Neurospora* enzyme is soluble and is recovered from the supernatant of the mycelial mat homogenate. This enzyme also differs from the animal enzyme by being relatively insensitive to nicotinamide. The latter is inhibitory only at very high concentrations (0.1 M).

The *Neurospora* enzyme cleaves NAD and NADP but does not catalyze the formation of analogues of NAD, in contrast to the animal enzyme.

Information regarding the NADase in plant material is meagre. Roberts (1959) demonstrated an enzyme from the crude extracts of wheat leaves which catalyzes the cleavage of NAD. He classified this enzyme as a pyrophosphatase rather than a NADase due to the absence of inhibition by nicotinamide and isonicotinic hydrazide (INH). In this case the possibility

of the occurrence of both a pyrophosphatase and a NADAse cannot be excluded altogether, because *Neurospora* NADase is also insensitive to nicotinamide.

EXPERIMENTAL METHODS

PREPARATION OF THE ENZYME

2.5 gm of wheat embryos were soaked in 5 ml of 0.05 M phosphate buffer, pH 7.2, and homogenized with an additional 45 ml of the same buffer. The coarse material was screened through four layers of cheesecloth and the supernatant was centrifuged at 3,000 rpm for 5 minutes. The precipitate was discarded and the supernatant centrifuged again at 20,000 rpm for 15 minutes. The precipitate contained a major portion of the activity.

ASSAY

Two methods were used for studying the rate of cleavage of NAD. One method consists of following the splitting in NAD by assaying it with a suitable dehydrogenase system. The second method is based on the cyanide reaction. Cyanide reacts with N^1-substituted nicotinamide compounds to form a dissociable complex showing absorption in the region of 340 mμ (Colowick

et al., 1951). Cyanide complexes with analogues of NAD at the double bond adjacent to the pyridinium nitrogen, thus abolishing the peak at 260 mμ. Ultraviolet absorption spectra for the cyanide addition compounds of NAD and analogues are available (Siegel *et al.*, 1959).

The cyanide reaction is specific for the presence of intact N-ribosyl bond and is not given by free nicotinamide. When NAD and NADP are used as substrates in a NAD/NADase reaction it is possible, of course, to follow the cleavage of the molecule by coupling with an appropriate dehydrogenase system and measuring remaining pyridine nucleotide by the total increase in optical density at 340 mμ. By using a dehydrogenase, however, it is not possible to ascertain the site of the cleavage on the molecule. A combination of the two procedures, i.e. dehydrogenase and cyanide reaction, can be used conveniently to distinguish between the amount of pyridine nucleotide cleaved by NADase, split by breakage at the N-glycosidic linkage and that at the pyrophosphate grouping.

In the cyanide reaction a mixture containing 0.25 ml phosphate buffer, pH 7.2, 0.5 μmole of pyridine

nucleotide and 0.15 ml of an enzyme preparation, was incubated for 20 minutes at 37°C. At the end of the incubation period 3.0 ml of KCN (IM) was added and O.D. at 325 mμ was recorded after 10 minutes. A reference control which lacked NAD was also maintained. The quantity of pyridine nucleotide cleaved in any particular reaction was calculated by reference to a standard curve prepared from known NAD, NADP and acetylpyridine-NAD concentrations.

DETERMINATION OF RIBOSYL GROUPS

The cleavage of pyridine nucleotides at the nicotinamide ribose linkage was also demonstrated by determination of free ribose groups before and after the cleavage. Modified Barfoed's reagent, prepared according to the procedure of Colowick *et al.* (1951) showed negligible reduction on reaction with NAD but on disruption of nicotinamide ribose linkage, showed as much reduction as an equivalent quantity of ribose. The ribose groups were determined in the following manner:-

A 0.5 ml aliquot from reaction mixture containing approximately 0.5 μmole reducing sugar was mixed with 0.5 ml of Barfoed's reagent (Hawk, Oser and Summerson, 1961) and heated at 100°C for 3 minutes. After cooling

0.5 ml of Nelson's arsenomolybdate reagent (Nelson, 1944) was added immediately followed by 0.5 ml of 1.0 M trisodium citrate (which helped to dissolve any precipitate formed by NAD and arsenomolybdate). The samples were then diluted and read in a Coleman Spectronic Colorimeter at 660 mμ. The quantity of ribose present in any one sample was calculated by reference to a standard curve.

BENZIMIDAZOLE ADENINE DINUCLEOTIDE FORMATION

The NAD/NAD nucleotidase reaction was used in experiments designed to test the possibility of the replacement of benzimidazole, at the nicotinamide position in the NAD molecule. It would be reasonable to expect that if a substitution reaction occurred less of the reducing sugar would be available for determination by the Barfoed reagent in the presence of benzimidazole than its absence. This criterion was used to evaluate the extent of BAD formation in any given system.

EXPERIMENTAL RESULTS

As mentioned earlier, a major portion of the NAD nucleotidase in the wheat embryos was associated with the particilate fraction obtained after centrifugation

at 20,000 rpm. No attempt was made to solubilize and purify the enzyme preparation and also a detailed study of the enzyme kinetics and the factors effecting the rate of the reaction was not carried out.

