QUINOLINIC ACID PHOSPHORIBOSYLTRANSFERASE IN CASTOR BEANS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
DAVID F. MANN
1972

This is to certify that the

thesis entitled

Quinolinic Acid
Phosphoribosyltransferase
in Castor Beans

presented by

David F. Mann

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

R. H. Burris

Major professor

Date December 18, 1972

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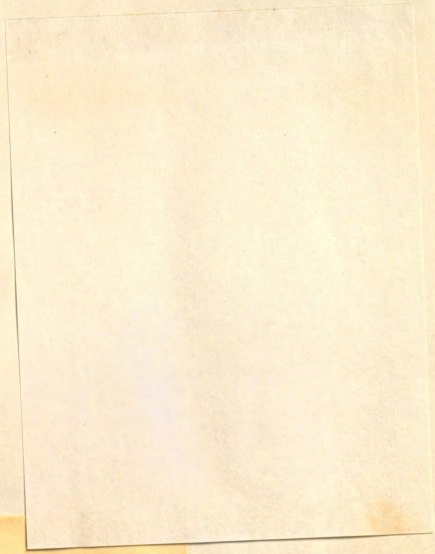

ABSTRACT

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IN CASTOR BEANS

By

David F. Kane

The purpose of this study was to purify quinolinic acid phosphoribosyltransferase and study its role in the de novo biosynthesis of NAD in the castor bean plant. Quinolinic acid phosphoribosyltransferase (E.C. 2.4.2.__) catalyzes the following reaction: quinolinic acid + 5-phosphoribosyl-1-pyrophosphate (PRPP) ----> nicotinic acid mononucleotide + pyrophosphate + CO_2. This enzyme was purified 500-fold from the cotyledons of etiolated seedlings of Ricinus communis L. by a procedure which included a heat denaturation step, DEAE Sephadex chromatography, hydroxyapatite chromatography, and isoelectric focusing to give a 20% overall yield. The enzyme appeared homogeneous after SDS-disk gel electrophoresis, but the native enzyme gave three closely migrating bands on analytical disk gels at pH 8.3. The three proteins were of the same molecular weight but differed slightly in charge, as determined by Ferguson plots of the R_f values

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The purpose of this study was to purify quinolinic acid phosphoribosyltransferase and study its role in the de novo biosynthesis of NAD in the castor bean plant. Quinolinic acid phosphoribosyltransferase (E.C. 2.4.2.__.) catalyzes the following reaction: quinolinic acid + 5-phosphoribosyl-1-pyrophosphate (PRPP) ⟶ nicotinic acid mononucleotide + pyrophosphate + CO_2. This enzyme was purified 500-fold from the endosperm of etiolated seedlings of Ricinus communis L. by a procedure which included a heat denaturation step, DEAE Sephadex chromatography, hydroxyapatite chromatography, and isoelectric focusing to give a 20% overall yield. The enzyme appeared homogeneous after SDS-disk gel electrophoresis, but the native enzyme gave three closely migrating bands on analytical disk gels at pH 8.3. The three proteins were of the same molecular weight but differed slightly in charge, as determined by Ferguson plots of the R_f values

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at different acrylamide concentrations. The purified enzyme was stable for several months when stored at 4°C or -90°C in 0.05 M potassium phosphate buffer (pH 7.0) which contained 50% sucrose (w/v) and 0.01 M dithioerythritol.

In addition to the substrates, the enzyme required Mg^{2+} for activity. Neither Ca^{2+} nor Ba^{2+} could replace Mg^{2+}; however, in the presence of Mn^{2+} in place of Mg^{2+}, the enzyme exhibited 28% of its normal activity. The divalent cations, Ni^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+}, and Pb^{2+}, all at 1×10^{-3} M, nearly completely inhibited the enzyme reaction. At 1×10^{-4} M, Cu^{2+} and Zn^{2+} each effected 80% inhibition. The reaction had a Q_{10} value of 2.6, and the energy of activation calculated from the Arrhenius equation was 17.5 Kcal. Quinolinic acid gave 50% protection from heat denaturation of the enzyme, but PRPP was ineffective. The enzyme displayed a broad pH optimum, ranging from pH 6.5 to pH 7.7. Its isoelectric point was pH 6.1.

Inhibition studies with analogues of quinolinic acid suggested that the enzyme needed both the 2- and 3-carboxyl groups on the pyridine ring for maximum activity, but that the carboxyl group at the 2 position was more important for effective competition. A series of reported and suspected inhibitors, nicotinic acid adenine dinucleotide, NMN, NADP, ATP, ricinine, and

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azaleucine, had no effect on the enzyme activity except for 15% inhibition by NAD at 1×10^{-3} M. Double reciprocal plots of the initial velocity studies indicated that the enzyme formed a ternary complex with quinolinic acid and PRPP.

The molecular weight of the enzyme was estimated by Sephadex G-200 chromatography and sucrose density gradient centrifugation to be 68,000 and 72,000 respectively. Electrophoresis on SDS-polyacrylamide gels demonstrated that the enzyme was composed of two subunits with a molecular weight of 35,000 each.

The level of quinolinic acid phosphoribosyltransferase was found to be 100-fold or more higher in the etiolated seedling than in the adult green castor bean plant. This large increase in enzyme activity in the de novo pathway for NAD biosynthesis correlated well with a rapid surge in ricinine biosynthesis. Regenerated roots of Nicotiana rustica, which synthesizes nicotine, were also shown to have elevated levels of quinolinic acid phosphoribosyltransferase. The elevation of the de novo pathway for NAD biosynthesis in castor beans and tobacco may represent a universal manner in which plants that synthesize pyridine alkaloids compensate for the loss of the pyridine nucleus.

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QUINOLINIC ACID PHOSPHORIBOSYLTRANSFERASE IN CASTOR BEANS

By

David Franklyn Mann

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1972

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and my wife, Dotty

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ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. Richard U. Byerrum for his counsel and guidance during this investigation. I would also like to thank Drs. Clifford Pollard, Paul Kindel, and N. Edward Tolbert for their suggestions and corrections they made in this manuscript. I, especially, want to thank my wife, Dotty, for her encouragement, tolerance, and help during our graduate study together and for her many suggestions in writing this thesis. The financial assistance from the National Institutes of Health is acknowledged.

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NAD

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POPOP

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BBOT

LIST OF ABBREVIATIONS

NAD	oxidized nicotinamide adenine dinucleotide
NaAD	nicotinic acid adenine dinucleotide
NMN	nicotinamide mononucleotide
NaMN	nicotinic acid mononucleotide
NADP	oxidized nicotinamide adenine dinucleotide phosphate
FAD	flavin adenine dinucleotide
(ADP)ribose	adenosine 5'-diphosphate ribose
ATP	adenosine 5'-triphosphate
PRPP	5-phosphoribose-1-pyrophosphate
UL	uniformly labeled ^{14}C
SDS	sodium dodecyl sulfate
DEAE	diethylaminoethyl
M Wt	molecular weight
Abs	absorbance
mA	milliampere
QA	quinolinic acid
POPOP	1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
BBOT	2,5-bis[2-(5-tert-butylbenzoxazolyl)]-thiophene

TEMED

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TEMED	N, N, N', N'-tetramethyl ethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Bicine	N, N-bis(2-hydroxyethyl)glycine
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Mes	2-(N-morpholino)ethanesulfonic acid
i.d.	internal diameter

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LITERATURE REVIEW

The pyridine nucleotides play an important role in cellular energy metabolism. They are involved in a large number of oxidation-reduction reactions which are catalyzed by dehydrogenases. Alkaloids such as ricinine, nicotine, and anabasine are derived from the same ring structure as is found in the pyridine nucleotides. Although these alkaloids are very intriguing, their importance in plant metabolism has not yet been established, even though their synthesis may be associated with the pyridine nucleotide cycle (14).

Cozymase was first reported in 1904 by Harden and Young (1), who found a heat stable factor in the dialyzate of boiled yeast which stimulated glucose conversion to alcohol in cell-free preparations of yeast. Later, in 1934, Warburg and Christian (2) isolated a similar factor from mammalian erythrocytes which was required in the anaerobic oxidation of glucose-6-phosphate. After much more work, cozymase was identified as nicotinamide adenine dinucleotide (NAD), and the closely related compound, coferment, of Warburg and

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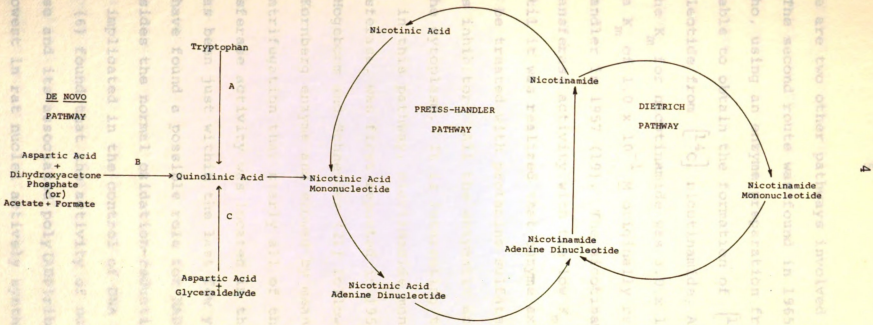
Christian was identified as nicotinamide adenine dinucleotide phosphate (NADP) (3,4). In 1954 Pullman et al (5) demonstrated that hydrogen was transferred to and from the fourth position of the pyridine ring.

About the time that biochemists were determining the importance of nicotinic acid and nicotinamide in cellular metabolism, nutritionists found that nicotinic acid and its amide could cure "canine black-tongue," which is analogous to human pellagra (10). Tryptophan was also shown to relieve the symptoms of pellagra and canine black-tongue (11). Niacin deficiency occurs when people eat diets consisting mainly of corn, which is especially low in tryptophan. However, the most common dietary form of the vitamin is nicotinamide.

It was not until 1958 that the now classic papers of Preiss and Handler (12, 13) first described the sequential synthesis of NAD from nicotinic acid. They incubated human erythrocytes with $[^{14}C]$ nicotinic acid and isolated the products. After identification of the products and the enzymatic reactions involved, they proposed the following pathway for NAD biosynthesis: nicotinic acid ⟶ nicotinic acid mononucleotide ⟶ nicotinic acid adenine dinucleotide ⟶ nicotinamide adenine dinucleotide. This sequence was also found to be operative in plants (14), bacteria (15), fungi (16), and avian tissue (17) (see Figure 1).

Figure 1.--The three dimensions of WAD discrimination. The de NOAC

Figure 1.--The three pathways of NAD biosynthesis. The de novo pathways A, B, and C have been found in animals, bacteria, and plants respectively. The Dietrich pathway has been demonstrated only in animals. Whereas the Preiss-Handler pathway is common to all three systems.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There are two other pathways involved in NAD biosynthesis. The second route was found in 1965 by Dietrich et al (18) who, using an enzyme preparation from rat liver, were able to obtain the formation of [^{14}C] nicotinamide mononucleotide from [^{14}C] nicotinamide, ATP, Mg^{2+}, and PRPP. The K_m for nicotinamide was 3.0×10^{-6} M as compared to a K_m of 1.0×10^{-1} M originally reported by Preiss and Handler in 1957 (19). The nicotinamide phosphoribosyltransferase activity with the low K_m had eluded detection until it was realized that enzyme extracts first had to be treated with protamine sulfate to remove an endogenous inhibitor. All the enzymatic activity was located in the cytoplasm. It is interesting that the other enzyme in this pathway, nicotinamide mononucleotide adenylyltransferase, was first reported in 1950 by Kornberg (20). Hogeboom and Schneider (21) followed up the work on the Kornberg enzyme and showed by means of differential centrifugation that nearly all of the NMN adenylyltransferase activity was located in the nucleus.

It has been just within the last few years that biochemists have found a possible role for NAD in the nucleus. Besides the normal oxidation-reduction reactions, NAD has been implicated in the control of DNA synthesis. Haines et al (6) found that the activity of nuclear NAD glycohydrolase and its associated poly(ADP)ribose polymerase was lowest in rat nuclei actively synthesizing DNA.

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This enzyme system cleaved NAD to form nicotinamide and ADP-ribose, which was then polymerized into chains of ADP-ribose, 25 residues or less long (7). Burzio and Koide (8, 9) showed that ADP-ribosylation of nuclear proteins was related to the inhibition of DNA synthesis in rat liver nuclei or chromatin preincubated with NAD.

There is some confusion in the literature as to the physiological role in mammals of the Preiss-Handler and the Dietrich pathways, starting with nicotinic acid or nicotinamide respectively. Hayaishi et al (23) feel that nicotinic acid is a better precursor of NAD than nicotinamide as determined by injection of equimolar quantities (78 nmoles) of the $[^{14}C]$ labeled compounds into the portal vein of mice and measurement of the appearance of $[^{14}C]$ labeled NAD in the liver. However, they found that large doses (82 to 164 μmoles) of nicotinamide resulted in a higher incorporation into NAD than equal doses of nicotinic acid. A large percentage of the injected nicotinic acid was excreted in the urine, whereas the nicotinamide was believed to be deamidated by intestinal bacteria over a period of several hours and the resulting nicotinic acid used as a source of NAD in the liver (24). On the other hand, Kaplan et al (25) and more recently Chaykin et al (26) demonstrated that nicotinamide rather than nicotinic acid was the more efficient precursor of NAD. As stated

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before, the dietary as well as the circulating form of the vitamin is usually nicotinamide (22). Chaykin et al (26) injected mice interperitoneally with 900 nmoles of either nicotinic acid or nicotinamide. This is considered the normal daily intake of the vitamin in mice. By surveying all the body tissues rather than just the liver, they were able to show that nicotinamide was the only precursor of NAD in the spleen, skeletal muscle, kidney, ovary, lung, heart, and brain tissue and that this synthesis of NAD was via the Dietrich pathway. In the liver and intestine, however, nicotinic acid was rapidly converted to NAD and then to nicotinamide by the Preiss-Handler pathway. The nicotinamide thus formed from nicotinic acid could be used by the peripheral tissue as a precursor of NAD. These findings are supported by the earlier in vitro work of Greenbaum and Pinder (27), who showed that the Dietrich pathway was the only operable pathway for NAD biosynthesis in rat mammary gland.

The first enzyme in both the Preiss-Handler pathway (nicotinic acid phosphoribosyltransferase) and the Dietrich pathway (nicotinamide phosphoribosyltransferase) as well as the microbial and plant nicotinamidases and the mammalian NAD glycohydrolase are subject to a variety of control mechanisms. Nicotinic acid phosphoribosyltransferase from various organisms spans the gamut from dependency on ATP to independency of ATP. The enzyme

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from Astasia longa was completely independent of ATP while the enzyme purified from bovine liver was activated by ATP and had a 10-fold lower K_m for nicotinic acid and PRPP (32, 33). The ATP used to activate the bovine liver enzyme was apparently stoichiometrically cleaved to ADP with the concomitant formation of nicotinic acid mononucleotide. Nicotinic acid phosphoribosyltransferase from B. subtilis and yeast required ATP for activity; Imsande (34) and Gholson et al (35) demonstrated that the ATP was stoichiometrically cleaved to ADP in both organisms.

Dietrich and his co-workers (18) studied nicotinamide phosphoribosyltransferase from rat liver and found that ATP was a positive allosteric effector of the reaction. Their enzyme synthesized NMN in the absence of ATP but only at elevated levels of PRPP and Mg^{2+}. The K_m for nicotinamide decreased from 1×10^{-1} M in the absence of ATP to 3.0×10^{-6} M in the presence of ATP. Nicotinamide phosphoribosyltransferase was inhibited 50% by 5×10^{-4} M NAD. It would therefore seem that this enzyme is an important control point in the biosynthesis of NAD in mammals.

Microbial nicotinamidase was also inhibited by NAD; however, the nicotinamidase from rabbit liver was not (70, 31). Nicotinamide itself had an inhibitory effect on mammalian nuclear NAD glycohydrolase and

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poly(ADP)ribose polymerase as well as rat liver microsomal NAD glycohydrolase (37). It seems therefore that the pyridine nucleotide cycle is no exception to the phenomenon that the end product of a specific metabolic sequence (NAD) inhibits the enzyme (nicotinamide phosphoribosyltransferase or nicotinamidase) catalyzing the first step in its formation.

The Preiss-Handler pathway is probably of much more physiological importance in plants, bacteria, and fungi which have an active nicotinamidase sensitive to NAD (28, 29, 30). The nicotinamidase allows the cycle to act as a true salvage pathway which can convert the nicotinamide formed from the breakdown of NAD to nicotinic acid. The nicotinamidase in mammals had a very high K_m for nicotinamide (1×10^{-2} M) (31) and probably is not very significant. From this and in vivo feeding experiments with nicotinamide (26), it would seem that the Dietrich pathway is of more physiological significance in mammals. Also, only two moles of ATP were needed for NAD biosynthesis from nicotinamide via the Dietrich pathway, whereas three moles of ATP were needed when starting from nicotinic acid via the Preiss-Handler pathway except in the case of Astasia longa (32), where nicotinic acid phosphoribosyltransferase was ATP independent.

The third route for NAD biosynthesis is the de novo pathway. As stated earlier, tryptophan was able to

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replace nicotinic acid in the diet of certain mammals (11), but investigators were unable to detect any nicotinic acid formation in cell-free extracts incubated with tryptophan (39). It was not until 1963 that Nishizuka and Hayaishi (39) using a rat liver homogenate found that (UL) $[^{14}C]$ 3-hydroxyanthranilic acid or (UL) $[^{14}C]$ quinolinic acid (previously thought to be an end product of tryptophan metabolism) could form $[^{14}C]$ nicotinic acid mononucleotide and $^{14}CO_2$ in a PRPP and Mg^{2+} dependent reaction. This finding established nicotinic acid mononucleotide rather than nicotinic acid as the compound made from tryptophan. Nishizuka and Hayaishi (40) demonstrated that nicotinic acid was not an intermediate in the reaction and that the decarboxylation of the α-carboxyl group of quinolinic acid was probably a concerted reaction involving the formation of a positive charge on the quaternary nitrogen adjacent to the departing carboxyl group.

Soon after quinolinic acid was established as an intermediate in the conversion of tryptophan to nicotinic acid mononucleotide in mammals, quinolinic acid phosphoribosyltransferase was also found in plants and bacteria (41, 42). Earlier work by Henderson, Byerrum, and co-workers (43) had established that tryptophan could not serve as a precursor of the pyridine ring in plants. Further in vivo work with tobacco plants by Byerrum et al

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(44) demonstrated that aspartic acid and some three carbon compound, probably glyceraldehyde or glyceraldehyde-3-phosphate, condensed to form the pyridine ring. Cell-free preparations from aerobic and anaerobic bacteria indicated two different pathways for quinolinic acid biosynthesis in these organisms. Aspartic acid was a common precursor to both systems, but the other three carbons came from dihydroxyacetone phosphate in aerobically grown E. coli (45) and glycerol and N-formyl-L-aspartate in anaerobically grown E. coli (63, 64). The anaerobic formation of nicotinic acid in E. coli appears to be a tetrahydrofolate dependent reaction while aerobic formation of the pyridine ring is an FAD dependent reaction (64, 45). Partially purified extracts from the obligate anaerobe, Clostridium butylicum, utilized aspartic acid, acetyl CoA, and formate to form quinolinic acid (46). In vivo studies with yeast showed that it was unique in that it could use tryptophan under aerobic conditions and glycerol and aspartic acid under anaerobic conditions for the biosynthesis of quinolinic acid (65). As yet none of the intermediates in quinolinic acid biosynthesis has been established. This work is hindered by low yields of quinolinic acid and a tedious assay procedure for the identification of quinolinic acid.

Quinolinic acid phosphoribosyltransferase, which is the one enzyme common to the various de novo pathways

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for NAD biosynthesis, was purified from bovine liver by two different groups (40, 47) as well as from a pseudomonad mutant (48, 49, 50) which used quinolinic acid as its sole carbon source. The crystalline bacterial enzyme had a molecular weight of 165,000 as determined by ultracentrifugation and could be irreversibly dissociated into inactive subunits of 54,000 M. Wt. using 4.8 M quanidine-HCl. Further proof of a concerted mechanism for the decarboxylation and formation of nicotinic acid mononucleotide was obtained when Packman and Jakoby (47) were unable to demonstrate any exchange reactions with their crystalline enzyme preparation.