The particulate fraction from the crude homogenate containing pyridine nucleotidase was capable of cleaving NAD, acetylpyridine-NAD and to a small extent NADP. In Table XIII data are presented from a typical experiment. It is seen that approximately 80 per cent of NAD, 53 per cent of acetylpyridine-NAD and 21 per cent NADP is split by the enzyme.

TABLE XIII. The cleavage of pyridine nucleotides by wheat embryo NAD nucleotidase

Pyridine Nucleotide	Optical density of the CN complex Before reaction	After reaction	μmoles cleaved
NAD	0.73	0.080	0.40
NADP	0.87	0.71	0.105
Acetylpyridine-NAD	0.60	0.320	0.265

In general a high rate of cleavage of NAD compared to a small quantity of NADP was encountered. Ribose values of the split product corresponded very closely to those obtained by the cyanide reaction.

The pH optimum for both NAD and NADP cleavage

was 7.3 using phosphate buffer. The curves showed broad maxima with a poorly defined peak at 7.3.

Substitution of benzimidazole for the nicotinamide moiety of NAD, NADP and acetylpyridine-NAD as measured by determination of free ribose groups before and after the reaction, is indicated in Table XIV. These values are averages of three experiments. There was no detectable free ribose in the presence of benzimidazole when NAD was split by NADase. With acetylpyridine and NADP the presence of benzimidazole produced no difference.

TABLE XIV. Reaction of benzimidazole with pyridine nucleotides.

Pyridine Nucleotide	μmoles Cleaved	Reducing Sugar Liberated (equivalent to μmoles of ribose) Without benzimidazole	With benzimidazole
NAD	0.45	0.403	-
NADP	0.777	0.804	.079
Acetylpyridine NAD	0.209	0.196	0.21

In order to isolate BAD in sufficient quantities and characterize it chemically, a bulk reaction mixture containing phosphate buffer (pH 7.3); NAD, 20 μmoles; and 20 ml of an enzyme preparation, was incubated at

$37^{o}C$ for three hours. The mixture was deproteinized and lyophillized. The lyophillized power was dissolved in water and chromatographed on silicic acid thin layer using propanol: chloroform (80:20) as the developing solvent.

Figure 25 is an ultraviolet photograph of the chromatogram. Both benzimidazole and the product appeared as fluorescent zones on the plate. Benzimidazole reference spot is also included on the right. It is of interest that there was virtually no separation when methanol:chloroform (80:20) was used as the developing solvent. It is interesting to recall that benzimidazole and BMN had separated remarkably well on this solvent, but had failed to separate in propanol: chloroform. This in itself is a good indication that BMN and the product of benzimidazole-NAD reaction were not identical.

The zones containing the product were scraped off about 10 chromatograms and eluted with the developing solvent as described for BMN. Ultraviolet absorption spectra were obtained at pH 2.0 and pH 11.0 by a procedure identical to that used for BMN (Figure 26). The spectrum at pH 2.0 showed peaks at 240, 267 and 273 mμ and at pH 11.0 at 250, 270 and 280 mμ. On chemical

analysis the compound was found to contain no free phosphate. It gave a positive spot tests for carbohydrate (Feigl, 1960), confirmed to be ribose by the orcinol test.

The product of the reaction between benzimidazole and NAD was anticipated to be BAD but instead benzimidazole riboside (BR) was isolated from the chromatogram. The only explanation for the formation of BR is the hydrolysis of BAD by a pyrophosphatase contaminating the DPNase preparation. This pyrophosphatase was, however, unable to attack NAD. If this explanation was correct then a second product, ADP, would have also been present on the chromatogram. For locating ADP the chromatogram was divided into four one inch sectors, then the adsorbent was collected from each of these sectors and eluted. Ultraviolet adsorption spectra were obtained for each of the four sectors. ADP was, indeed, located in the first sector, a major portion of it remaining very close to the point of application (Figure 27).

The sequence of reactions catalyzed by the wheat embryo DPNase preparation has been summarized in Figure 28. BAD formed by the reaction is immediately broken down by a pyrophosphatase yielding BR and ADP as con-

trasted to that catalyzed by beef spleen enzyme. In the wheat embryo preparations a phosphatase, which is specific for BAD, appears to be present. Since it does not attack NAD, its presence may have some important metabolic implications.

Figure 25. The chromatographic separation of benzimidazole and BAD.

The faint band at the bottom is BAD, and the band on top is benzimidazole.

The spot is a benzimidazole reference.

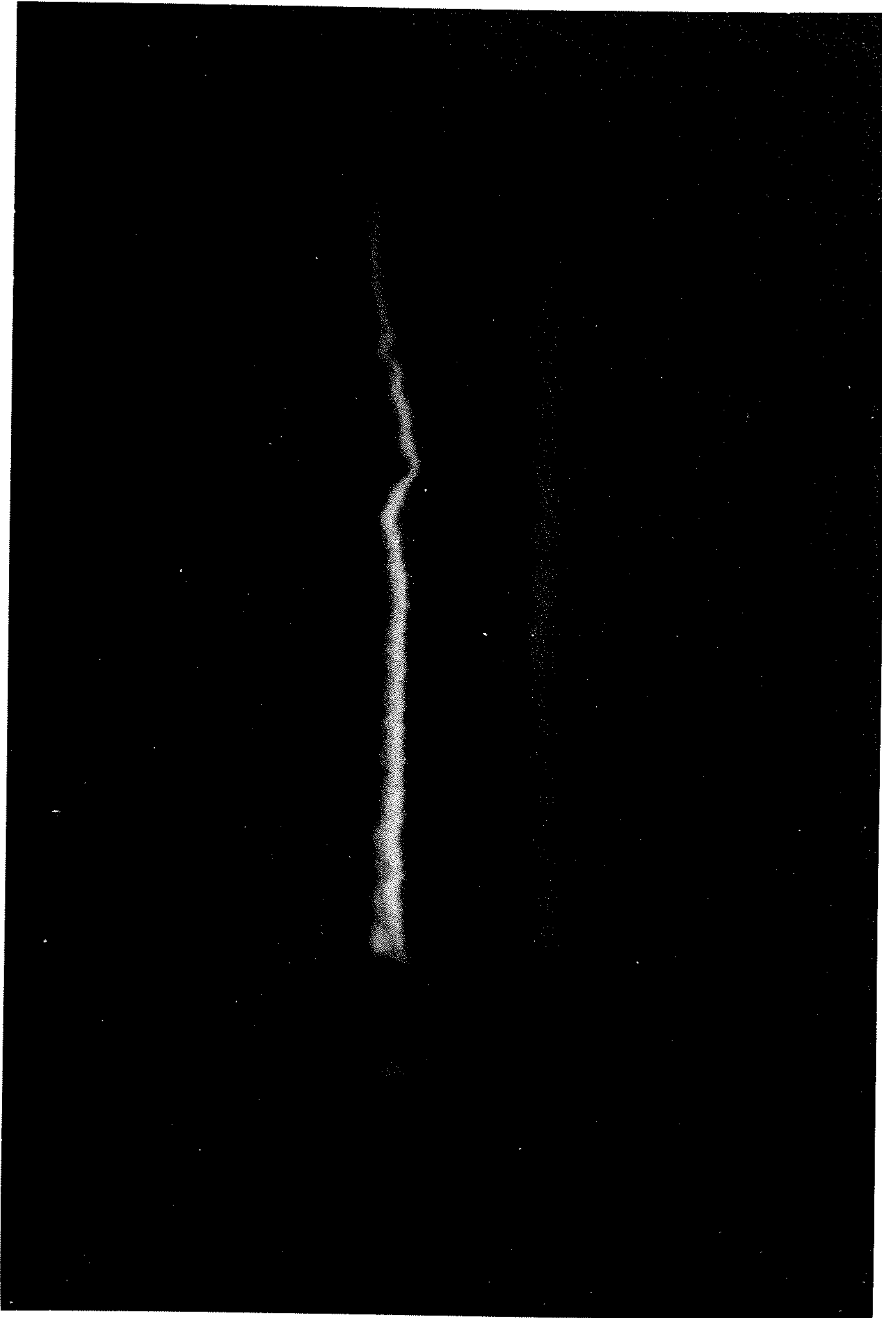

B
BAD
B

Figure 26. The UV absorption spectra of benzimidazole riboside at pH 2 (continuous line) and pH 11 (interrupted line).

BR was one of the products obtained from BAD.