Some tentative evidence has been presented indicating the existence of possible control mechanisms modulating the activity of quinolinic acid phosphoribosyltransferase, whereby the de novo pathway would be integrated with the salvage pathway. Gholson et al (47) observed an 88% inhibition of activity with 1×10^{-3} M ATP using their purified bovine liver enzyme, and Hadwiger et al (41) observed a 77% inhibition of activity with 2.5×10^{-2} M ATP using crude preparations from castor bean cotyledons. Kahn and Blum (32) showed that nicotinic acid at 1×10^{-3} M inhibited quinolinic acid phosphoribosyltransferase by 33% in crude enzyme preparations from Astasia longa. However, no inhibition with nicotinic acid was observed with the bovine liver enzyme

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or the castor bean cotyledon preparation. As stated before, nicotinic acid phosphoribosyltransferase needed ATP for activity (33); therefore one could invoke a control mechanism for the two pathways based on ATP levels.

As can be seen from the three pathways for NAD biosynthesis briefly outlined here and pictured in Figure 1, it is a very complex process. To date the Dietrich pathway has been established only in mammals and therefore awaits further investigation to see how widely spread it actually is. The Preiss-Handler pathway has been found in most organisms studied, although the entire cycle is limited to the liver and intestine in mice (26). The de novo route uses tryptophan in mammals and is limited to the liver and kidney (40). In plants and bacteria, however, the de novo route for NAD biosynthesis uses aspartic acid and various other three-carbon precursors to form quinolinic acid, which is then converted to nicotinic acid mononucleotide and NAD via the Preiss-Handler pathway.

The turnover of NAD and NADP is not a perpetual cycle but involves some loss of the pyridine nucleus. Nicotinamide and nicotinic acid are excreted in man, while the former is also excreted as its N-methyl derivative or as the 2 or 6 pyridone of its N-methyl derivative (51). Normal adults on a normal diet excrete 3.0 to 12.5 mg of N-methyl nicotinamide daily (66).

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Plants also form N-methyl derivatives of nicotinic acid and nicotinamide; however, N-methyl nicotinic acid, also called trigonelline, predominates, probably because of the much more active nicotinamidase present in plants (29, 52).

Little is known of the complete metabolism of the pyridine nucleus in plants as they cannot excrete excess nicotinamide as is the case in animals. Various bacteria are able to break the ring down to compounds like propionic and acetic acid (72) or malic acid (73), which can then easily enter general metabolism again. Certain plants, however, use pyridine compounds in additional reactions to form pyridine alkaloids such as ricinine in the castor bean plant (1 mg ricinine/gm fresh weight), nicotine in the tobacco plant (5% of the dry weight of Nicotiana rustica) and anabasine, also in the tobacco plant. Free pyridine was found in the rayless goldenrod, Aplopappus hartwegi (Gray) Blake, where it made up 2.04% of the dry weight (53). The reader should be aware, however, that alkaloids are not end products of plant metabolism. To the contrary recent work showed that administered [^{14}C] ring labeled ricinine was actively metabolized to $^{14}CO_2$ in the castor bean plant (71).

Two recent papers (29, 52) on NAD biosynthesis in wheat and barley shed some light on the pyridine nucleotide cycle in plants. Using physiological amounts

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of [^{14}C] labeled compounds, they established by in vivo methods that the Preiss-Handler pathway was operative and that NAD and NADP were broken down by way of NAD ⟶ NMN ⟶ Nicotinamide riboside ⟶ Nicotinamide. Another important concept established in this work is that NAD and NADP levels remain constant even when the plant is given high nonphysiological doses of pyridine nucleotide precursors (52). This is in direct contrast to long-accepted results with rats and mice where injections of nicotinamide lead to a several fold increase in hepatic NAD concentrations (60). This finding would argue for the existence of tighter controls on the biosynthesis of NAD in plants than in animals. Constant pyridine nucleotide levels were also established for E. coli. Using an E. coli mutant lacking quinolinic acid phosphoribosyltransferase and therefore requiring nicotinic acid, Lundquist and Olivera (61) showed that when the media contained a large excess of ^{14}C nicotinic acid, the level of the pyridine nucleotides remained constant. These experiments were, of course, performed at a certain stage of development or growth and should not be misconstrued to mean that the pyridine nucleotide pool is constant throughout development, for in fact it is not. Yamamoto (62) showed that there were variations in the pool levels of NAD/NADH and NADP/NADPH during

germination, growth, and subsequent senescence in beans, sunflower, corn, watermelon, rice, and wheat.

The constant NAD pool becomes even more interesting when coupled with the fact that the excess nicotinic acid, nicotinamide, and quinolinic acid (supposedly after one trip through the cycle) are stored as trigonelline. Ryrie and Scott (29) demonstrated this in their work with barley leaves. Normally they obtained a 4.9% incorporation of [^{14}C] nicotinamide into trigonelline when feeding 5 nmoles of this precursor per gram of fresh weight of tissue. However, when nonradioactive nicotinic acid was co-fed with the [^{14}C] nicotinamide a larger proportion of the label was funnelled into trigonelline. Five nmoles of nicotinic acid per gram of fresh weight gave 9.3% conversion of the [^{14}C] nicotinamide to trigonelline, while 25 nmoles of nicotinic acid per gram of fresh weight lead to 31.0% conversion. Godavari and Waygood (52) obtained similar results using wheat leaves. However, they routinely used a rather high dose, 55 nmoles, of either [^{14}C] nicotinic acid or [^{14}C] nicotinamide per gram of fresh weight. This led to an average incorporation of approximately 80% of the labeled precursor into trigonelline. These workers stated that nicotinic acid is toxic to plants while trigonelline is not; therefore trigonelline biosynthesis appears to be an excellent detoxification mechanism (62). Mothes

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(74) also suggested that methylation generally makes a compound less reactive or "metabolically stabilized."

The interconnection between the pyridine nucleotide cycle and the biosynthesis of ricinine and nicotine was first suggested by Leete and Leitz (54), who proposed that NADH might serve as a possible precursor in pyridine alkaloid biosynthesis. To date most of the evidence regarding the actual incorporation of a pyridine nucleotide into one of the alkaloids is circumstantial, even though all the precursors of the pyridine nucleotides themselves (quinolinic acid, nicotinic acid, and nicotinamide) are readily incorporated into ricinine and nicotine (41, 57, 67, 68). Waller et al (14) using $[^{14}C]$ labeled intermediates of the pyridine nucleotide cycle (i.e. nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, and nicotinamide adenine dinucleotide) showed that these compounds were incorporated into ricinine at about the same level as nicotinic acid. Similar results using $[^{14}C]$ labeled NAD in tobacco plants were obtained for nicotine biosynthesis (75). There is some question regarding the meaning of these results, since it is doubtful that the pyridine nucleotides could have been transported across the cellular membrane intact (55, 56).

Several jumping off points have been proposed for ricinine biosynthesis. Yang and Waller (58) suggested

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two possible pathways, the first of which would involve quinolinic acid and the pyridine nucleotide intermediates (i.e., nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide) resulting from the entry of quinolinic acid into the pyridine nucleotide cycle. The second route would begin with nicotinamide because previous data showed that the carboxyamide group of nicotinamide undergoes intramolecular dehydration to form the nitrile group of ricinine (57). Hiles and Byerrum (59) suggested an alternative route for ricinine biosynthesis based on results from competitive feeding studies using $[^{14}C]$ quinolinic acid and a ten-fold molar excess of non-radioactive NAD. The assumption made was that if NAD were an obligatory intermediate, the $[^{14}C]$ quinolinic acid incorporated into ricinine should be diluted by the NAD. Their results showed, however, that when $[^{14}C]$ quinolinic acid was co-fed with NAD, there was in fact a three-fold increase in incorporation of the quinolinic acid into ricinine. They therefore suggested that quinolinic acid need not go through the pyridine nucleotide cycle at all to be incorporated into ricinine.

Waller et al (14) performed some experiments to assess the efficiencies of nicotinic acid and quinolinic acid as ricinine precursors. They found that quinolinic acid incorporation into ricinine was twice that of nicotinic acid or nicotinamide, all administered at the

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same level of 600 nmoles per 7-day-old seedlings. This finding was followed with co-feeding studies. When a nine-fold excess of nonradioactive quinolinic acid was fed with four μmoles of $[^{14}C]$ nicotinic acid, there was a 40% decrease in nicotinic acid incorporation into ricinine, suggesting that the quinolinic acid diluted the label from the $[^{14}C]$ nicotinic acid entering ricinine. They also did the converse experiment, that is of feeding thirty-six μmoles of nonradioactive nicotinic acid with four μmoles of $[^{14}C]$ quinolinic acid per plant. In this case they obtained 4.9% incorporation of $[^{14}C]$ quinolinic acid into ricinine and 5.4% incorporation when fed with the nicotinic acid. These results indicate that quinolinic acid is used in preference to nicotinic acid; this is not incongruous with the data from the barley and wheat experiments where excess nicotinic acid was converted to trigonelline. If this conversion to trigonelline had been occurring in the castor bean plants, then the co-fed nicotinic acid would have been unable to dilute the labeled quinolinic acid, since much of the nicotinic acid may never have entered the cycle. These experiments should be repeated using shorter time periods and physiological quantities of quinolinic acid and nicotinic acid.

Yang *et al* (58) published a very interesting experiment in 1965 in which they showed that the

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percentage of labeled ricinine decreased as the amount of [^{14}C] nicotinic acid or [^{14}C] quinolinic acid fed increased. Quinolinic acid again served as the more efficient precursor at all levels tested. This experiment indicates that there are definite controls on ricinine biosynthesis. Recent work by Johnson et al (69) suggested a possible feedback control. They found that a two- or three-fold excess of unlabeled ricinine decreased the incorporation of labeled quinolinic acid into ricinine.

Preliminary evidence in our laboratory indicated that castor beans had considerably more quinolinic acid phosphoribosyltransferase than nicotinic acid phosphoribosyltransferase. This might help explain the reason that quinolinic acid served as a better precursor of ricinine than did nicotinic acid. In order to obtain a better understanding of the pyridine nucleotide cycle in plants and of quinolinic acid incorporation into ricinine, the purification and characterization of quinolinic acid phosphoribosyltransferase were undertaken. It was also decided to establish the level of several of the key enzymes in the pyridine nucleotide cycle to determine their influence in channeling precursors into ricinine.

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MATERIALS AND METHODS

Chemicals

We are indebted to Dr. Richard Hiles who made the nicotinic acid mononucleotide and the nicotinic acid adenine dinucleotide. Dr. Hiles also synthesized the following compounds which were used in the inhibitory studies with quinolinic acid phosphoribosyltransferase.

Methyl 3-carboxypyridine-2-carboxylate

Methyl 3-amidopyridine-2-carboxylate

Methyl 3-cyanopyridine-2-carboxylate

3-Cyanopicolinic acid

3-Amidopicolinic acid

4-Hydroxyguinolinic acid

2-Hydroxynicotinic acid

3-Cyanopyridine-2-carboxylate

The other chemicals used in this research were reagent grade. Co-factors and enzymes were for the most part the purest available.

Aldrich Chemical Company

2,4-Pyridinedicarboxylic acid	Nicotinic acid
3,4-Pyridinedicarboxylic acid	Nicotinamide
2,6-Pyridinedicarboxylic acid	Trigonelline
2,5-Pyridinedicarboxylic acid	Picolinic acid
8-Hydroxy quinoline	

Amersham/Sear

[6-^{14}C] Qu

New England

[7-^{14}C] Ni

[7-^{14}C] Ni

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[^{14}C] BaCO

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Amersham/Searle Corporation

[6-^{14}C] Quinolinic acid

New England Nuclear

[7-^{14}C] Nicotinic acid
[7-^{14}C] Nicotinamide
UL [^{14}C] Aniline·HCl
[^{14}C] $BaCO_3$
[^{14}C] Toluene (standard)

K & K Laboratories, Inc.

DL-4-azaleucine di HCl

Kontes Glass Company

Rubber septa
Center well (for CO_2 collection)

Packard Instrument Company, Inc.

Hydroxide of Hyamine 10-X, p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide

POPOP, 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene (scintillation grade)

PPO, 2,5-diphenyloxazole (scintillation grade)

BBOT, 2,5-bis [2-(5-tert-butylbenzoxazolyl)]-thiophene (scintillation grade)

P-L Biochemicals, Inc.

Adenosine 5'-triphosphate
Nicotinamide-adenine dinucleotide
Nicotinamide-adenine dinucleotide phosphate
5-phosphoribose 1-pyrophosphate (magnesium salt)

Canalco

Acrylamide
N,N'-methylene-bis-acrylamide
TEMED, N,N,N',N'-tetramethyl ethylenediamine
Ammonium persulfate

Pharmacia Fine Chemicals, Inc.

Sephadex G-200
DEAE Sephadex A-50
Blue Dextran 2000

Mallinckrodt Chemical Works

Sodium dodecyl sulfate 95%
Toluene

Clarkson Chemical Company

Hydroxyapatite C

Sigma Chemical Company

Bovine serum albumin
Dithioerythritol
Ovalbumin
α-chymotrypsinogen

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Quinolin
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Quinolinic acid
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Catalase
MES, 2-(N-morpholino) ethanesulfonic acid
HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid
Bicine, N,N-bis(2-hydroxyethyl) glycine
Diaphorase (pig heart)
Acid phosphatase
Snake venom phosphatase

Eastman Kodak Company

p-Dioxane

Matheson Coleman & Bell

Naphthalene
Sand (Standard Ottawa)

Plants

Ricinus communis L., variety Hale, was selected as the biological material used in most of this thesis. These seeds were very graciously supplied by Mr. Walter Domingo, Director of the Oil Seeds Production Division of the Baker Castor Oil Company, La Mesa, California 92041. The seeds were treated with a 10% solution of Orthocide (50% N-[(trichloromethyl)thio]-4-cyclohexene-1, 2-dicarboximide, "captan") and germinated in moist vermiculite for four or five days at 30°C. The flats were tightly covered with aluminum foil. Etiolated seedlings of approximately the same physiological age were used throughout (i.e., the endosperm was firm and the secondary root structure was well developed).

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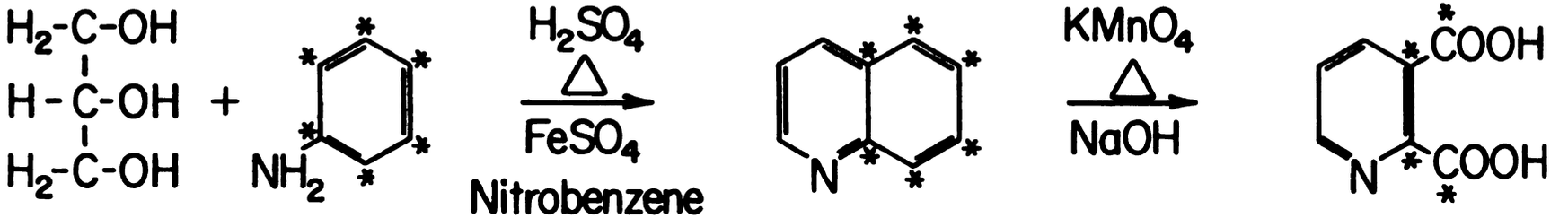
The tobacco plants used were Nicotiana rustica L. The roots were removed from plants grown in the greenhouse just prior to flowering and new roots were allowed to regenerate according to the method of Byerrum et al (43). All other plants were grown under standard conditions unless otherwise stated in the text.

Substrates

Labeled [2,3,7,8-^{14}C] quinolinic acid was synthesized by the method of Fleeker and Byerrum (79). Using the Skraup synthesis (80), (UL) [^{14}C] aniline was condensed with glycerol to form quinoline, which was then oxidized to quinolinic acid by means of an alkaline permanganate oxidation (see Figure 2). The following reagents were added in order: 87 mg of pulverized $FeSO_4 \cdot 7H_2O$, 0.84 ml of anhydrous glycerol, 227 mg redistilled aniline, 3.23 mg (UL) [^{14}C] aniline (0.25 mCi), 0.18 ml redistilled nitrobenzene, and 0.48 ml concentrated H_2SO_4. The flask, equipped with an air-cooled reflux condenser, was placed in a 140°C Wood's metal bath for 6 hr. The reaction mixture was then steam distilled to remove all the nitrobenzene. After the addition of 9 ml of 40% KOH, the reaction mixture was steam distilled again. This time the distillate, which contained the [^{14}C] quinoline, was collected in 0.1 M NaOH and 150 ml of 5% $KMnO_4$ were added dropwise. The flask with the $KMnO_4$ solution was

Figure 2.--Synthesis of [2,3,7,8-^{14}C] quinolinic acid from glycerol and (UL) [^{14}C] aniline.



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fitted with a water cooled reflux condenser and placed in a steam bath for 16 hr. The MnO_2 formed was removed by filtration; absolute ethanol was used to convert excess $KMnO_4$ to MnO_2, which was again removed by filtering the solution. The solution was applied to a Dowex 1X8 (50-100 mesh) formate column (2.7 cm i.d. x 30 cm) and the quinolinic acid was eluted with a 1000 ml linear formic acid gradient (0.0 to 0.4 M). The quinolinic acid fraction was evaporated to dryness on a rotary evaporator and recrystallized giving a 31% yield. The [2,3,7,8-^{14}C] quinolinic acid contained 117 dpm per nmole.

The synthesized product was 100% radiochemically pure as determined by autoradiography and co-chromatography with standard quinolinic acid in three solvent systems (A,B,D). The α-carboxyl group contained one-quarter of the total radioactivity as determined by decarboxylation technique of Scott et al (81).

The other radioactive substrates, [6-^{14}C] quinolinic acid, [7-^{14}C] nicotinic acid and [7-^{14}C] nicotinamide, were obtained commercially and checked for purity by co-chromatography on Whatman No. 1 paper in two different solvent systems (A and B). The magnesium salt of PRPP was obtained from P-L Biochemicals and used in the characterization studies whereas the sodium salt of PRPP (90% purity) was obtained from Sigma Chemical Company (for 1/26th the cost of the magnesium salt)

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and used in the assay procedures throughout the purification. Under comparable conditions the magnesium salt gave 10% more activity than the sodium salt.

Enzyme Assays

Quinolinic acid phosphoribosyltransferase activity was determined either by quantitating the $^{14}CO_2$ released or by measuring the formation of $[6\text{-}^{14}C]$ nicotinic acid mononucleotide. The standard assay mixture for the enzyme contained, in a total volume of 0.8 ml, the following reagents at the final concentrations given: 0.5 to 5 milliunits of enzyme;* 115 mM potassium phosphate (pH 7.0); 12.5 mM $MgCl_2$; 0.187 mM $[2,3,7,8\text{-}^{14}C]$ quinolinic acid and 0.375 mM PRPP. The 25 ml erlenmeyer reaction flasks were equipped with a rubber septum and a polyethylene hanging well which contained 0.2 ml of hydroxide of hyamine 10-X and a folded paper wick. The flasks, minus the PRPP, were equilibrated for 5 min with shaking in a 30°C water bath; the reaction was started by the addition of PRPP. After 20 min, the reaction was stopped by the addition of 0.3 ml of 10% perchloric acid through the rubber septum. To insure complete absorption of the $^{14}CO_2$ by the hydroxide of hyamine,

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*A unit of enzyme activity is defined as that amount of enzyme needed to catalyze the formation of 1 μmole of product per min under the given assay conditions.

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the flasks were allowed to stand for one hr. The entire well was then placed in a counting vial with 6 ml of the scintillation fluid A and placed in a scintillation spectrometer. The vials were counted after a period of four or five hr equilibration in the scintillation spectrometer. This assay procedure using hydroxide of hyamine was initially checked using standard $[^{14}C]$ $BaCO_3$. The results indicated that 0.2 ml of hydroxide of hyamine with a wick was sufficient to quantify up to 200 μmoles of CO_2. The results also indicated that one hr was ample time to allow for complete absorption of the released $^{14}CO_2$.

The second, more sensitive, assay procedure measured the formation of nicotinic acid mononucleotide using paper chromotography. The assay mixture contained the following reagents at the final concentrations given in a total volume of 0.2 ml: 115 mM potassium phosphate (pH 7.0), 5 mM dithioerythritol, 12.5 mM $MgCl_2$, 0.375 mM PRPP, and 10 to 30 microunits of enzyme. The reaction was started by adding 0.3 mM $[6\text{-}^{14}C]$ quinolinic acid (4.15 μCi/μmole) and it was allowed to proceed for 2 hr at 30°C. The reaction was stopped by heating the flask in a boiling water bath for one min. Unlabeled quinolinic acid (1.6 μmoles) was added to each assay to serve as a chromatographic standard and the protein was removed by centrifugation in a clinical centrifuge for

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3 min. Approximately 10 µl of the assay mixture were spotted on Whatman No. 1 paper and developed by descending chromatography with solvent system A. Quinolinic acid (R_f.36) was detected under a mineralight UVS·11 lamp; the $[^{14}C]$ labelled nicotinic acid mononucleotide (R_f .14), as well as the quinolinic acid, were located by scanning 1.5 inch wide strips on a Packard Radiochromatogram Scanner. Three-inch sections of the strips corresponding to the two radioactive peaks, were cut out and placed in counting vials. The vials were filled with scintillation fluid B and counted. The percent quinolinic acid converted to nicotinic acid mononucleotide was determined by dividing the cpm, corrected for background, associated with the product by the total number of cpm (product and quinolinic acid) on the paper strip. Since the specific activity of the labeled quinolinic acid used and the percent conversion to nicotinic acid mononucleotide were known the amount of product could be calculated.

Nicotinic acid phosphoribosyltransferase was assayed by measuring the formation of $[^{14}C]$ nicotinic acid mononucleotide from $[7\text{-}^{14}C]$ nicotinic acid. The assay mixture contained the following reagents at the final concentrations given in a total volume of 0.2 ml: 25 mM potassium phosphate (pH 7.0), 4 mM dithioerythritol, 20 mM tris·potassium phosphate (pH 8.0), 60 mM ATP,

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1.24 mM PRPP, and 10 to 30 microunits of enzyme. The reaction was started by adding 0.148 mM [7-^{14}C] nicotinic acid (6.8 μCi/μmole) and was allowed to proceed for 2 hr with shaking in a 30°C water bath. The assay was stopped by heating the flask in a boiling water bath for one min. Standard nicotinic acid and trigonelline (4 μmoles each) were added to the assay mixture before the protein was removed by centrifugation. Trigonelline was a side product in this reaction and had to be accounted for in order to determine the percentage of nicotinic acid mononucleotide formed. From this point on, the assays were treated in the same way as the chromatographic assay of quinolinic acid phosphoribosyltransferase.

Nicotinamidase activity was determined by measuring the formation of [7-^{14}C] nicotinic acid from [7-^{14}C] nicotinamide. The reaction mixture contained the following reagents at the final concentrations given in a total volume of 0.15 ml: 36 mM potassium phosphate (pH 7.0), 7.3 mM dithioerythritol, and approximately 100 microunits of enzyme. The assay was started by the addition of 0.73 mM [7-^{14}C] nicotinamide (5.13 μCi/μmole) and was allowed to proceed for one hr in a shaking water bath at 30°C. The assays were stopped by heating the flasks in a boiling water bath for two min. Standard nicotinic acid and nicotinamide (4 μmoles each) were added to the assay mixture and the protein was removed by

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centrifugation. Approximately 10 µl of each assay mixture was spotted on Whatman No. 1 paper and developed by descending chromatography with solvent system C. The assay mixtures were then treated the same as those for quinolinic acid phosphoribosyltransferase.

Paper Chromatography

Descending paper chromatography was performed with several solvent systems: (A) 1-butanol-acetic acid-water (4-1-2 v/v); (B) 1 M ammonium acetate (pH 7.0)-95% (v/v) aq. ethanol (3-7 v/v); (C) upper phase of 1-butanol-acetone-water (9-1-10 v/v); and (D) 600 gm $(NH_4)_2SO_4$ per liter of 0.1 M potassium phosphate (pH 6.8), plus 2% (v/v) 1-propanol.

Scintillation Fluids

Three scintillation fluids were used in this work. A: This toluene-based scintillation fluid was used to quantitate $^{14}CO_2$ trapped in hydroxide of hyamine and was prepared by adding 1.265 gm of POPOP and 19.0 gm of PPO to 3.79 liters of toluene. B: This scintillation fluid was used to count paper strips and contained 4 gm of BBOT per liter of toluene. After counting, the paper strips were removed and the vials of fluid were recounted. If the fluid was not contaminated (i.e. if the radioactivity equalled the background) it was reused. C: Aqueous samples (0.5 ml) were counted in

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Bray's scintillation fluid (5.0 ml) which was made by adding 289.8 gm of naphthalene, 28.98 gm of PPO, and 0.7225 gm of POPOP to 3 kgm of p-dioxane. All the samples were counted in a Packard Tri-Carb scintillation spectrometer.

Preparation of Materials for Ion Exchange Chromatography

Diethylaminoethyl Sephadex (A-50, 40-120μ particle size) was suspended in water and allowed to swell for 24 hr. The resin was "defined" by suspending the gel in two volumes of water and allowing it to settle for 8 to 10 min after which time the material still suspended was siphoned off. After repeating this process 10 times, the gel was allowed to equilibrate with 10 volumes of 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol for two days. Fresh buffer was added twice daily.

Hydroxyapatite C (modified calcium phosphate) packed in 0.001 M phosphate buffer was suspended in 10 volumes of 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol. The buffer with no 2-mercaptoethanol was changed twice daily for three days to ensure complete equilibration.

Dowex 1X8 (50-100 mesh) was converted from the chloride form to the formate form by washing the resin with 40 volumes of 3 N ammonium formate (or until no

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chloride was eluted as determined with a 1% (w/v) solution of $AgNO_3$). The resin was then made ready for use by washing it with deionized water to remove the excess ammonium formate.

Polyacrylamide Gel Electrophoresis

The method of Ornstein and Davis (88, 89) was followed for disk gel electrophoresis. The gels were prepared using four stock solutions made in the following manner: solution A consisted of 48 ml 1 N HCl, 36.6 gm tris, and 0.23 ml TEMED; solution B consisted of 28 gm acrylamide and 0.735 gm N,N'-methylenebisacrylamide; solution C consisted of 0.14 gm ammonium persulfate; solution D consisted of 48 ml 1 N HCl and 5.98 gm tris. A standard 7% gel was made by mixing 2.5 ml solution A, 5.0 ml solution B, 10 ml solution C, and 2.5 ml water. The gel solution was transferred to glass tubes (9.0 x 0.5 cm) and a small amount of water (3 mm) was layered on top of each gel.

Samples for electrophoresis contained 12 µl solution D, 20% glycerol, protein (10 to 50 µgm) and water to a final volume of 100 µl per gel. The electrode buffer, pH 8.3, contained 0.605 gm tris and 2.85 gm glycine per liter of water. The anode was at the bottom of the apparatus. Electrophoresis was carried out at 3 mA per tube using bromophenol blue

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as the tracking dye. Gels were stained in amido schwartz (2 gm amido schwartz, 250 ml water, 250 ml 95% ethanol, and 10 ml glacial acetic acid) and destained in 7% acetic acid or they were stained in Coomassie blue (0.05% in 12.5% trichloroacetic acid) overnight and destained in 10% trichloroacetic acid.

Gels were assayed for enzyme activity by sectioning them with a Canalco gel slicer into 1.4 to 1.6 mm sections or slicing by hand with a razor blade into 8 to 9 sections per cm. Slices were immediately dropped into a test tube containing the regular assay components for the CO_2 assay for quinolinic acid phosphoribosyltransferase. The reaction was started by the addition of PRPP and the assays were allowed to proceed for 45 min at room temperature.

The isoelectric focusing experiments were done with a LKB 8100 electrofocusing column of 110 ml capacity. The carrier ampholytes were in the 3-10 or the 5-7 pH range. The procedure for running the column was that specifically recommended for the LKB apparatus (86).

Molecular Weight Estimation

The estimated molecular weight of quinolinic acid phosphoribosyltransferase was determined by chromatography on Sephadex G-200 by the method of Andrews (83). Another estimate of the molecular weight

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was determined by sucrose gradient centrifugation using the method of Martin and Ames (84). Electrophoresis in SDS-polyacrylamide gels was performed according to the procedure of Shapiro et al (85).

Protein Determination

Protein determinations were done by the method of Lowry et al (76) on crude fractions while the protein in more purified preparations was determined by measuring the absorbance values at 280 and 260 nm with a Hitachi spectrophotometer by the method of Warburg and Christian (77).

Protein solutions were concentrated using either a 10 or 200 ml capacity Amicon Ultrafiltration cell equipped with a PM-10 filter (10,000 molecular weight exclusion limit). At least 98% of the activity was recovered each time after concentration of the protein solution by this method.

Ricinine Determination

Ricinine was extracted from castor beans by homogenizing the entire plant in a Sorvall Omni-mixer with 5 ml of hot water ($\simeq$ 80°C) per gram of tissue for two min at top speed. The homogenate was then filtered and the residue was re-extracted. The combined filtrates were extracted with 1/4 volume of ether at least three times, or until no more lipid could be removed. The

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aqueous phase was concentrated almost to dryness on a rotary evaporator and then diluted to volume with water in either a 10 or 25 ml volumetric flask. The material (500 ul) was streaked on Whatman No. 1 paper, 22.5 x 8.5 in., and the chromatogram was developed overnight in solvent system A. The chromatograms were examined by visual ultra violet quenching with a Mineralight UVS·11 lamp and the portion exhibiting a fluorescent band at an R_f of standard ricinine was eluted with water using spin-thimbles (Reeve Angel Company, New York, N.Y.). The eluted ricinine was adjusted to a final volume of 10 ml and the absorbance of an aliquot was measured at 260 nm with matched quartz cuvettes. The amount of ricinine was determined from a Beer's Law plot of standard ricinine. By using paper chromatography to isolate the ricinine, interfering ultraviolet absorbing compounds were eliminated.

For the identification of nicotinic acid mononucleotide, phosphate was determined by the micro method of Ames and Dubin (81) and ribose was measured with orcinol (82). The NAD content of the plant tissue was determined by the method of Yamamoto (62).

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RESULTS

Purification of Quinolinic Acid Phosphoribosyltransferase

Enzyme Isolation

Endosperm tissue (120 gm) from which the cotyledons were removed was harvested from etiolated 5-day-old castor bean seedlings and immediately placed in an ice bucket. The endosperm tissue was weighed, rinsed with distilled water, and blotted dry. The remaining procedures were carried out at 4°C. The tissue was first ground with sand in a cold mortar using two volumes of 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol. The crude extract was strained through four layers of cheese cloth and then centrifuged at 27,000xg for 20 min. The supernatant fraction, which contained the enzyme activity was adjusted to pH 7.0 with 0.40 M NaOH-glycine (pH 10.0).

The enzyme extract was then made 1 mM in quinolinic acid with 0.08 M quinolinic acid and allowed to equilibrate for 10 min in the ice bucket. The enzyme, 100 ml at a time, was heated in a 1 liter flask which was swirled in an 85°C water bath until the solution

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reached 60°C. The flask was immediately transferred to a 60°C water bath, incubated for one min, and then cooled by swirling in an ice bath. The precipitated protein was removed by centrifugation at 27,000xg for 15 min and the supernatant was dialyzed overnight at 40°C against four 4 liter portions of grinding buffer to remove the quinolinic acid.

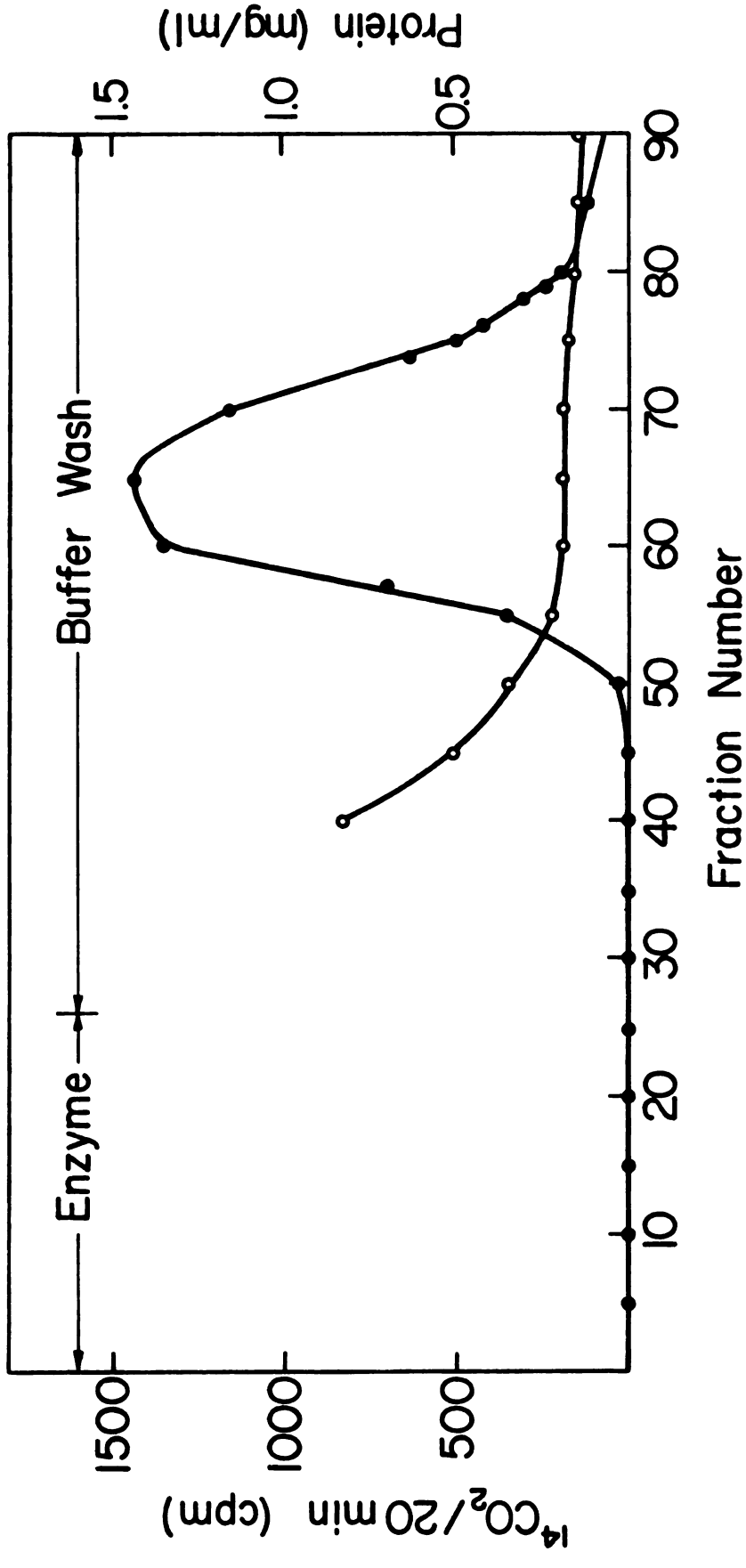
The precipitate which formed during dialysis was removed by centrifugation at 27,000xg for 15 min. This enzyme preparation (280 ml) was then placed on a DEAE Sephadex (A-50) column (2.7 cm i.d. x 16.0 cm) and the quinolinic acid phosphoribosyltransferase activity was removed by eluting with approximately 800 ml of grinding buffer. The elution pattern may be seen in Figure 3. The fractions from the column were assayed for quinolinic acid phosphoribosyltransferase activity as described in the methods section and protein was determined by 280/260 nm readings (77). Those tubes with the highest specific activity were pooled and concentrated to 30 ml using an Amicon Ultrafiltration apparatus equipped with a 62 mm PM-10 membrane.

The concentrated enzyme was then placed on a hydroxyapatite C column (1.7 cm i.d. x 16.5 cm), washed with grinding buffer and eluted from the column with a 900 ml linear gradient of 0.05 to 0.225 M potassium phosphate (pH 7.0). The flow rate was approximately

Figure 3.--Elution profile of quinolinic acid phosphoribosyltransferase from a typical DEAE Sephadex (A-50) column. The enzyme was assayed as described in the methods section using 0.2 ml aliquots of each fraction. The fractions prior to number 40 contained yellow material which interfered with protein determinations by the method of Warburg and Christian (77). The flow rate was 44 ml per hr and 11 ml fractions were collected. Those fractions (#57-76) which had a high specific activity were pooled and concentrated.

Protein (o——o)

Enzyme activity (●——●)


$^{14}CO_2$/20 min (cpm)
1500
1000
500
Enzyme
Buffer Wash
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20
30
40
50
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70
80
90
Fraction Number
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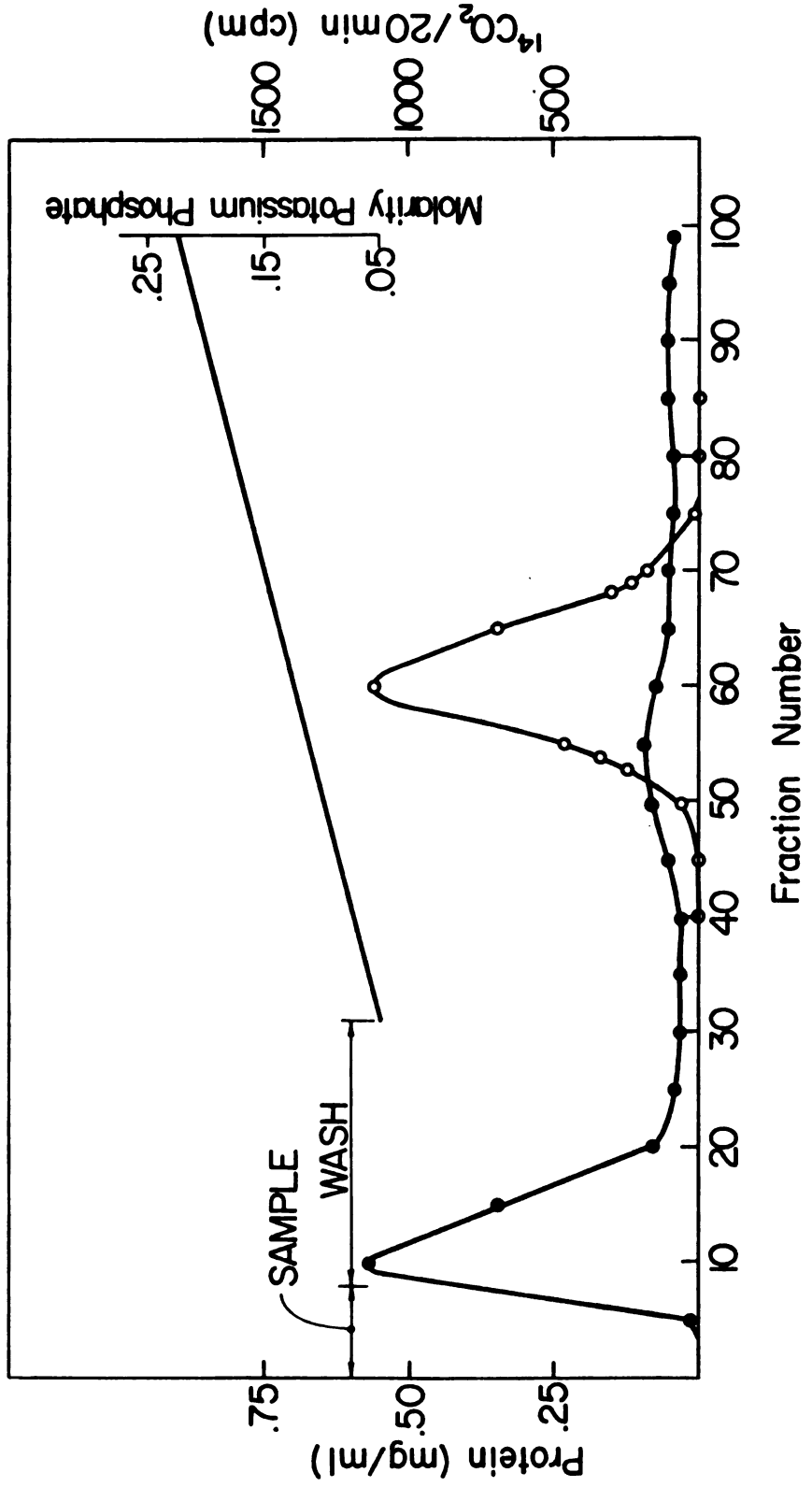
30 ml per hr and 8 ml fractions were collected. Figure 4 shows the elution pattern from this column. After assaying the fractions for quinolinic acid phosphoribosyltransferase activity and protein concentration as before, those fractions (#55-68) having the highest specific activity were pooled. An Amicon Ultrafiltration apparatus was again used to concentrate the pooled fractions. The concentrated enzyme (6 ml) was then dialyzed overnight against 600 volumes of 1% glycine solution (pH 7.0) to remove excess potassium phosphate acquired during elution from the hydroxyapatite column.