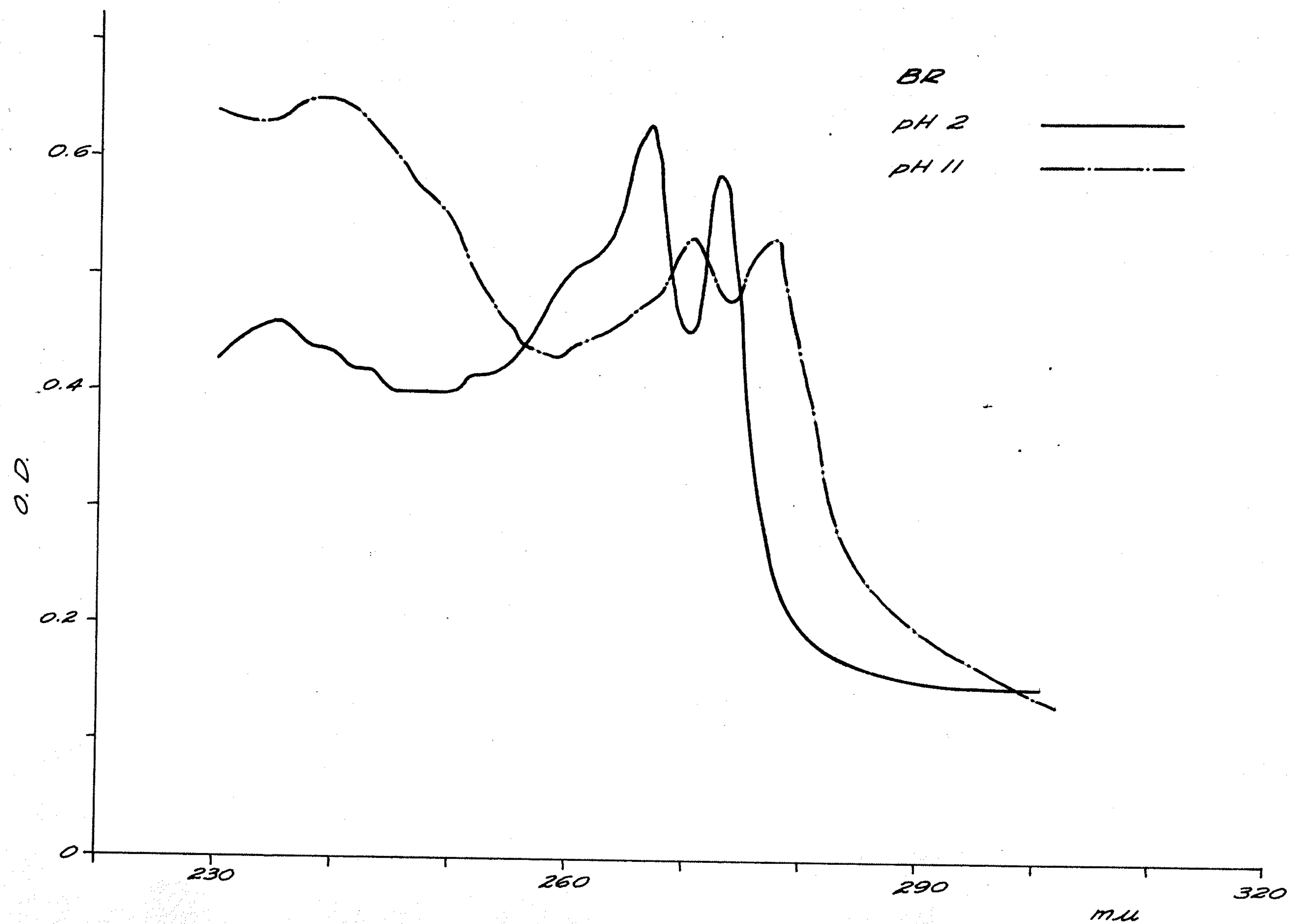

BR
pH 2
pH 11
0.6
0.4
0.2
0
O.D.
230
260
290
320
mμ

Figure 27. UV absorption spectrum of the second product obtained from BAD (identified as ADP)