The last step in the purification procedure utilized an isoelectric focusing column. For this column carrier ampholytes (LKB) were used to establish a pH gradient from 5-7. The stepwise sucrose gradient was prepared by dilution of 24 aliquots of solution A with solution B to a final volume of 4.6 ml. Solution A contained 47% sucrose and 1.25% ampholyte and solution B contained 0.4% ampholyte. The enzyme (6 ml) in 1% glycine solution (pH 7.0) from the hydroxyapatite column was substituted for an equal amount of solution B in the middle three fractions. The voltage was increased stepwise over a three-hour period to 700 volts and was held there until the current had stabilized around 0.50 mA (44 hours). Then the column was slowly drained and 2 ml fractions were collected and assayed for quinolinic acid

Figure 4.--Elution profile of quinolinic acid phosphoribosyltransferase from a typical hydroxyapatite column. After the enzyme was absorbed onto the column, the column was washed with 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol until the 280/260 nm ratio was constant. The wash was followed by a 900 ml linear gradient of potassium phosphate (0.05 to 0.225 M). The enzyme was assayed as described in the methods section using 0.2 ml aliquots of the various fractions. The protein concentrations were determined by the method of Warburg and Christian (77). When larger amounts of protein were to be chromatographed, as was the case when several DEAE Sephadex columns were pooled, a hydroxyapatite column (2.7 cm i.d. x 42.0 cm) was used with results similar to those shown here.

Protein (●——●)

Enzyme activity (o——o)


Protein (mg/ml)
.75
.50
.25
SAMPLE
WASH
Molarity Potassium Phosphate
.25
.15
.05
$^{14}CO_2$/20min (cpm)
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phosphoribosyltransferase and protein. Figure 5 shows the elution pattern obtained with the isoelectric focusing column. The fractions containing enzyme activity were pooled and exhaustively dialyzed against 0.05 M sodium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol to remove the ampholytes. The enzyme solution was then made 50% (w/v) in sucrose and 0.01 M in dithioerythritol and stored at either -90°C or 4°C. Table 1 summarizes the complete purification procedure.

Discussion of the Purification Scheme

The pH of the initial extract played an important role in the recovery of enzyme activity during the heating step. When the enzyme preparation was heated at pH 6.5, only 50 to 60% of the activity could be recovered. However, when the extract was adjusted to pH 7.0 before heating 80 to 90% of the original activity could be recovered. Heating the enzyme preparation for periods of time longer than one min at 60°C resulted in a loss of enzyme activity, yet did not appear to cause any further protein precipitation.

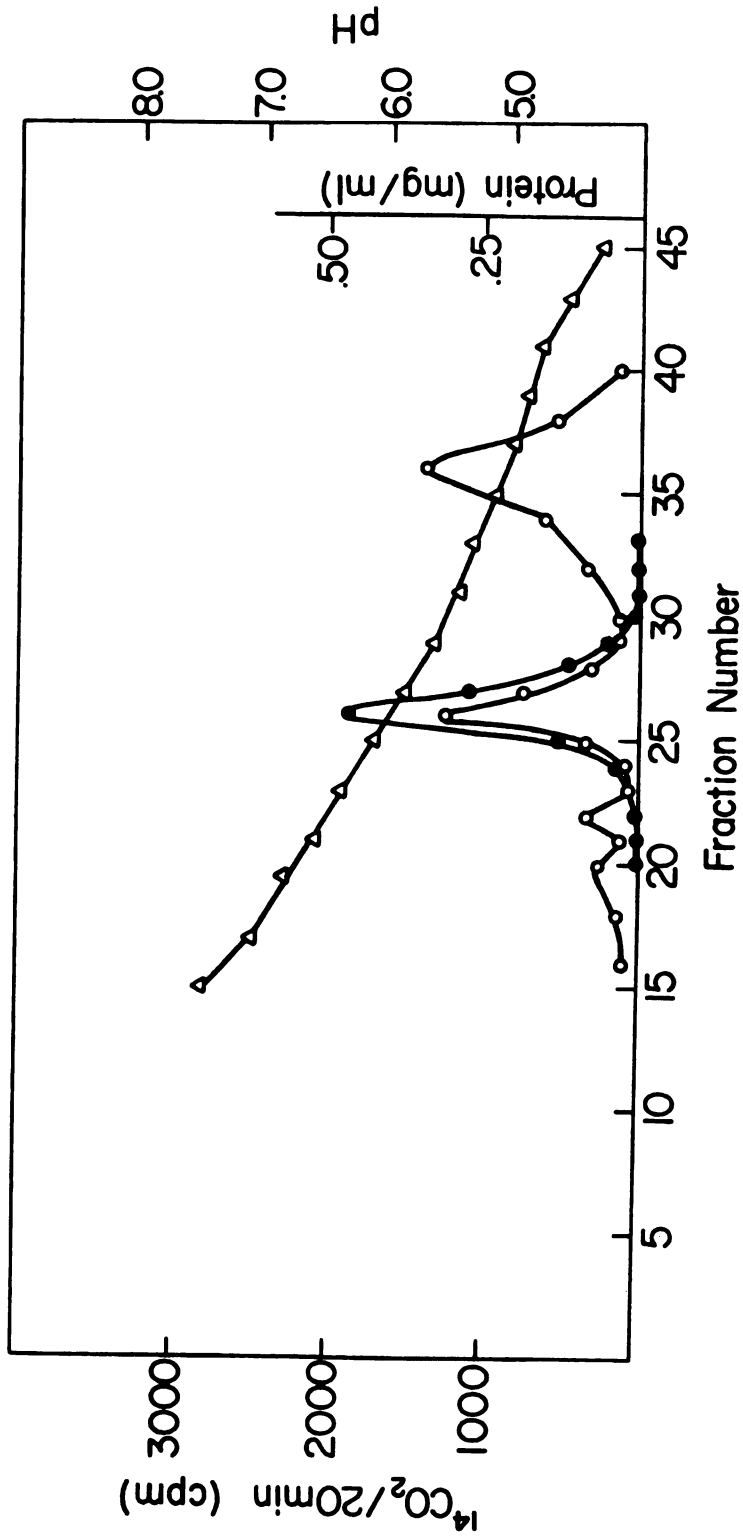
The DEAE Sephadex column (2.75 cm i.d. x 16.0 cm) gave the best results when 1000 to 3000 mg protein were applied. When columns were run with less than 1000 mg protein, all the quinolinic acid phosphoribosyltransferase activity was invariably lost. On the other hand, when

Figure 5.--Elution profile of quinolinic acid phosphoribosyltransferase from an isoelectric focusing column. A pH gradient from 4.5 to 7.5 was established using the narrow range (pH 5-7) ampholytes. The 6 ml enzyme from the hydroxyapatite column were added to the middle three fractions as described in the text. The pH of each 2 ml fraction was determined at room temperature using a Sargent-Welch Model NX pH meter. The enzyme was assayed as described in the methods section using 10 ul of each fraction. The protein concentration was determined using the method of Warburg and Christian (77).

Protein (o——o)

Enzyme activity (●——●)

pH (Δ——Δ)


pH
8.0
7.0
6.0
5.0
Protein (mg/ml)
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Fraction Number

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TABLE 1.--Purification of quinolinic acid phosphoribosyltransferase from castor bean endosperm.

Step	Protein (mg)	Units[a]	Specific Activity milliunits x mg^{-1} protein	Yield %
Extraction	3,580	5.6	1.5	100
Heating	2,800	4.4	1.6	79
DEAE	54	3.3	60.0	59
Hydroxyapatite	11	2.2	200.0	39
Isoelectric Focusing	1.5	1.1	750.0	20

[a]1 unit = 1 μmole of nicotinic acid mononucleotide formed per min.

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large amounts of protein (> 3000 mg) were used, the specific activity of quinolinic acid phosphoribosyltransferase was not as high. Therefore, when working with larger amounts of enzyme, the enzyme preparation was divided in half and two columns were run simultaneously.

Many variations on the elution of quinolinic acid phosphoribosyltransferase from the hydroxyapatite column were tried in an effort to separate it from the protein which started to elute just prior to the enzyme activity (see Figure 4). None of the various gradients tried worked any better than the original linear gradient; it was, therefore, used in all the purifications.

In order to obtain a large amount of enzyme for the characterization studies, eight individual preparations of the enzyme carried through the DEAE Sephadex column step were pooled and concentrated with an Amicon Ultrafiltration apparatus. This solution was then treated as one preparation during the last two steps. This was necessary because two full days were required for harvesting 150 to 180 gm endosperm, isolating the enzyme, chromatographing the enzyme on DEAE Sephadex, and assaying the column(s).

Enzyme Stability

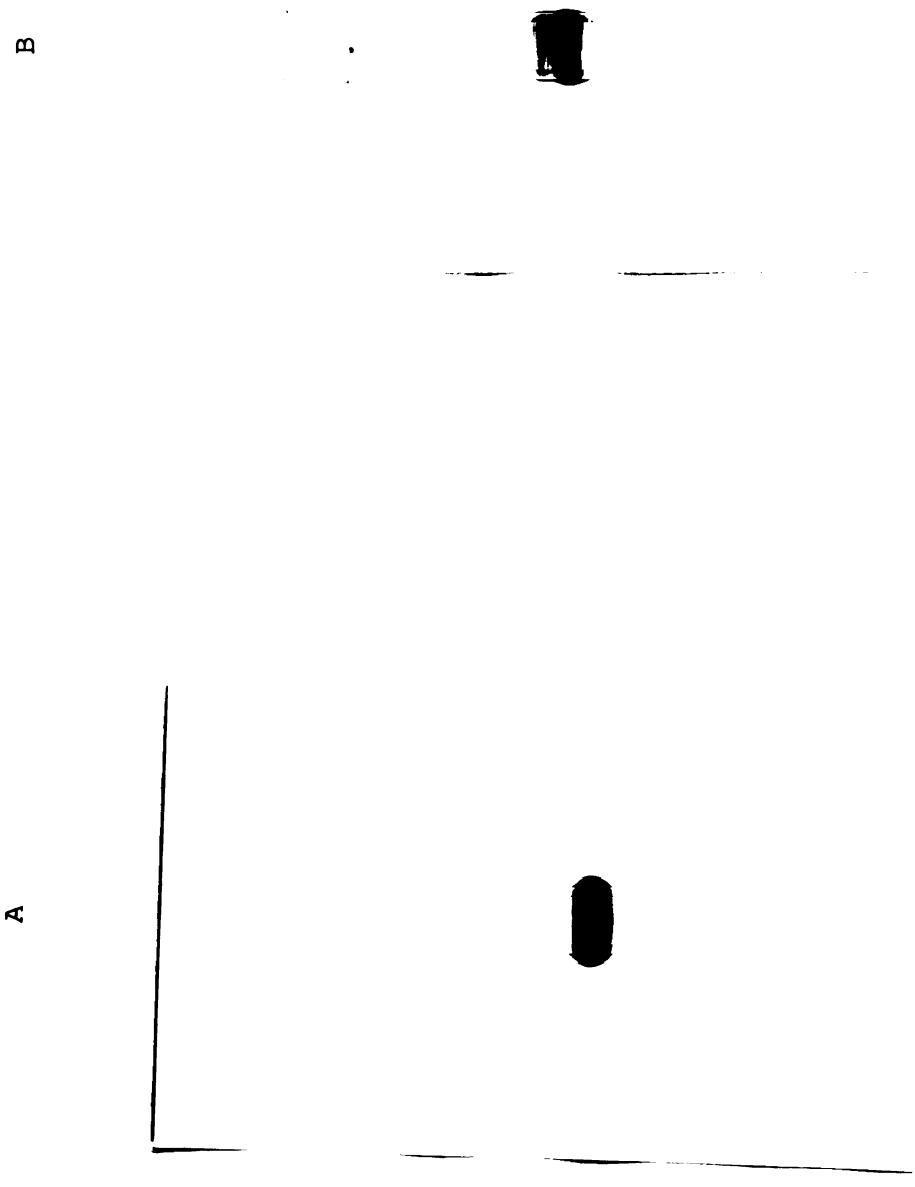
Quinolinic acid phosphoribosyltransferase from castor bean endosperm can be considered a very stable enzyme. However, the enzyme was completely inactivated

when frozen at -20°C in 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol. It lost no activity when stored in the same buffer at room temperature for over 24 hr. When the 0.05 M potassium phosphate (pH 7.0) was made 50% (w/v) in sucrose and 0.01 M in dithioerythritol, the enzyme was found to be completely stable for over four months at either 4°C or -90°C. However, when it was stored in 0.05 M potassium phosphate (pH 7.0) containing 50% (w/v) sucrose but not dithioerythritol, 35% of the original activity was lost after only one month at either 4°C or -90°C. One-third of this lost activity was recovered by making the enzyme solution 0.01 M in dithioerythritol.

Enzyme Purity

A portion of the enzyme isolated from the isoelectric focusing column was denatured by treatment with sodium dodecyl sulfate (SDS) as described on page 97. SDS-polyacrylamide gel electrophoresis (85) of this denatured enzyme resulted in only one band of protein as seen in Figure 6, A. The electrophoresis was carried out using up to 20 μgm of protein and the gels were stained with Coomassie blue. The molecular weight of this band as determined by comparison with protein standards was one-half that of native quinolinic acid phosphoribosyltransferase, as will be discussed later.

Figure 6.--Polyacrylamide gel electrophoresis of quinolinic acid phosphoribosyltransferase from the isoelectric focusing column. A. A solution of the enzyme (20 µg protein per 10 µl) containing 1% SDS, 1% 2-mercaptoethanol, 0.1 M sodium phosphate (pH 7.1), and 10% glycerol was heated for 10 min at 100°C. The enzyme solution was layered on the gel surface and electrophoresis was performed for 3.5 hr at 24°C and at a constant current of 8 mA per tube. The protein was stained with a solution of Coomassie blue overnight and destained in 10% trichloroacetic acid. B. Quinolinic acid phosphoribosyltransferase (18 µg) was layered on the gel surface in a 20% glycerol solution using the method of Ornstein and Davis (88, 89). Electrophoresis (pH 8.3) was performed in 5% gel for 1.5 hr at 40°C and at a constant current of 3 mA per tube. The protein was stained with a solution of Coomassie blue overnight and destained in 10% trichloroacetic acid.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Although the enzyme appeared to be pure by SDS gel criteria, analytical disk gels of the native enzyme resulted in three closely spaced bands as seen in Figure 6, B. Calculations from the densitometer tracing of a typical gel (Figure 7) indicates the relative abundance of the three bands: 70, 25, and 5 per cent for band 1, 2, and 3 respectively. Disk gel electrophoresis at pH 7.0 gave a profile similar to that obtained at pH 8.3, but electrophoresis at pH 4.3 resulted in a smear with no discrete bands of protein.

Even though the three bands of protein obtained with analytical disk gel electrophoresis were very close, it was possible by slicing the gels with a Canalco Gel Slicer or with a razor blade to obtain two peaks of quinolinic acid phosphoribosyltransferase activity. The two peaks were not always resolved and sometimes the enzyme activity profile had a main peak with a definite shoulder. Both cases are illustrated in Figure 8. Three peaks of activity were never observed and it is not known which protein bands on the gel corresponded to the peaks of enzyme activity observed.

The Ferguson plots in Figure 9 lend further support to the contention that all three protein bands observed on analytical gels were of the same molecular weight. The log of the relative electrophoretic mobility (R_f) of the three protein bands in gels of

Figure 7.--Densitometer tracing of polyacrylamide gel electrophoresis of quinolinic acid phosphoribosyltransferase. The enzyme (18 ug) isolated from the isoelectric focusing column was layered on the gel surface in a 20% glycerol solution. Electrophoresis (pH 8.3) was performed in 5% gel for 1.5 hours at 4°C and at a constant current of 3mA per tube. The gel was scanned at 660 nm after destaining.

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5.5
6.0
6.5
7.0
⊕
Distance from Origin (cm)
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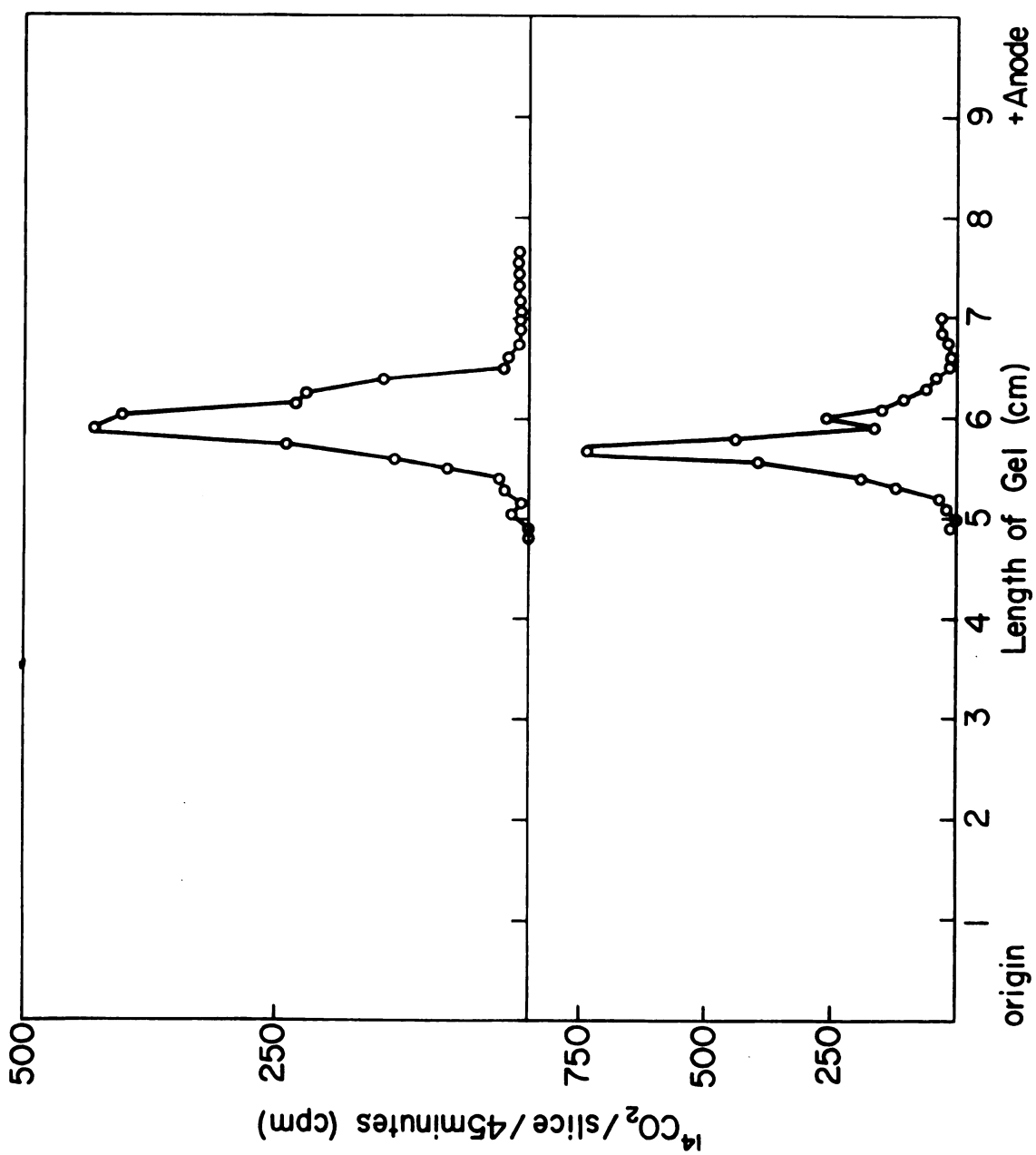
.2
.3
Absorbance at 660nm

Figure 8.--Quinolinic acid phosphoribosyltransferase activity from a 4%, pH 8.3 acrylamide gel. The gels were sliced using a Canalco Gel Slicer (1.4-1.6 mm) or by hand with a razor blade (1.2 - 1.4 mm) and assayed immediately as described in the methods section. Electrophoresis was performed at 3 mA per tube using 30 µg of protein per gel. It was not possible to correlate the enzyme activity to any particular one of the three bands as seen in Figure 6.