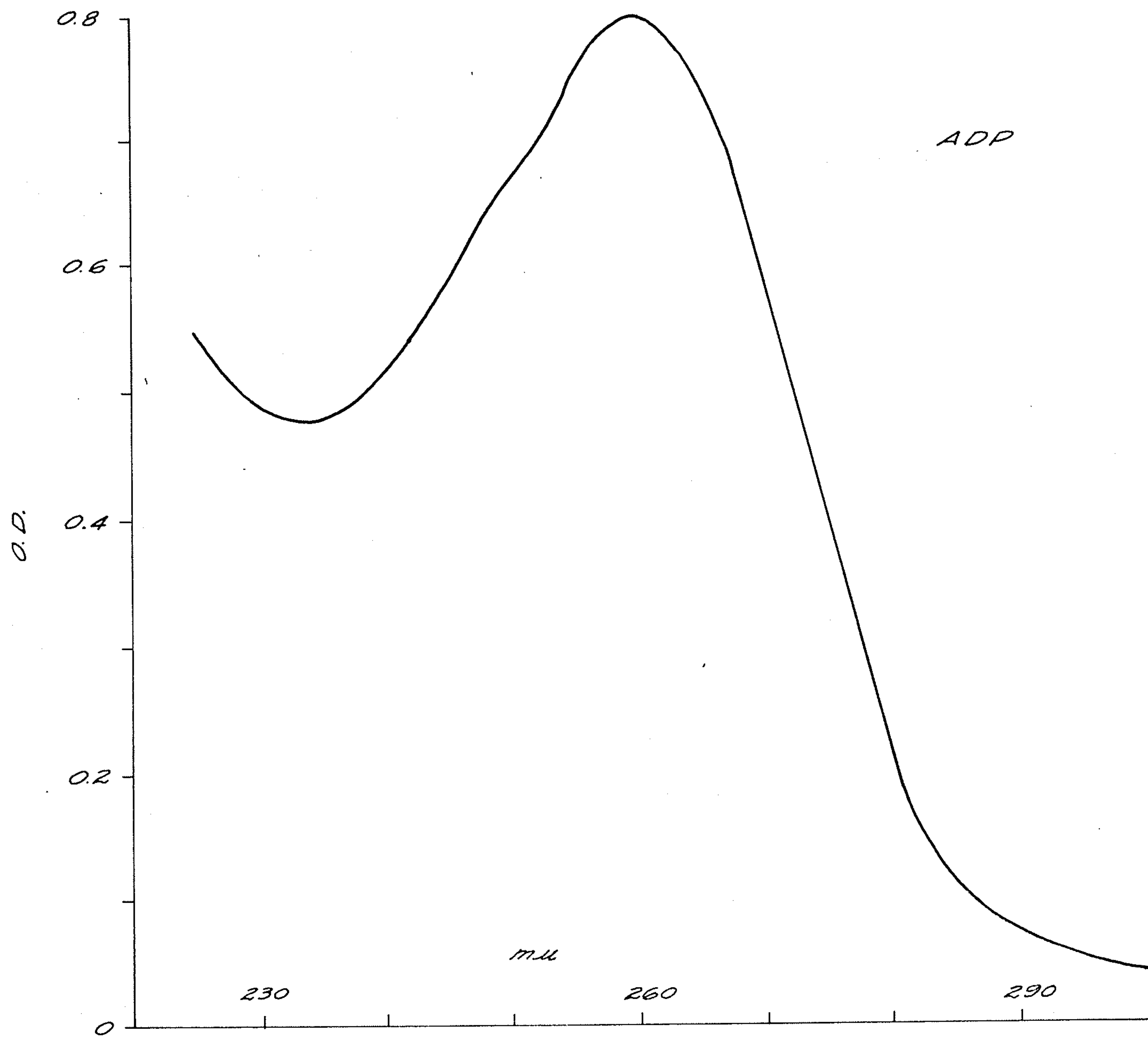
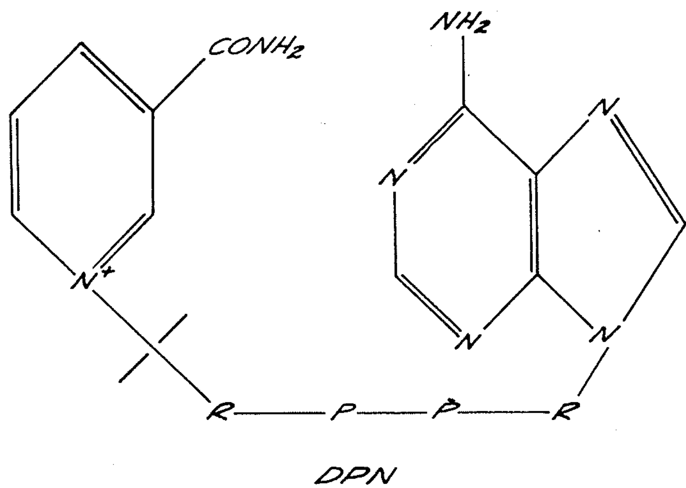
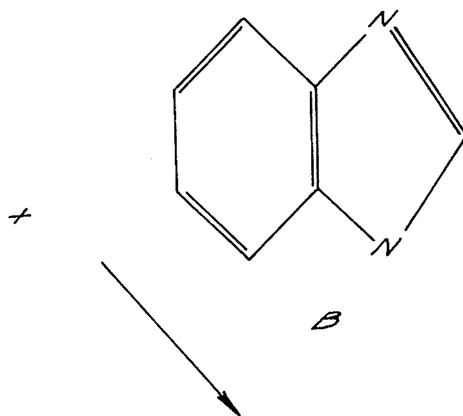
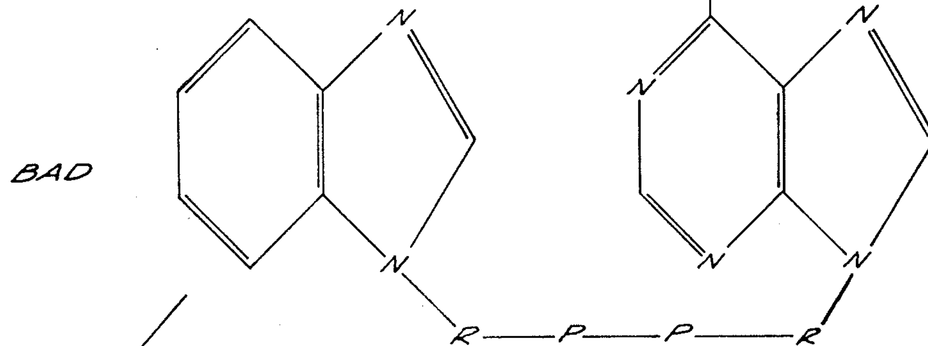

ADP
0.8
0.6
0.4
0.2
0
O.D.
mμ
230
260
290

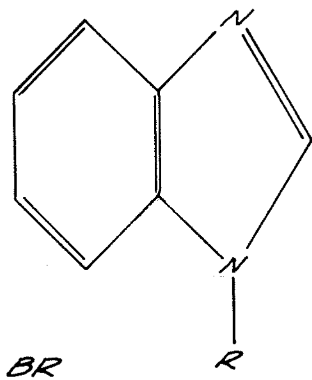
Figure 28. A schematic representation of the reaction between NAD and benzimidazole.


CONH2
NH2
N
N
N
N
N+
R—P—P—R
DPN

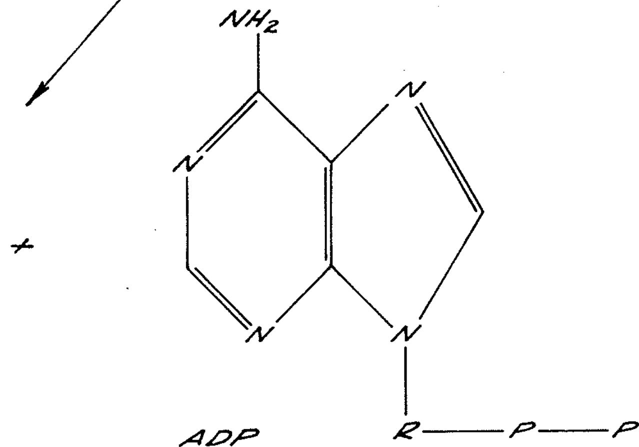
+


N
N
B


BAD
N
N
NH2
N
N
N
N
R—P—P—R


N
N
R
BR

+


NH2
N
N
N
N
ADP
R—P—P

D. DISCUSSION

Numerous theories have been advanced to account for the changes occuring during senescence, a natural and universal phenomenon, ranging all the way from easily recognizable alterations in the gross physical processes and a host of more subtle and less easily discernible ones. A comparative study of the metabolism of normal, growing and aging tissues in both plant and animal kingdom has suggested the occurrence of a gradual shift in a number of metabolic sequences, during the stage of transition from a growing to a senescing tissue. Explanations of this shift have been provided both in terms of accumulation of inhibitory substances and equally frequently, by more speculative and philosophical ideas.

The loss of cellular vitality evident in senescence is terminated by a complete loss of the capacity to perform enzymatic reactions and synthesis. The periods of transition and that of initiation of the process of aging have often been used for a study of metabolic processes. Isolated leaves of plants, mainly, Wheat and _Xanthium_ have been the object of such investigations. As is shown by common experience the changes

leading to aging of cells and organs are irreversible and internally regulated (liable to modification by environmental changes, of course). Regulatory mechanisms operating in a cell manifest themselves at all levels of organization and complexity. In a broad sense two general levels can be recognized, the operational or the metabolic level and the genetic or the molecular level. Regulation at the operational level is exemplified by the control of enzymatic reaction rates by inhibitors or feedback, and at the molecular level by the specific construction of enzymic sites, and induction and repression of protein synthesis. The control mechanisms at the genetic level operate by regulating the activity of the structural gene. It is generally agreed upon that DNA carries the necessary information for the synthesis and regulation of enzymes controlling the performance of various metabolic cycles and consequently, the proportioning of these pathways at different stages in the life history of an organism. It must be made clear at the very outset, however, that regulatory problems posed to and by differentiated systems are of an order of complexity, far removed from and of a greater magnitude than those in microorganisms. Probably they are different and in higher organisms may

consist of mechanisms not known to exist in the micro-organisms. One example of such a regulatory mechanism is the hormonal control of growth processes in higher plants and animals which is not known to exist in the bacteria.

The process of senescence can be studied in isolated systems both in plants and animals. In plants, as has been mentioned before, excised leaves have provided a very flexible model system which allows both for experimental accuracy and convenience in handling. Due to these advantages offered by detached leaves, a large number of external regulators have been explored during the last few years for their effect on metabolic sequences within the cells. Benzimidazole is one of the regulators involved in the aging of leaves which has attracted a fair amount of attention and is of major interest in the present study. Although its mechanism of action is not very well understood, benzimidazole is known to exert a profound influence on the protein synthesis,degradation, chlorophyll synthesis and in the excised leaves of certain wheat varieties on the expression of the property of rust resistance. In an attempt to investigate into the metabolism of benzimidazole, some specific reactions were studied. Phos-

phoribosyl pyrophosphate has been shown to react enzymatically with naturally occurring purines (Remy et al., 1955), pyrimidines (Liebermann et al., 1955), pyridines (Preiss and Handler, 1957) and also with precursors involved in the de novo pathway of purine biosynthesis (Goldthwait et al., 1957; and Hartman et al., 1956). In the present study it has been demonstrated that this phosphoribosyl donor reacts with benzimidazole, a purine antagonist with a non-purine structure. The same reaction has also been shown to occur with the amino and methyl- derivatives of benzimidazole at the 2-position and also with kinetin, which has a purine structure with a substitution at 6-position. The fact that kinetin and benzimidazole exert a similar control on chlorophyll and protein metabolism too, would suggest that this control may be through competition with the natural substrates for the pyrophosphorylases in the cells. Kinetin is known to stimulate nuclear and cell divisions in tissue cultures; it is quite likely that a phosphoribosyl derivative of kinetin rather than kinetin itself, mediates a step leading to increased mitotic activity of the nucleus. Handerson et al. (1962) have proposed that the regulatory effect of kinetin is exercised through interference with purine degradation, more specifically

through inhibition of xanthine oxidase activity.