500
250
750
500
250
$^{14}CO_2$/slice/45minutes (cpm)
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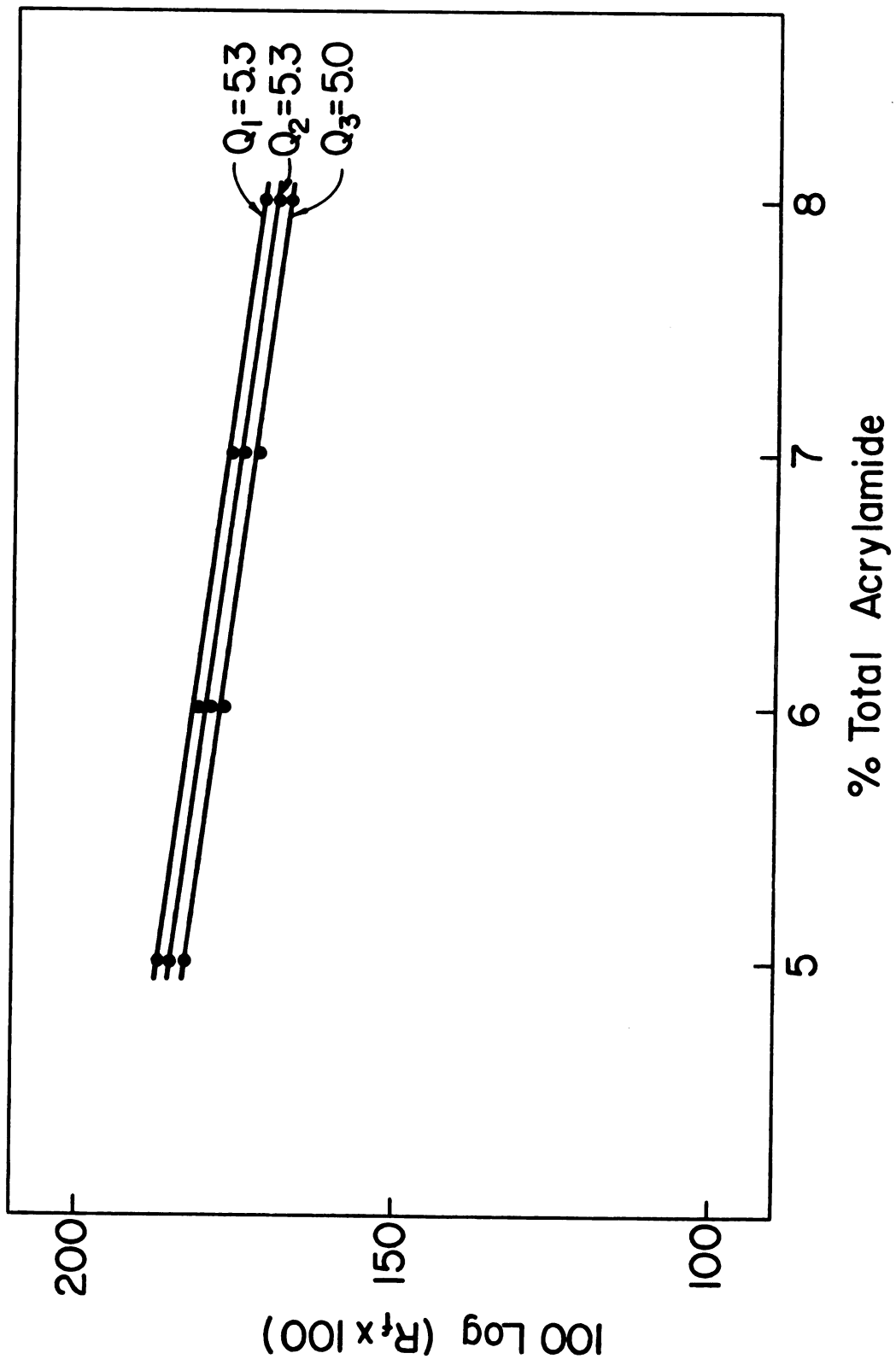
2
3
4
5
6
7
8
9
+Anode
Length of Gel (cm)

Figure 9.--Ferguson plots of the log of the relative electrophoretic mobility, R_f, versus the polyacrylamide concentration of the protein (Q_1, Q_2, and Q_3) isolated from the isoelectric focusing column. The negative slope of the curves may be noted in the figure. The different percentages of acrylamide were obtained by varying the amounts of solution B and water used to make the gels as described in the methods section. Thirty µg protein were added to each gel. Each point is the mean of two R_f measurements.


Q_1=5.3
Q_2=5.3
Q_3=5.0
200
150
100
100 Log (R_f x 100)
5
6
7
8
% Total Acrylamide

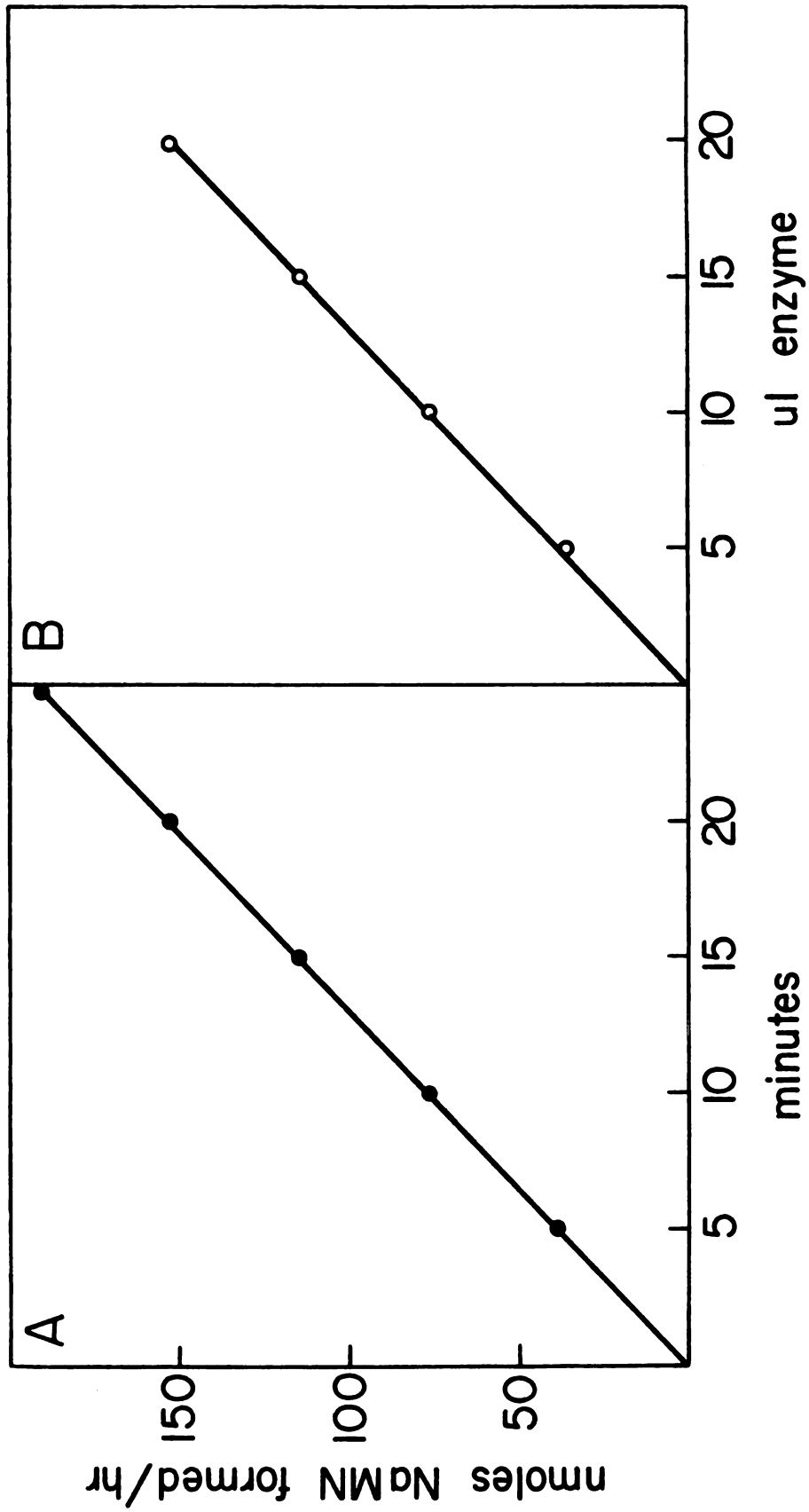
varying porosity plotted against the percentage acrylamide in the gel gave three parallel lines of equal slope. This indicated that the proteins were of the same molecular weight and differed in charge only. This is in agreement with the results of the SDS gels, which indicated that the proteins were composed of subunits of the same molecular weight.

Assay Characteristics of Quinolinic Acid Phosphoribosyltransferase

Quinolinic acid phosphoribosyltransferase activity was linear with both time and enzyme concentration under the conditions described in the methods, as seen in Figure 10. This linearity was checked after every step in the purification. The only time when a deviation from the above results was noticed was after the heating and subsequent dialysis of the enzyme preparation. It was assumed that this nonlinearity with enzyme concentration was due to residual unlabeled quinolinic acid which diluted the labeled quinolinic acid used in the enzyme assay.

The requirements for the conversion of quinolinic acid and PRPP to nicotinic acid mononucleotide as catalyzed by quinolinic acid phosphoribosyltransferase are summarized in Table 2. The reaction was dependent on Mg^{2+} and PRPP as well as the enzyme and quinolinic acid. Removal of all potassium phosphate from the enzyme by

Figure 10.--Formation of nicotinic acid mononucleotide by quinolinic acid phosphoribosyltransferase as a function of time (A) and amount of enzyme (B). In part A, the enzyme was used at a level of 20 µl. In part B, the assays proceeded for 20 min. The assays for both plots were performed under the standard conditions described in the methods section, by measuring the $^{14}CO_2$ produced.


nmoles NaMN formed/hr
150
100
50
A
5
10
15
20
minutes
B
5
10
15
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ul enzyme

TABLE 2.--Requirements for quinolinic acid phosphoribosyltransferase activity purified from castor bean endosperm. The enzyme was assayed as described in the methods section, except that the Na^{+} salt of PRPP was used. Tris·HCl (0.05 M, pH 7.0) was used in place of potassium phosphate (0.05 M, pH 7.0) in the assays with Ba^{2+}, Mn^{2+}, and Ca^{2+}.

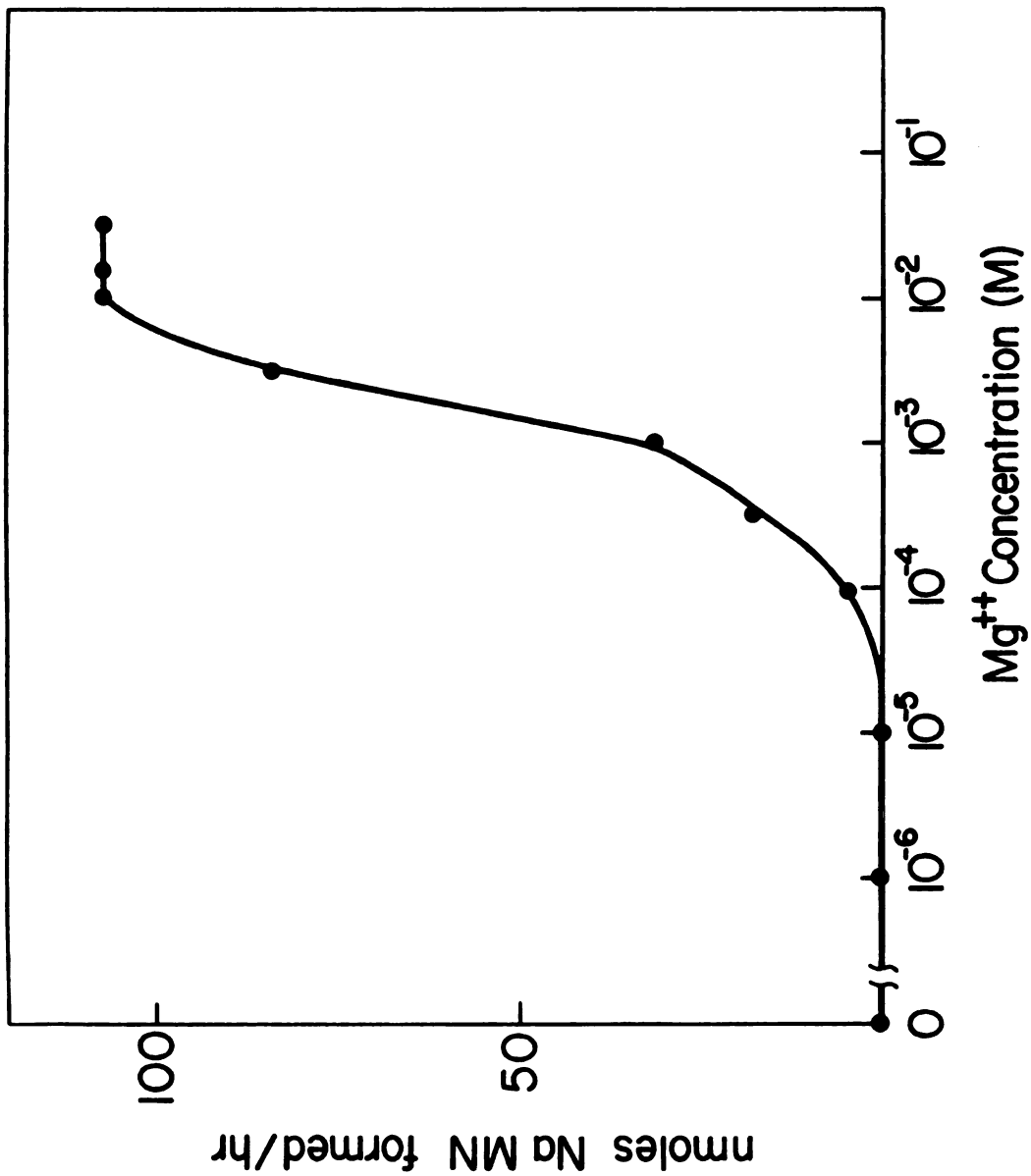
Reaction System	Nicotinic Acid Mononucleotide nmoles/hr
Complete	127.5
-Enzyme	0.0
$-Mg^{2+}$	0.0
-PRPP	0.0
-Potassium Phosphate + Tris·HCl	121.2
$-Mg^{2+}$, + Mn^{2+} 1×10^{-2} M	35.7
$-Mg^{2+}$, + Ca^{2+} 1×10^{-2} M	0.0
$-Mg^{2+}$, + Ba^{2+} 1×10^{-2} M	0.0

dialysis against 0.05 M tris·HCl (pH 7.0) containing 0.01 M 2-mercaptoethanol did not result in any significant decrease in enzyme activity. This was in contrast to those results with quinolinic acid phosphoribosyltransferase from a pseudomonad in which all enzymatic activity was lost by replacing the potassium phosphate with tris·HCl. Subsequent addition of potassium phosphate to the dialyzed bacterial enzyme did not reactivate it (50).

Metal Requirements

Quinolinic acid phosphoribosyltransferase displayed a requirement for magnesium, which is the case for all known reactions using PRPP. The Mg^{2+} requirement in this reaction could not be replaced by equal molar quantities of either Ca^{2+} or Ba^{2+}. On the other hand, Mn^{2+} was one-third as effective as Mg^{2+} at equimolar concentrations. The sodium salt of PRPP was used in these experiments to insure that no Mg^{2+} was present when determining the effect of the divalent cations. Figure 11 shows the effects of Mg^{2+} concentrations under the standard assay conditions. With a PRPP concentration of 0.375 mM, optimal activity was obtained with Mg^{2+} concentrations between 10 mM and 50 mM. Gholson et al (47) reported the quinolinic acid phosphoribosyltransferase from bovine liver was stimulated by such monovalent cations as K^+, Li^+, or NH_4^+ at levels of

Figure 11.--The effect of Mg^{2+} concentration on quinolinic acid Phosphoribosyltransferase. Standard assay conditions for quinolinic acid phosphoribosyltransferase were used, except that the Mg^{2+} salt of PRPP was replaced by the Na^{+} salt.


nmoles Na MN formed/hr
100
50
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Mg^{++} Concentration (M)

20 mM. The effect of the monovalent cations Li^+, K^+, Na^+, and NH_4^+ was investigated with the enzyme from castor bean endosperm and the reaction was not significantly affected. These assays were done under standard conditions except that the 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercatoethanol was replaced with 0.05 M tris·HCl (pH 7.0) also containing 0.01 M 2-mercaptoethanol.

Product Identification

In order to identify the product of the reaction, nicotinic acid mononucleotide, the following were incubated at room temperature for five hr: 1.5 units of enzyme from the hydroxyapatite column, 111 mM potassium phosphate (pH 7.0), 5.4 mM 2-mercatoethanol, 12.5 mM $MgCl_2$, 3.1 mM PRPP, 2.5 mM unlabeled quinolinic acid, and 2.1 mM [6-^{14}C] quinolinic acid (3.82 μCi/μmole) in a total volume of 8 ml. The reaction was stopped by adding 1 ml of 10% perchloric acid and the solution was adjusted to pH 6.7 with KOH. After centrifugation at 10,000xg for 10 min to remove the potassium perchlorate, the supernatant was chilled; the additional potassium perchorate which formed was again removed by centrifugation. The supernatant was then applied to a Dowex 1X2 (50-100 mesh) formate column (1 cm i.d. x 40 cm) along with 10 μmoles of authentic nicotinic acid used as a column marker. The column was eluted with a differential formic acid

gradient as described by Ijichi et al (90) and 7 ml fractions were collected. The elution profile is shown in Figure 12. The nicotinic acid mononucleotide fraction was concentrated on a rotary evaporator, streaked on citrate-washed paper, and chromatographed in solvent system B. Table 3 summarizes the comparisons made between authentic nicotinic acid mononucleotide and the reaction product obtained from the column. Authentic nicotinic acid mononucleotide (0.5mg) and the reaction product (60,000 dpm) were hydrolyzed in 0.1 M NaOH for 10 min in a 100°C water bath. Alkaline hydrolysis under these conditions completely destroys the pyridinium linkage (12), and as shown in Table 3, the products of alkaline hydrolysis migrated with standard nicotinic acid. Molar ratios for nicotinic acid, ribose, and phosphate were determined for the reaction product and are as follows: 100, 82, and 104 respectively. Nicotinic acid was determined by its specific activity, ribose by the orcinol reaction (82) and inorganic phosphate by the micro method of Ames and Dubin (81).

pH Optimum and Isoelectric Point

The enzyme was active over a very wide pH range as illustrated in Figure 13. The optimum pH was centered between 6.5 and 7.7. This was in contrast to quinolinic acid phosphoribosyltransferase from both bovine liver

Figure 12.--Chromatographic separation of the products formed by quinolinic acid phosphoribosyltransferase using a Dowex 1X2 (50-100 mesh) formate column eluted with a differential formic acid gradient. The column had a flow rate of 40 ml per hour and 7 ml fractions were collected at room temperature. Standard nicotinic acid (10 μmoles) was added with the reaction mixture. The radioactivity in every second fraction was determined by counting 0.5 ml of the fraction in 5 ml scintillation fluid C. The NaMN fractions were evaporated and used in identification procedures.