It does not appear plausible that such a broad spectrum of effects (at least in the case of benzimidazole) leading up to the maintenance of an intricate relationship between different metabolic pathways could be explained on basis of inhibition of a specific reaction occurring in one pathway. It is perhaps more likely that substances like kinetin and benzimidazole manifest themselves through participation in or modification of metabolic sequences at the level of a common denominator. One example of effects at the level of a common denominator is interference at the level of a redox system, i.e. NAD/reduced NAD, NADP/red NADP, or a flavoprotein/red flavoprotein. Interference or control at this level is one way of explaining convincingly the influence on diverse pathways. It has been demonstrated by the work of Alivisatos _et al._ (1962) as well as in the present work that benzimidazole reacts with NAD and to a small extent with NADP (but not red NAD and thereby replaces the nicotinamide moiety of the pyridine nucleotide molecule. It would be of great interest and significance to know whether it would also react with FAD and replace the isoalloxazine ring

in the molecule. Reaction at such a level as this also, could lead to two distinct, possible consequences:

a) a physiological modification, or

b) a chemical and molecular modification

A chemical modification with attendant metabolic implications is illustrated by the coenzymic efficiency of NAD-analogues in catalyzing the dehydrogenase reactions and in addition (and necessarily preceding this), in activation of the binding sites on the enzyme surface. Up-to-date a large number of NAD analogues have been synthesized chemically and by means of enzymatic reactions, by introducing substituent groups into the adenine moiety or else in place of the adenine moiety itself, the same being true of the nicotinamide portion of the pyridine nucleotide molecule.

Investigations into the role of functional groups of NAD molecule have resulted in the postulation that the ARPPR moiety of NAD is responsible for fulfilling the structural requirements of the coenzyme whereas the ribose of the quaternary riboside predisposes the dinucleotide toward nucleophilic additions at the position 4 of the pyridine ring (Lamborg *et al.*, 1957). No definite function had been attributed to the adenine

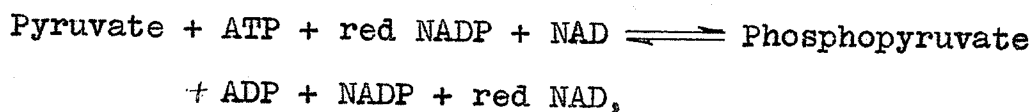
ribonucleotide portion. From the studies of Windmueller and Kaplan (1961) using N-6 alkylated adenine, it turned out that the adenine amino group does not appear to be essential, although it is believed that this portion acts as a link to the enzyme. Generalizations cannot be made regarding the essentiality and nonessentiality of the individual groups of NAD molecule for all the dehydrogenases. An illustration of individual differences between enzymes is provided by the observation that a modification in the purine moiety of NAD (by substitution with UMP or hypoxanthine) reduces the coenzymic activity of the analogue in yeast alcohol dehydrogenase system but the liver alcohol dehydrogenase is insensitive to this substitution (Fawcett and Kaplan, 1962). The data of Fawcett and Kaplan further suggest interference by ADP and ribosylnicotinamide diphosphoribose (NRPPR) in horse liver alcohol dehydrogenase. These two fragments of NAD compete to a small extent with NAD for the active sites on the enzyme surface. For the binding to the enzyme surface, however, a complete molecule of NAD appears to be essential. It is not clearly understood whether this is due to each of the heterocyclic rings contributing individually to the links with the enzyme or because

it is the overall configuration of the NAD molecule that is essential for a complete system of links with the enzyme surface. The replacement of benzimidazole for nicotinamide would introduce a difference of charges in the molecule and also alter the configurational relationships of the molecule to the enzyme surface. Unfortunately, BAD has not been tested for coenzyme activity in either of the alcohol dehydrogenase systems. The information concerning the effect of this substitution of the activity in a redox system would also be of significance.

At the physiological level a reaction with NAD brings about alterations in the NAD/red NAD ratio and hence modifies the metabolic patterns. A manipulation of NAD/red NAD, NADP/red NADP and flavoprotein/red flavoprotein ratios can be very easily affected by either supplying one of them externally or else withdrawing one of the members of a couple, from the system. The first is extremely simple and can be achieved by adding the required substance to the system under study, in the form of a solution. In studies with isolated leaves the leaves can merely be floated on a dilute solution of one of the substances. The removal of these compounds might require a slightly more involved but

nevertheless a simple procedure. Coupling with a system which would utilize the compound of interest usually does the trick (unless side reactions complicate the picture).

In this connection it is of interest to recall that a high ATP/ADP can lead to a reversal of glycolysis and cause a net increase in glycogen synthesis (Davies, 1961). Maintenance of a low ATP/ADP ratio is essential to drive the glycolytic sequence forward. A potential regulatory mechanism in respiratory rates is thus available, since a competition exists between glycolysis and the oxidative pathway for ADP (Beevers, 1962). Similarly, NAD/red NAD ratio determines significantly the extent to which a particular pathway could dominate over another. A consideration of the reaction:



Pyruvate + ATP + red NADP + NAD $\rightleftharpoons$ Phosphopyruvate + ADP + NADP + red NAD,

in the conversion of pyruvate the phosphopyruvate shows that in the presence of a NAD generating system the ratio NAD/red NAD would be high and the reaction will proceed towards the formation of phosphopyruvate. The regulatory effect of NAD/red NAD ratio is evident not only at the level of individual reactions but also it is

important in determining which pathway would operate at a particular stage.