Absorbance at 260 nm (●——●)

Radioactivity x 10^{-3} (o——o)

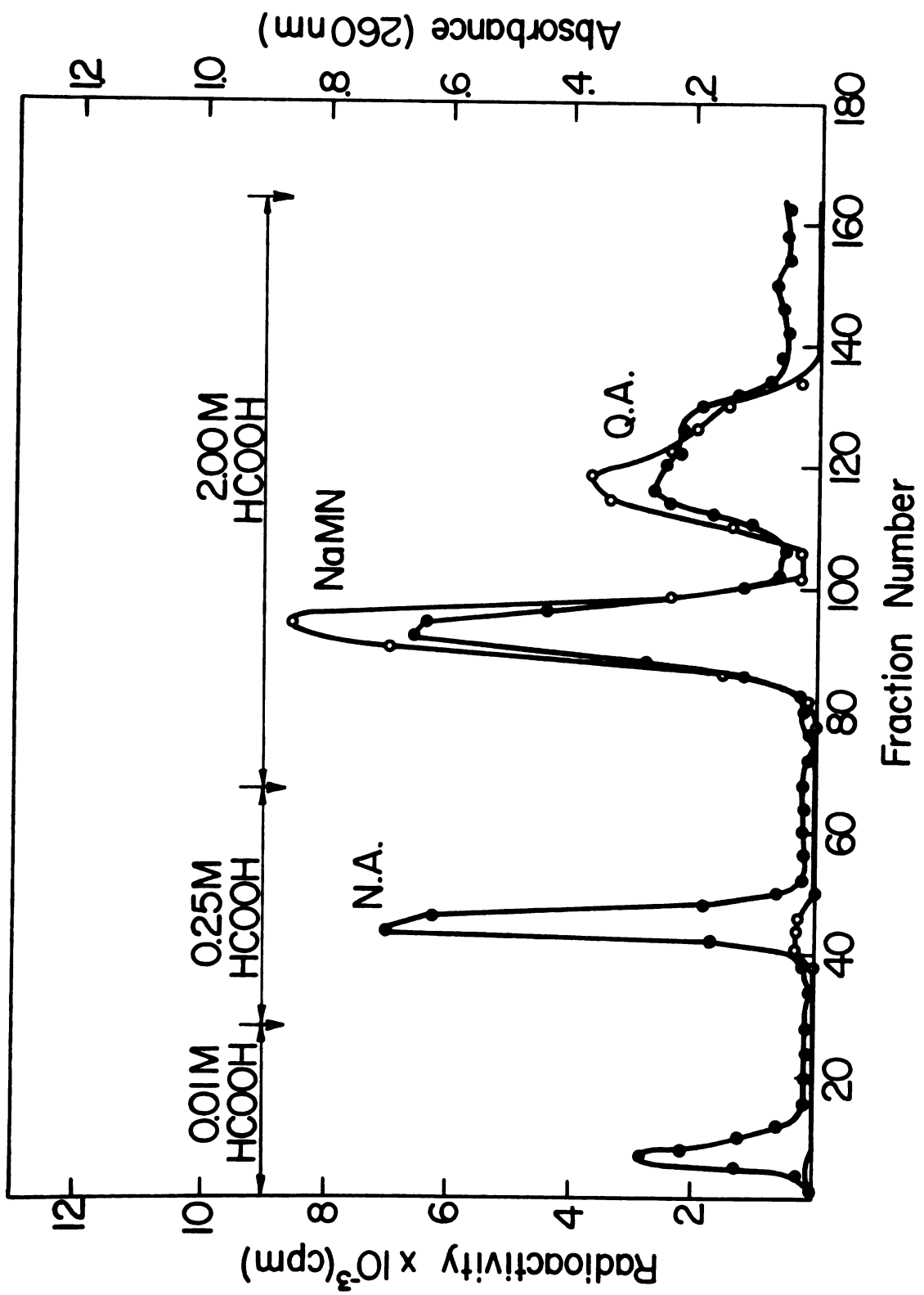
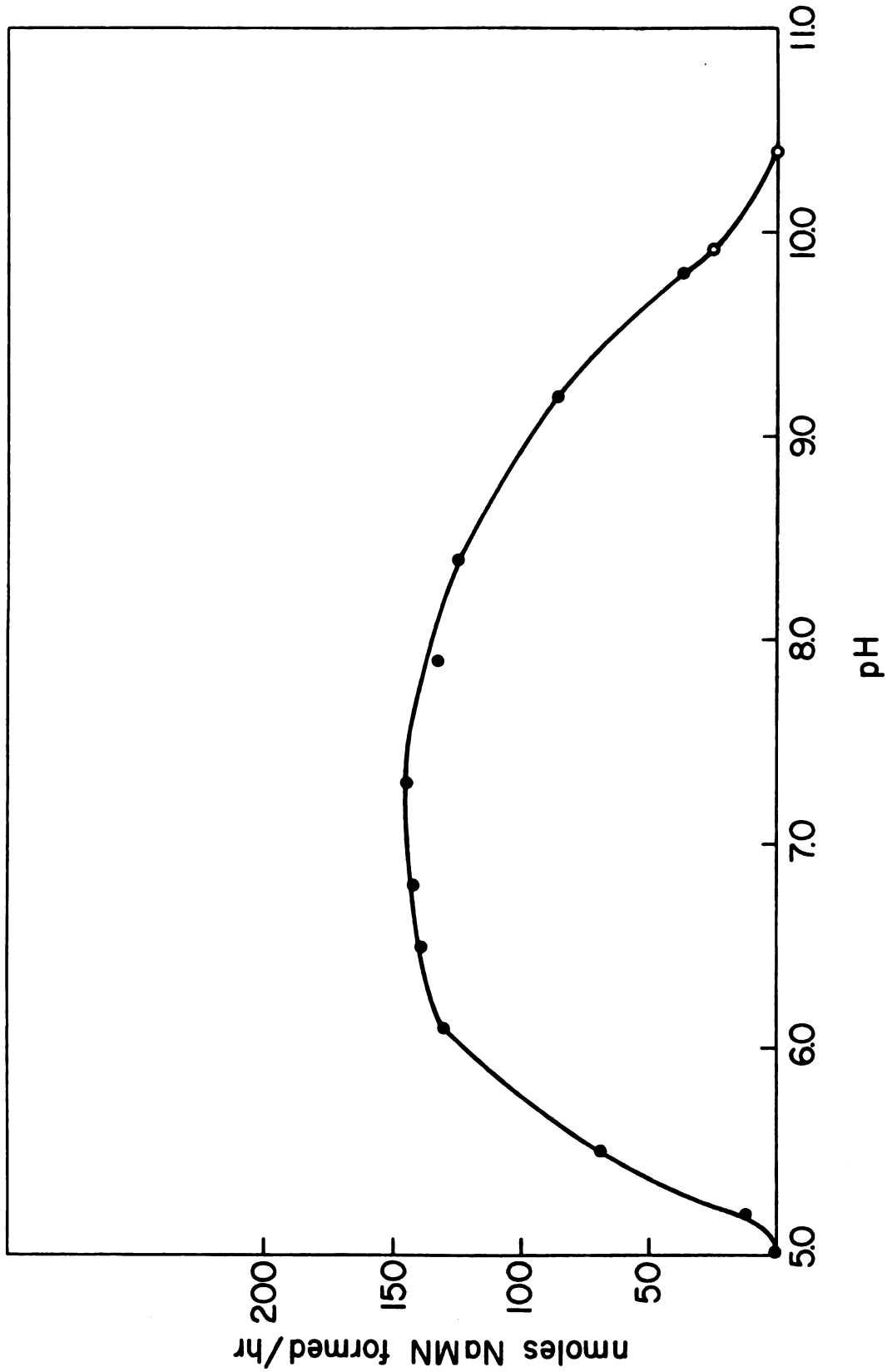

Radioactivity x 10^{-3}(cpm)
Absorbance (260nm)
Fraction Number
0.01M HCOOH
0.25M HCOOH
2.00M HCOOH
N.A.
NaMN
Q.A.

TABLE 3.--Quinolinic acid phosphoribosyltransferase product identification. The compounds were spotted on Whatman no. 1 paper and developed by descending chromatography in the three solvent systems. The enzyme reaction product was identified by scanning its chromatogram with a Packard Radiochromatogram Scanner. All other compounds were detected visually with a Mineralight UVS·11 lamp. Alkaline hydrolysis was done in 0.1 M NaOH for 10 min in a 100°C water bath.

Compound	R_f in Solvent System		
	A	B	D
Authentic Nicotinic Acid Mononucleotide	.77	.24	.10
Reaction Product	.77	.27	.12
Alkaline Hydrolysis of Authentic Nicotinic Acid Mononucleotide	.37	.73	.65
Alkaline Hydrolysis of Reaction Product	.37	.73	.65
Authentic Nicotinic Acid	.37	.75	.66

Figure 13.--Activity of quinolinic acid phosphoribosyltransferase as a function of pH. Standard assay conditions were used as described in the methods section, except that the phosphate buffer was replaced by 100 mM MES, HEPES, and Bicine (●——●) and by 100 mM glycine-NaOH (o——o).


nmoles NaMN formed/hr
200
150
100
50
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

and the pseudomonad which exhibited well-defined optima at pH 6.2 and 7.1 respectively. The isoelectric point of the enzyme was 6.0 and was determined as described earlier in the methods section (Figure 5). This was the average of four determinations in which enzyme isolated from the hydroxyapatite column was used.

Temperature Activation

Nicotinic acid mononucleotide formation was measured as a function of temperature (Figure 14). The reaction rate increased until 45°C after which point it sharply declined, indicating that some enzyme inactivation must have taken place. An Arrhenius plot of this data (Figure 14, A) indicated that inactivation was also occurring at 40°C and 45°C. The Q_{10} at these two temperatures did not agree with the value of 2.6, which was the Q_{10} of the reaction between 15°C and 35°C. The energy of activation calculated from the Arrhenius equation was 17.5 Kcal.

Substrate Protection Against Heat Inactivation

Quinolinic acid was quite effective in protecting quinolinic acid phosphoribosyltransferase from heat inactivation under the same conditions in which the controls lost 48 per cent of their activity when heated at 55°C for 10 min (Table 4). The protection derived from quinolinic acid was probably due to its binding to

Figure 14.--The effect of temperature on the reaction rate of quinolinic acid phosphoribosyltransferase. A. This is an Arrhenius plot of the data given in part B of this figure. The log of the reaction rate (log K) is plotted against the reciprocal of the absolute temperature. B. This is the reaction rate of quinolinic acid phosphoribosyltransferase at different temperatures. Each point is the average of four values. The assays were performed as stated in the methods section, except that the reaction was started by the addition of 20 µl enzyme which was at room temperature.

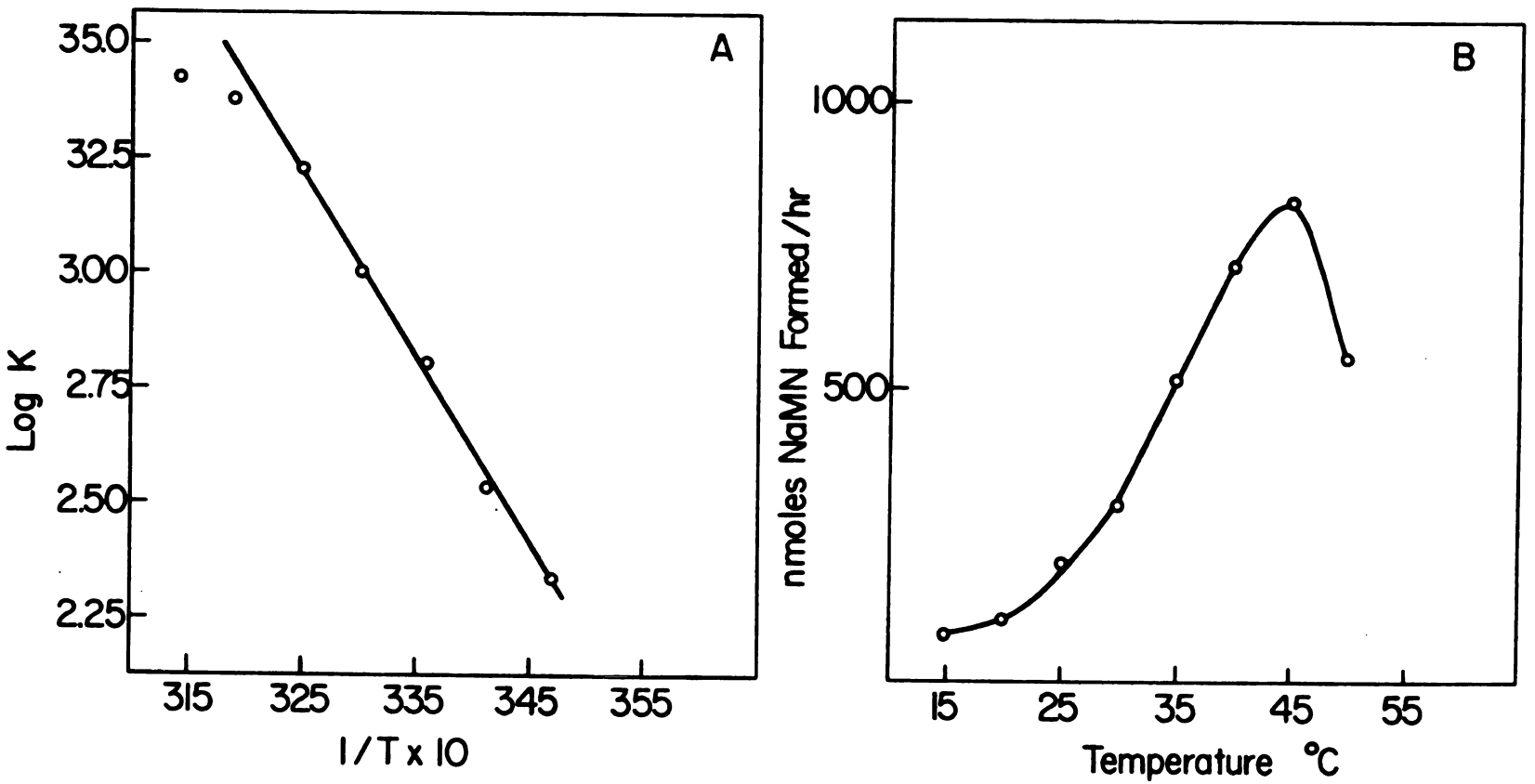

A
35.0
32.5
3.00
2.75
2.50
2.25
Log K
315
325
335
345
355
1/T x 10
B
1000
500
nmoles NaMN Formed/hr
15
25
35
45
55
Temperature °C

TABLE 4.--Substrate protection against heat inactivation. The enzyme was heated for 10 min at 55°C in 0.05 M potassium phosphate (pH 7.0) containing 0.01 M dithioerythritol with and without 0.8 mM quinolinic acid, 1.6 mM PRPP, 1.0 mM $MgCl_2$ and with combinations thereof. The samples were then cooled and diluted for assaying. Both the Mg^{2+} and the Na^{+} salt of PRPP were used and no difference was observed between the two. Control samples (0% protection) retained 48% of their original activity.

Additions Prior to Heating	Percentage Protection
Quinolinic Acid	52
Quinolinic Acid + Mg^{2+}	38
PRPP	4
PRPP + Mg^{2+}	8
Mg^{2+}	-10

the enzyme, although it cannot be said that the binding was at the active site. Neither PRPP (1.6 mM) or its biologically active form, PRPP-Mg^{2+} (1.6 mM and 1.0 mM, respectively) were significantly effective in protecting the enzyme from heat inactivation. Magnesium (1.0 mM) slightly promoted enzyme inactivation by heat (10%). When quinolinic acid (0.8 mM) was added in addition to the Mg^{2+} (1.0 mM), the percentage protection was decreased by 14% from that obtained with quinolinic acid alone. When the effectiveness of PRPP as a protective agent against heat inactivation was tested, both its sodium and its magnesium salts gave essentially the same results whether or not additional Mg^{2+} was present.

Effect of Divalent Cations

The effect of a series of divalent cations on quinolinic acid phosphoribosyltransferase is given in Table 5. The assays were performed in 0.05 M tris·HCl rather than the equivalent phosphate buffer, as the latter tended to form precipitates with some of the metal ions used. The metals were incubated for 15 min with the enzyme before starting the reaction with PRPP. Ca^{2+}, Ba^{2+}, and Sr^{2+} had hardly any effect on the enzyme activity even at 1.0 mM. On the other hand Ni^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+}, and Pb^{2+} completely inhibited the enzyme at 1.0 mM and caused about 40% inhibition at 0.1 mM. While Cu^{2+} and Zn^{2+} at 0.1 mM inhibited the enzyme by 80%. The percentage inhibition caused by Mn^{2+} might have

TABLE 5.--The effect of various cations on quinolinic acid phosphoribosyltransferase activity. The assay conditions were the same as described in the methods section, except that the enzyme was dialyzed against 0.05 M tris·HCl (pH 7.0) with no mercaptan present. There was no appreciable loss in activity. The following salts of the cations were used: Ca^{2+}, Ba^{2+}, Sr^{2+}, Fe^{3+}, and Zn^{2+} chloride; Mn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{2+} sulfate; and Pb^{2+} acetate.

Metal	Relative Activity Percentage			
	1×10^{-5}	1×10^{-4}	1×10^{-3}	1×10^{-2}
Ca^{2+}			104	90
Ba^{2+}			105	110
Mn^{2+}			59	33
Sr^{2+}			100	83
Ni^{2+}	100	88	2	0
Co^{2+}	81	79	20	1
Cu^{2+}	68	18	0	0
Cd^{2+}	69	61	4	0
Fe^{2+}	96	64	4	0
Fe^{3+}			64	0
Pb^{2+}	99	n.d.	1	0
Zn^{2+}	82	21	0	0

been competitive as previous results indicated that Mn^{2+} could partially replace the requirement of the enzyme for Mg^{2+}. Quinolinic acid phosphoribosyltransferase was stabilized over long periods of time with the mercaptan, dithioerythritol. Studies with the classical sulfhydryl inhibitors, NEM, and p-CMB at 0.1 mM, however, did not lead to any significant inhibition.

Effect of Quinolinic Acid Analogues

The effect of quinolinic acid analogues on the rate of quinolinic acid phosphoribosyltransferase activity when assayed together with quinolinic acid is shown in Table 6. The assays were carried out in triplicate as stated in the methods, except that lower levels of quinolinic acid (37.5 μM) and PRPP (62.5 μM) were used. The inhibitors were incubated with the enzyme for 15 min at 30°C before adding the quinolinic acid to start the assay. The assays were stopped after 5 min.

From the data given, it was apparent that a compound having a carboxyl group, a carboxylamide group, or even a cyano group at the 3 position of the pyridine ring was an ineffective inhibitor of the enzyme. However, if the latter two compounds had a carboxyl group added at the 2 position, they were very effective inhibitors of the reaction. When the added carboxyl group at the 2 position was converted to its methyl ester, it no

TABLE 6.--The effect of substrate analogues on the rate of nicotinic acid mononucleotide formation. The standard assay for quinolinic acid phosphoribosyltransferase as described in the methods was used, except that lower levels of quinolinic acid and PRPP were present. The assay contained the following reagents at the final concentrations given in a total volume of 0.8 ml: 2 milliunits of enzyme; 115 mM potassium phosphate (pH 7.0) with 2-mercaptoethanol; 12.5 mM $MgCl_2$; 37.5 μM [2,3,7,8-^{14}C] quinolinic acid; 62.5 μM PRPP and 1.0 and 10.0 mM additions of the various compounds listed in the table. The assays were equilibrated for 15 min at 30°C, started by the addition of quinolinic acid, and then stopped after 5 min. The average value for three reactions are reported as percentages of the control.

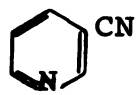
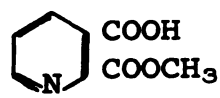
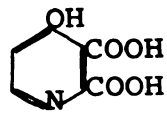
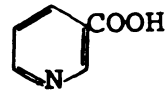
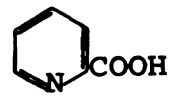
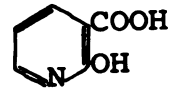
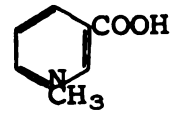
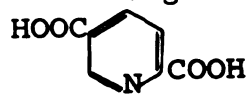
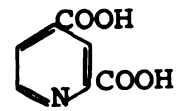
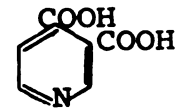
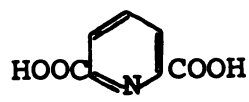
Compound	Structure	Percentage of Control	
		10^{-3} M	10^{-2} M
Nicotinamide		106	102
3-Amidopyridine-2-carboxylate		39	5
Methyl 3-amidopyridine-2-carboxylate		86	43
Nicotinonitrile		101	91
3-Cyanopyridine-2-carboxylate		52	22
Methyl 3-cyanopyridine-2-carboxylate		95	85
Methyl 3-carboxypyridine-2-carboxylate		69	30

TABLE 6.--Continued.

Compound	Structure	Percentage of Control	
		10^{-3}M	10^{-2}M
4-Hydroxyquinolinic Acid		48	12
Nicotinic Acid		101	91
Picolinic Acid		20	8
2-Hydroxynicotinic Acid		78	50
Trigonelline		90	88
2,5-Pyridinedi-carboxylic Acid		100	90
2,4-Pyridinedi-carboxylic Acid		88	73
3,4-Pyridinedi-carboxylic Acid		61	20
2,6-Pyridinedi-carboxylic Acid		43	6

longer served as an effective inhibitor. In fact, methyl 3-cyanopyridine-2- carboxylate was about as ineffective as its parent compound, 3-cyanopyridine (101% and 95% relative activity at 1mM and 19% and 85% relative activity at 10 mM respectively). The 2-methyl ester of quinolinic acid inhibited more effectively than either methyl 3-amidopyridine-2-carboxylate or methyl 3-cyano-pyridine-2-carboxylate, which indicated that the enzyme showed some preference for the group at the 3 position ($COOH > CONH_2 > C \equiv N$).

Picolinic acid (2-pyridine carboxylate) proved to be one of the best inhibitors tried. The 2-hydroxy-nicotinate inhibited to some extent (50% at 10mM), but it was not as effective as the 2-carboxy analogues. The 4-hydroxyquinolinic acid was also one of the more effective inhibitors, which might imply that a certain degree of steric interference at the 4 position did not exclude the molecule from the active site. The four positional isomers of quinolinic acid tested can be divided into two groups based on their effectiveness as inhibitors of this reaction. The 2,5- and 2,4-pyridinedicarboxylate were relatively ineffective, whereas the 3,4- and, to a greater extent, the 2,6-pyridine dicarboxylate were more effective.

Effect of Potential Inhibitors on Quinolinic Acid Phosphoribosyltransferase

The effects of a series of possible inhibitors including the pyridine nucleotides (NAD, NADP, NMN, and NaAD), the pyridine nucleotide precursors (nicotinic acid and nicotinamide), the pyridine alkaloids (ricinine and trigonelline), and two other reported inhibitors of the enzyme (ATP and azaleucine) are listed in Table 7. The possible effectors were assayed using lower levels of PRPP and quinolinic acid, as were used with the quinolinic acid analogues. None of the compounds tried inhibited the enzyme to a great degree; NAD, however, caused a slight inhibition of about 19% and 15% at 1mM and 10 mM levels respectively. ATP was reported to significantly inhibit the bovine liver enzyme (47) and to partially inhibit the enzyme in crude preparations of castor bean cotyledons (41), but it had no effect on the purified enzyme from castor bean endosperm. Waller et al (69) recently suggested that azaleucine might act as an inhibitor of quinolinic acid phosphoribosyltransferase in castor beans based on the results of in vivo studies. The purified enzyme used in these experiments was not inhibited even at 10 mM levels of azaleucine.

Kinetics

Double reciprocal plots of the initial velocity versus the concentration of one substrate at a series of

TABLE 7.--The effect of possible inhibitors of quinolinic acid phosphoribosyltransferase. The standard assay for quinolinic acid phosphoribosyltransferase as described in the methods section was used except that lower levels of quinolinic acid and PRPP were present. The assay contained the following reagents at the final concentrations given in a total volume of 0.8 ml: 2 milliunits of enzyme; 115 mM potassium phosphate (pH 7.0) with 2-mercaptoethanol; 12.5 mM $MgCl_2$; 37.5 μM [2,3,7,8-^{14}C] quinolinic acid; 62.5 μM PRPP and 0.1, 1.0, and 10.0 mM additions of the various compounds listed in the table. The assays were equilibrated for 15 min at 30°C, started by the addition of quinolinic acid, and then stopped after 5 min. The average value for three reactions are reported as percentages of the control.

Compound	Percentage of Control		
	10^{-4} M	10^{-3} M	10^{-2} M
NAD	94	81	85
NADP	91	94	89
NMN	94	101	110
NaAD	99	90	
Nicotinic Acid	98	101	91
Nicotinamide	114	106	102
Trigonelline	102	90	88
Ricinine	99	95	100
Azaleucine		100	105
ATP	96	89	90

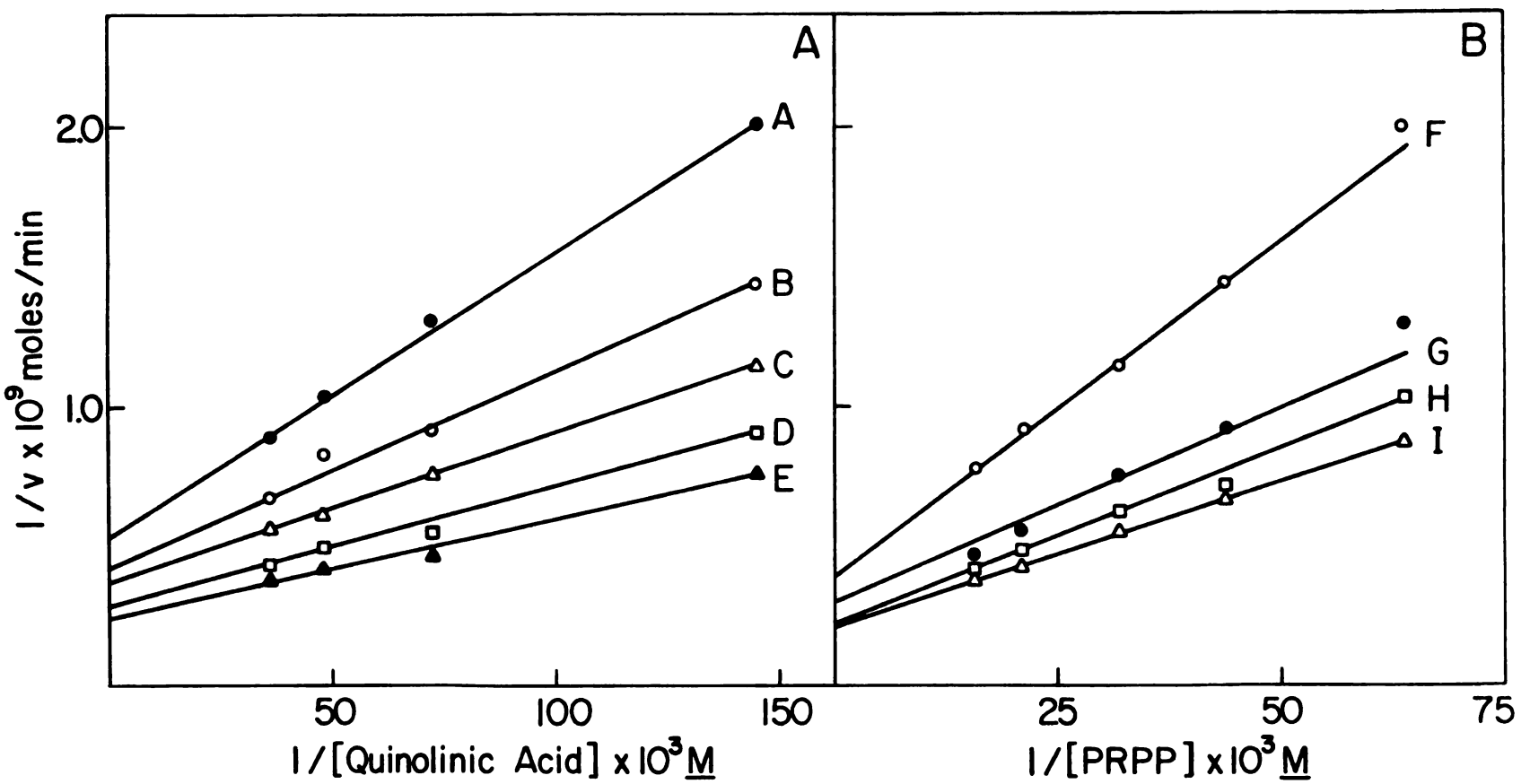
fixed concentrations of the second substrate are presented in Figure 15, A and B. The assays were performed in the usual manner described in the methods section, except that lower substrate concentrations as given in the legend of Figure 15, were used and the reaction time was only 3 min. The results showed that the double reciprocal plots were linear and nonparallel. It followed then that the reaction mechanism was sequential, i.e., both substrates add before either product can be released; however, a distinction between the ordered Theorell-Chance or rapid-equilibrium random type (94) could not be made, as both mechanisms have the same form of the initial velocity equation.

The velocity of the reaction, v, is expressed in Equation 1 in which a, b; K_a, $\bar{K}_a$; and V stand for the substrate concentrations; their Michaelis (K_a) and dissociation ($\bar{K}_a$) constants; and the maximum velocity at a given enzyme concentration respectively. This equation is derived from the Michaelis-Menton equation.

$$\frac{V}{v} = 1 + \frac{K_a}{a} + \frac{K_b}{b} + \frac{\bar{K}_a K_b}{ab} \qquad (1)$$

In order to obtain the concentration independent constants for Equation 1 from the data in Figure 15, secondary plots (16, A and B) were constructed. By replotting the vertical intercepts versus the reciprocal of the

Figure 15.--Double reciprocal plots of initial velocity against substrate concentration. Standard assay conditions were varied as given below and the reaction time was only 3 min. Because of the limitations inherent in an endpoint assay, separate experiments were performed to insure that the assays were linear for the time period used at all concentrations and combinations of quinolinic acid and PRPP. Each point is the average of three assays. The velocity, v, is expressed as nmoles of nicotinic acid phosphoribosyltransferase per minute. The PRPP concentrations were 1.25×10^{-5} M, 1.875×10^{-5} M, 2.5×10^{-5} M, 3.75×10^{-5} M, and 5.0×10^{-5} M, for the curves A, B, C, D, and E respectively. The quinolinic acid concentrations were 0.625×10^{-5} M, 1.25×10^{-5} M, 1.875×10^{-5} M, and 2.5×10^{-5} M for curves F, G, H, and I respectively.


A
B
2.0
1.0
1/v x 10^9 moles/min
A
B
C
D
E
F
G
H
I
50
100
150
25
50
75
1/[Quinolinic Acid] x 10^3 M
1/[PRPP] x 10^3 M

changing fixed substrate concentrations, $-1/K_a$ and $-1/K_b$ can be read from the abscissa and 1/V is the intercept on the ordinate. The slope of the curves in Figure 15, A and B, were determined and are plotted in Figure 16, B, as a function of the reciprocal of the changing fixed substrate concentrations. The two curves obtained with this replot had identical slopes equal to $\overline{K}_a K_b/V$. The values of these kinetic constants are given in Table 8. The K_m values were very similar to those observed with the bovine liver enzyme, 6×10^{-5} M and 5×10^{-5} M for quinolinic acid and PRPP respectively (47). The same was true for the bacterial enzyme with respect to PRPP, 7.4×10^{-5} M, but the K_m of this enzyme for quinolinic acid was significantly higher, 1.2×10^{-4} M (50).

Physical Characteristics

Molecular Weight Estimation Using a Sephadex G-200 Column

The molecular weight of quinolinic acid phosphoribosyltransferase in 0.05 M potassium phosphate containing 0.01 M 2-mercaptoethanol and 0.1 M KCl was estimated by the method of Andrews (83) with a Sephadex G-200 column. The enzyme had been purified through the hydroxyapatite column step as described previously. The molecular weight of castor bean endosperm quinolinic acid phosphoribosyltransferase was estimated by this method to be 68,000. The standard curve is shown in Figure 17.

Figure 16.--Secondary plots of the initial velocity data. A. Secondary plot of the reciprocal of the maximal reaction velocity obtained from the ordinate of Figure 15, A and B, versus the reciprocals of the molar concentrations, 1/[S], of PRPP (o——o) and quinolinic acid (●——●). B. Secondary plot of the slopes of the changing fixed substrate concentrations of Figure 15, of PRPP (Δ——Δ) and quinolinic acid (o——o).

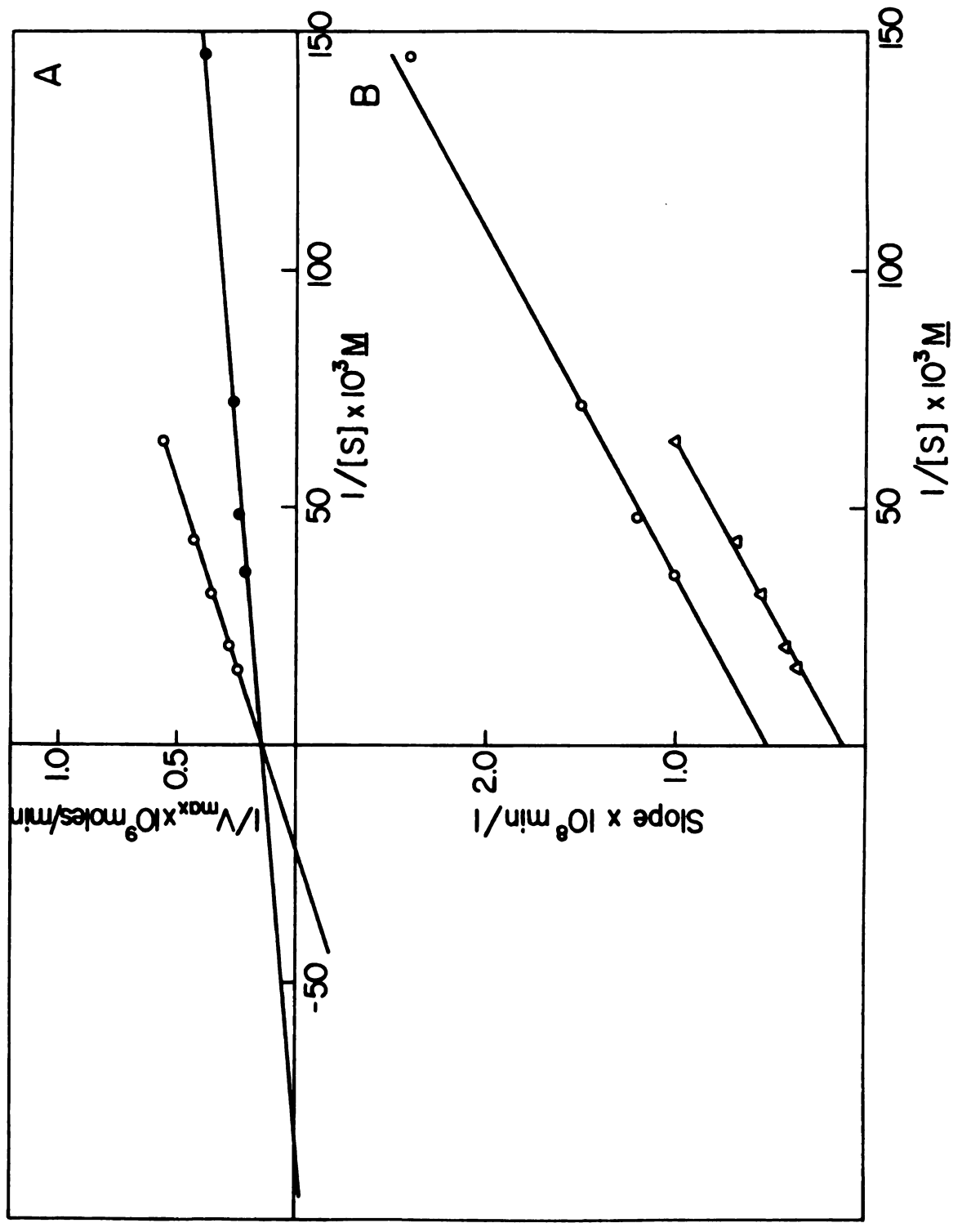
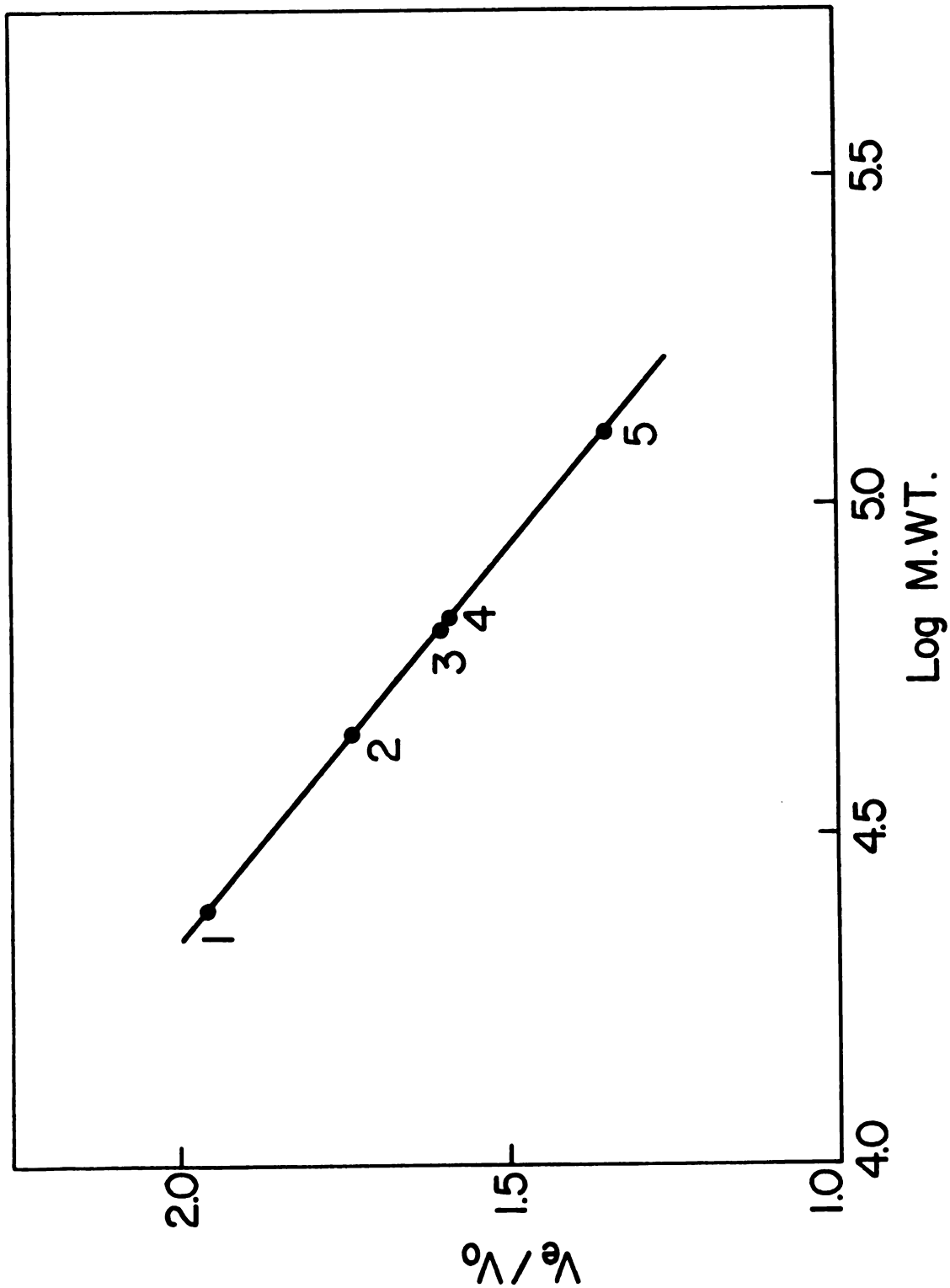

A
B
1.0
0.5
$1/V_{max} \times 10^9$ moles/min
-50
50
100
150
$1/[S] \times 10^3$ M
2.0
1.0
Slope x 10^8 min/l

TABLE 8.--Kinetic data for quinolinic acid phosphoribosyltransferase as determined from initial velocity studies in the absence of product.

Constant	Values
V	7.1 nmoles/min
K_{QA}	1.2×10^{-5} M
K_{PRPP}	4.5×10^{-5} M
$\bar{K}_a K_b$	1.1×10^{-9} M^2

Figure 17.--Plot of relative elution volume V_e/V_o, where V_e is the elution volume and V_o is the void volume, against the log of the molecular weights of various standard proteins eluted from a Sephadex G-200 column (2.5 cm i.d. x 85.5 cm). The column had been equilibrated with 0.05 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol, and 0.1 M KCl. The following standards prepared in 20% (w/v) sucrose in a total volume of one ml were applied to the column two at a time in various combinations for a total of three determinations: (1), 7.0 mg α-chymotrpsinogen (25,000); (2), 10 mg ovalbumin (45,000); (3), 8 mg bovine serum albumin (65,000); (4), 0.2 units of quinolinic acid phosphoribosyltransferase; and (5) bovine serum albumin dimer (130,000). A 0.2% (w/v) blue dextran solution was used to determine the void volume each time the column was run. Two-milliliter fractions were collected and assayed for protein at 230 nm or enzyme activity.