Experiments with ripening bananas have provided evidence for a shift from the pentose-phosphate to the Embden-Meyerhoff-Parnas pathway (Tager and Biale, 1957). On the other hand Gibbs and Beevers (1955) demonstrated that in embryonic root tissue the EMF pathway is the dominant glucose degrading mechanism but in progressively older tissue its importance declines and it is gradually replaced by the pentose-phosphate pathway. A similar shift in the metabolic pathway of the pea root is suggested by changes in activities of aldolase, 6-phosphoglucose dehydrogenase, ribose-5-phosphatase and fructose-1, 6-diphosphatase. Gibbs and Earl (1959) have explained the relative distribution of these pathways on basis of the controlling NAD/red NAD ratios.

The possibility that NAD/red NAD ratio might cause a shift in metabolism during senescence in wheat leaves is strengthened by the actual demonstration of higher NAD levels in water floated leaves than in benzimidazole treated ones (Mishra, Unpublished data). The experiments of Samborski _et al_. (1959) showed a higher rate of respiration in water floated wheat leaves as com-

pared to those treated with benzimidazole. Higher respiratory rates encountered here could safely be considered to be a consequence of higher NAD levels.

The relation of NAD to initiation of the process of senescence as evidenced by chlorophyll degradation has been demonstrated in a very elegant manner by Yoshida (1961), using excised _Elodea_ leaves as a model system. The leaves were plasmolysed in 0.2 M calcium chloride solution, this treatment resulted in the division of the protoplasm of some of the cells into two halves, one half containing the nucleus and the other lacking it. It was found that chlorophyll breakdown occurred much earlier in the nucleated halves compared to the enucleated ones. It was further shown that in instances where the two halves were joined by a cytoplasmic bridge, senescence started in both the halves at the same time. On testing the effect of various substances on the induction of senescence Yoshida came to the conclusion that NAD was the main factor responsible for initiation of chlorosis. He postulated that NAD was synthesized in the nucleus and transported outside, where it brought about degradation of chlorophyll in the chloroplasts.

If the above postulation was correct, it was argued that it should be possible to prevent the onset of chlorosis by removing NAD from the system. The NAD-removing mechanisms of the cell include, diphosphopyridine nucleotidase, PN pyrophosphatase system and transhydrogenase reactions. In addition to all these benzimidazole is now known to remove NAD from the system by formation of BAD. Hence, if indeed NAD was responsible for chlorosis, benzimidazole would be anticipated to counteract this effect by 'inactivating' NAD. This actually was demonstrated by true not only in Elodea but also in wheat leaves. NAD-induced senescence was shown to be arrested on benzimidazole treatment.

Although it has been shown that both animal (Alivisatos et al., 1962) and wheat embryo enzymes (the present study) are capable of catalyzing the substitution of nicotinamide moiety of NAD by benzimidazole, the reaction leads to the formation of different products in animal and plant tissue. Whereas BAD formed in the presence of beef spleen enzymes is stable, that formed by wheat embryo preparations is immediately acted upon by a pyrophosphatase resulting in the formation of ADP and BR. Adenosine diphosphate formation

may have some metabolic implications of the kind already discussed, but at present there is no definite evidence for the same.

The second product of BAD hydrolysis, namely BR could be incorporated into factors resembling vitamins or coenzymes of group B_{12} by analogy with bacterial systems (Friedman and Harris, 1962). It has been proved that vitamin B_{12}-like factors exist in plants, at least in pea, wheat and Lupins (Fries, 1962). An investigation into the mechanism of biosynthesis of B_{12} coenzymes in plants would clarify whether benzimidazole is incorporated in the form of the free base, the ribonucleoside or as a mononucleotide.

SUMMARY

V. SUMMARY

1. Some reactions, participating in the synthesis of nucleotides of purines, pyrimidines and benzimidazole in viable wheat embryos, have been investigated.
2. In the initial reactions of the biosynthetic pathway for purines, i.e., formation of GAR, asparagine was found to be the most effective donor of the amide group as compared to glutamine, carbamyl phosphate and ammonium chloride.
3. Dihydroorotic dehydrogenase and OMP pyrophosphorylase, two enzymes of orotic acid metabolism were demonstrated in wheat embryo preparations.
4. DHO dehydrogenase and OMP pyrophosphorylase were shown to differ from the same enzymes in bacterial and yeast preparations by being inhibited by orotate at higher concentrations.
5. Evidence has been obtained for a reaction between benzimidazole and PP-ribose-P resulting in the formation of benzimidazole mononucleotide. The enzyme catalyzing this reaction has been referred to as BMN pyrophosphorylase by analogy with OMP pyrophosphorylase.
6. Benzimidazole and BMN have been successfully separated by thin layer chromatography on silicic acid.
7. The molecular weight of BMN was found to be 392 and

on hydrolysis it yielded benzimidazole, phosphate and ribose in the ratio, 1:1:1.

8. A reaction between NAD and benzimidazole leading to the formation of BAD, has also been shown to occur. BAD thus formed, however, was acted upon by a phosphatase to yield BR and ADP. The possible significance of the above reactions is discussed.

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