2.0
1.5
1.0
V_e / V_o
4.0
4.5
5.0
5.5
Log M.WT.
1
2
3
4
5

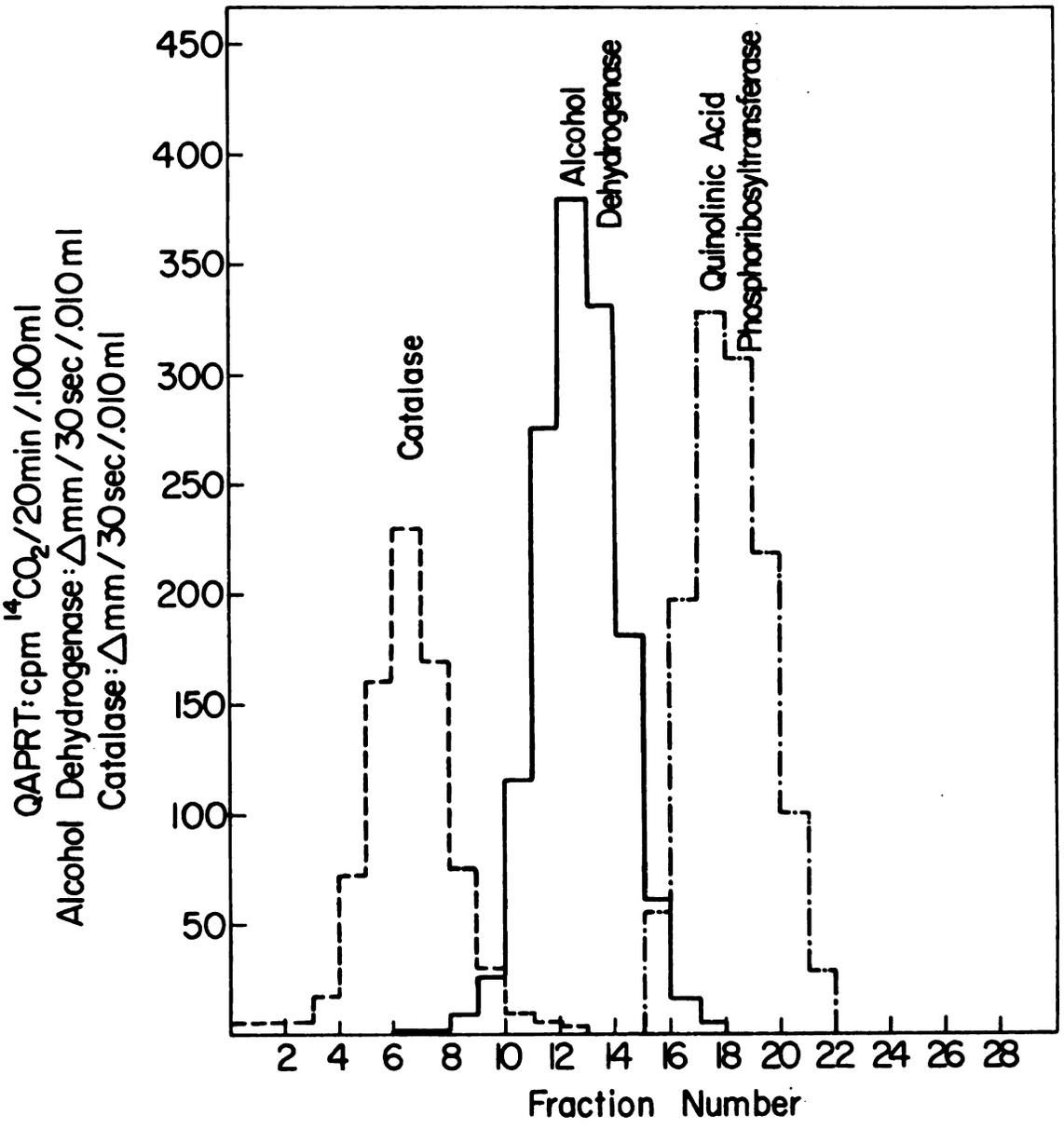
Sucrose Density Gradient Centrifugation

Another estimate of the molecular weight of quinolinic acid phosphoribosyltransferase was obtained by sucrose density gradient centrifugation according to the method of Martin and Ames (84). Five per cent and 20% sucrose solutions containing 0.05 M potassium phosphate (pH 7.0) were used to make 5 ml linear gradients. Quinolinic acid phosphoribosyltransferase in 0.1 ml containing, either together or separately, bovine liver catalase and yeast alcohol dehydrogenase was layered on top of each gradient. The gradients were centrifuged for 15 hr at 4°C in a SW-39L rotor at 38,000 rpm. Twenty-six 6 drop fractions were then collected and assayed for enzymatic activity as seen in Figure 18. The molecular weight of castor bean endosperm quinolinic acid phosphoribosyltransferase as determined by this method was 72,000 with a range of $\pm$2000. The enzyme used for this determination had also been purified through the hydroxyapatite column chromatography step.

Electrophoresis in SDS-Polyacrylamide Gels

It was shown by Shapiro et al (85) that during electrophoresis in SDS-polyacrylamide gels proteins are separated into individual subunits which migrate as a function of their molecular weight. Enzyme from the isoelectric focusing column containing 1% SDS, 1%

Figure 18.--Sedimentation pattern of quinolinic acid phosphoribosyltransferase, bovine liver catalase, and yeast alcohol dehydrogenase. Quinolinic acid phosphoribosyltransferase isolated from the hydroxyapatite column (20 milliunits per 0.1 ml) in a mixture with catalase (0.3 mg) and alcohol dehydrogenase (0.7 mg) were layered on gradient no. 1 pictured below. Two other gradients contained quinolinic acid phosphoribosyltransferase and catalase and quinolinic acid phosphoribosyltransferase and yeast alcohol dehydrogenase. The experimental conditions are given in the text. The assumed molecular weights were 250,000 for catalase and 150,000 for alcohol dehydrogenase (81).


QAPRT: cpm $^{14}CO_2$/20min/.100ml
Alcohol Dehydrogenase: Δmm/30sec/.010ml
Catalase: Δmm/30sec/.010ml
450
400
350
300
250
200
150
100
50
Catalase
Alcohol Dehydrogenase
Quinolinic Acid Phosphoribosyltransferase
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Fraction Number

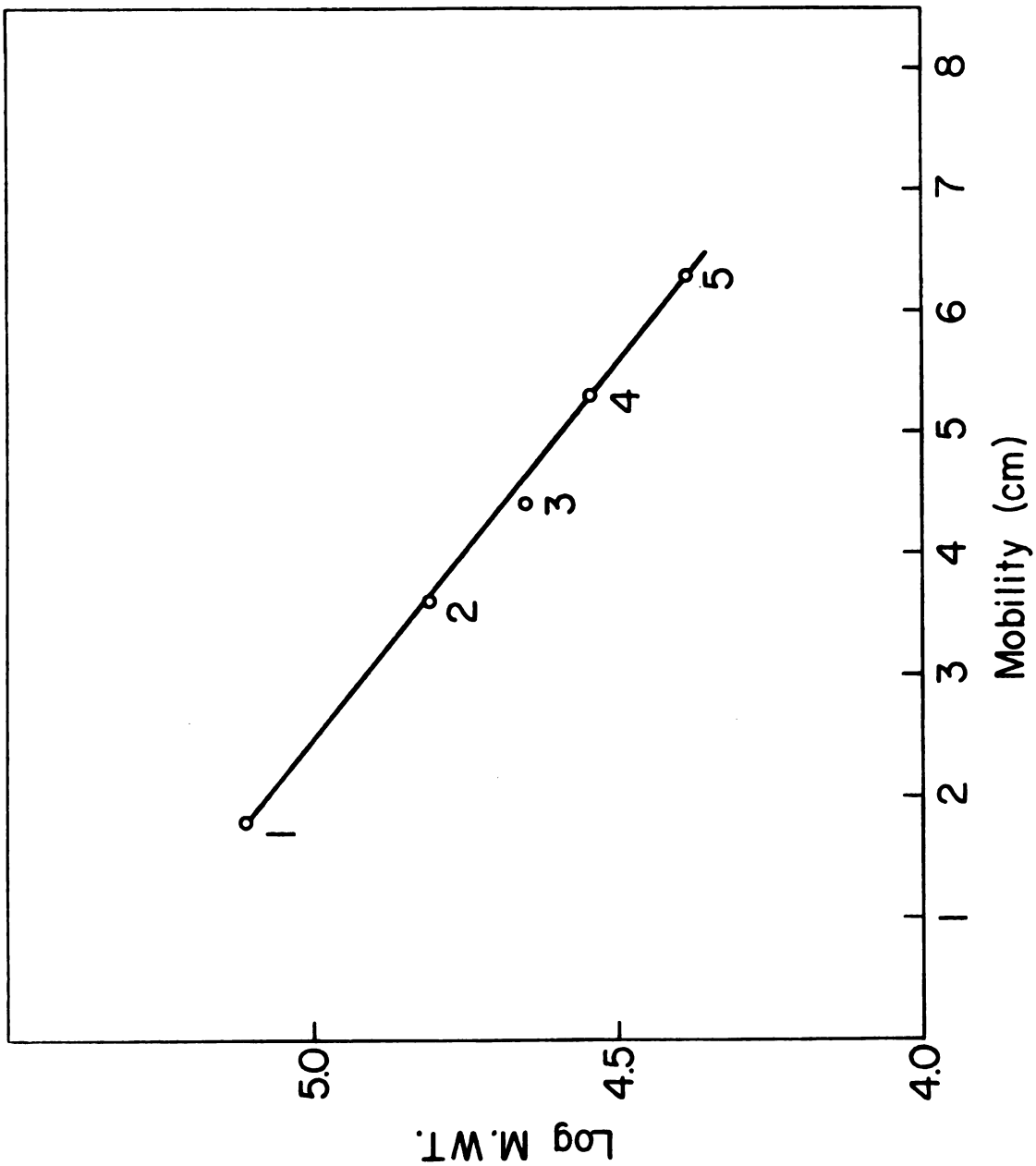
2-mercaptoethanol, 0.1 M sodium phosphate (pH 7.1), and 10% glycerol was incubated at 100°C for 10 min. The enzyme (12 μg protein), along with the similarly treated standards α-chymotrypsinogen, ovalbumin, bovine serum albumin, and bovine serum albumin dimer, was electrophoresed in 5% acrylamide gels containing 0.1% SDS for 3.5 hr at 8 mA per gel tube and stained in 0.4% Coomassie blue as described in the methods. A plot of the log of the molecular weight of the standards against the distance of migration gave a straight line as illustrated in Figure 19. The molecular weight of quinolinic acid phosphoribosyltransferase as determined from this and from the data of two other similar experiments gave an average value of 35,000 with a range of ±2000. This indicated that the enzyme was probably composed of two subunits, since a molecular weight of 70,000 would be in very close agreement with the molecular weight obtained by the two previously described methods.

Physiological Studies

Ricinine Biosynthesis

In the interval of six to seven days in a warm moist environment, the germinating castor bean mobilizes the energy stored in the fatty endosperm tissue for use by the developing seedling attached to it. Accompanying

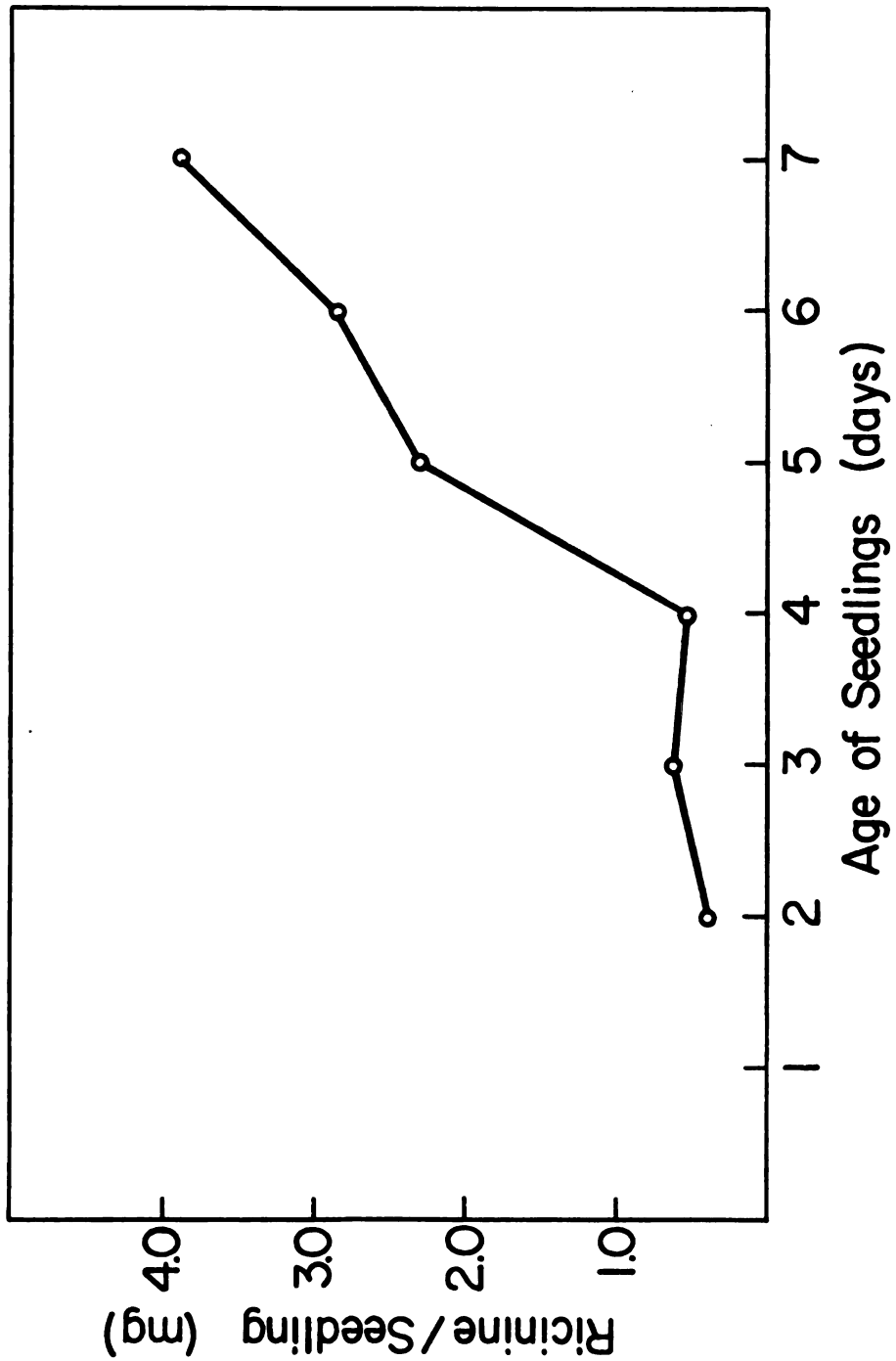
Figure 19.--Log of the molecular weights of standard proteins plotted against their distance of migration on SDS polyacrylamide gels. The experimental conditions are given in the text. The proteins depicted are as follows: (1), bovine serum albumin dimer; (2), bovine serum albumin; (3), ovalbumin; (4), quinolinic acid phosphoribosyltransferase; and (5) α-chymotrypsinogen. The assumed molecular weights used in plotting this data were the same as those reported in the legend of Figure 15.


Log M.WT.
5.0
4.5
4.0
1
2
3
4
5
Mobility (cm)
1
2
3
4
5
6
7
8

this period of active development are many diverse biochemical phenomena, not the least of which is the biosynthesis of the alkaloid ricinine. Its rate of production during early seedling development is shown in Figure 20. This data agreed well with the results of Waller (91), who found that the ricinine content of castor seedlings increased 30-50 fold during a 48-72-hour period between the third and sixth day following planting in moist sand at 30°C. After this period of rapid formation, the absolute amount of the alkaloid increased; however, its concentration (mg ricinine per gm fresh weight) remained constant (92).

To date, much of the experimental investigation of ricinine biosynthesis has involved feeding experiments with intact plants. Little work has been done on the biochemical processes related to ricinine biosynthesis. *In vivo* studies showed that the pyridine nucleus of quinolinic acid, nicotinic acid, and nicotinamide was directly incorporated into ricinine; however, the actual precursor remains unknown (14, 57). These three compounds are related in that the quinolinic acid formed by the *de novo* pathway is eventually converted to nicotinamide and nicotinic acid via the Preiss-Handler pathway. Waller *et al* (14) demonstrated that quinolinic acid was two times more efficient than either nicotinamide or nicotinic acid as an *in vivo* precursor of ricinine.

Figure 20.--Ricinine content of germinating castor bean seedlings. The ricinine was extracted from castor seedlings grown in moist vermiculite at 30°C and quantitated as stated in the methods section. Each value was based on the alkaloid content of three intact seedlings at the various stages of physiological development described in Table 11.


Ricinine / Seedling (mg)
4.0
3.0
2.0
1.0
1
2
3
4
5
6
7
Age of Seedlings (days)

Because a large amount of ricinine was made in the seedling (7 μmoles per day per seedling for a three-day period), it was of interest to see if any corresponding elevation in pyridine ring biosynthesis occurred via the de novo pathway. The enzymatic events of the de novo pathway have not yet been elucidated except for the last step, i.e., the PRPP dependent conversion of quinolinic acid to nicotinic acid mononucleotide (39). It was therefore decided to assay the enzyme responsible for this conversion, quinolinic acid phosphoribosyltransferase, as an indicator of the activity of the de novo pathway both during germination and maturation. The level of activity in the Preiss-Handler pathway was monitored by assaying nicotinic acid phosphoribosyltransferase and nicotinamidase.

Nicotinic Acid Phosphoribosyltransferase Assay

Nicotinic acid phosphoribosyltransferase was assayed as described in the methods section. Under these conditions, nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide were produced. The radioactivity incorporated into both nucleotides was measured in order to determine the total nicotinic acid phosphoribosyltransferase activity. The products of the reaction were identified by paper chromatography with both authentic nicotinic acid mononucleotide and

nicotinic acid adenine dinucleotide in solvent systems A, B, and D. Table 9 shows the dependency of activity on ATP, Mg^{2+}, PPRP, and the enzyme. These results were in agreement with those found for nicotinic acid phosphoribosyltransferase in B. subtilis (34) and yeast (35), which required the presence of ATP for the demonstration of any activity. The linearity of the assay with respect to time and enzyme concentration was checked on each sample, as illustrated in Figure 21. The assay was always linear with time and enzyme concentration, except at the lower enzyme concentrations used here, the activity was depressed. At the present time no explanation can be given for this. Values reported here were always taken from the linear portion of the curve.

Nicotinamidase

Nicotinamidase was assayed as described in the methods section. The enzyme activity was dependent on only the substrate and enzyme. As depicted in Figure 22, the formation of product was linear with respect to time and enzyme concentration. Nicotinic acid was first identified as the product by co-chromatography with authentic nicotinic acid in solvent system C. To provide additional proof that the product was nicotinic acid, a large-scale reaction was performed using 2.0 ml castor bean seedling extract along with 0.30 mM [7-^{14}C]

TABLE 9.--Requirements for nicotinic acid phosphoribosyltransferase activity in castor bean extracts. The complete assay contained the following reagents at the final concentrations given in a total volume of 0.2 ml: 25 mM potassium phosphate (pH 7.0), 4 mM dithioerythritol, 20 mM tris·potassium phosphate (pH 8.0), 60 mM ATP, 1.24 mM PRPP, and 10 to 20 microunits enzyme extract (approximately 1 mg protein). The reaction proceeded for two hr in a 30°C shaking water bath and was terminated by immersion of the reaction flask in a boiling water bath for one min.

Reaction System	Nicotinic Acid Mononucleotide and Nicotinic Acid Adenine Dinucleotide formed nmoles/hr/mg protein
Complete	3.33
- Enzyme	0
- Mg^{2+}	0
- PRPP	0
- ATP	0

Figure 21.--Formation of nicotinic acid mononucleotide by nicotinic acid phosphoribosyltransferase as a function of time and crude castor bean homogenate. A. The enzyme was assayed as described in the methods section using a crude castor bean homogenate as the enzyme preparation; the reaction was stopped by placing the reaction flask in a boiling water bath for one min at the appropriate time. B. The assays contained the same enzyme preparation in the amounts indicated. After two hr the assays were stopped by placing the reaction flask in a boiling water bath for one min.

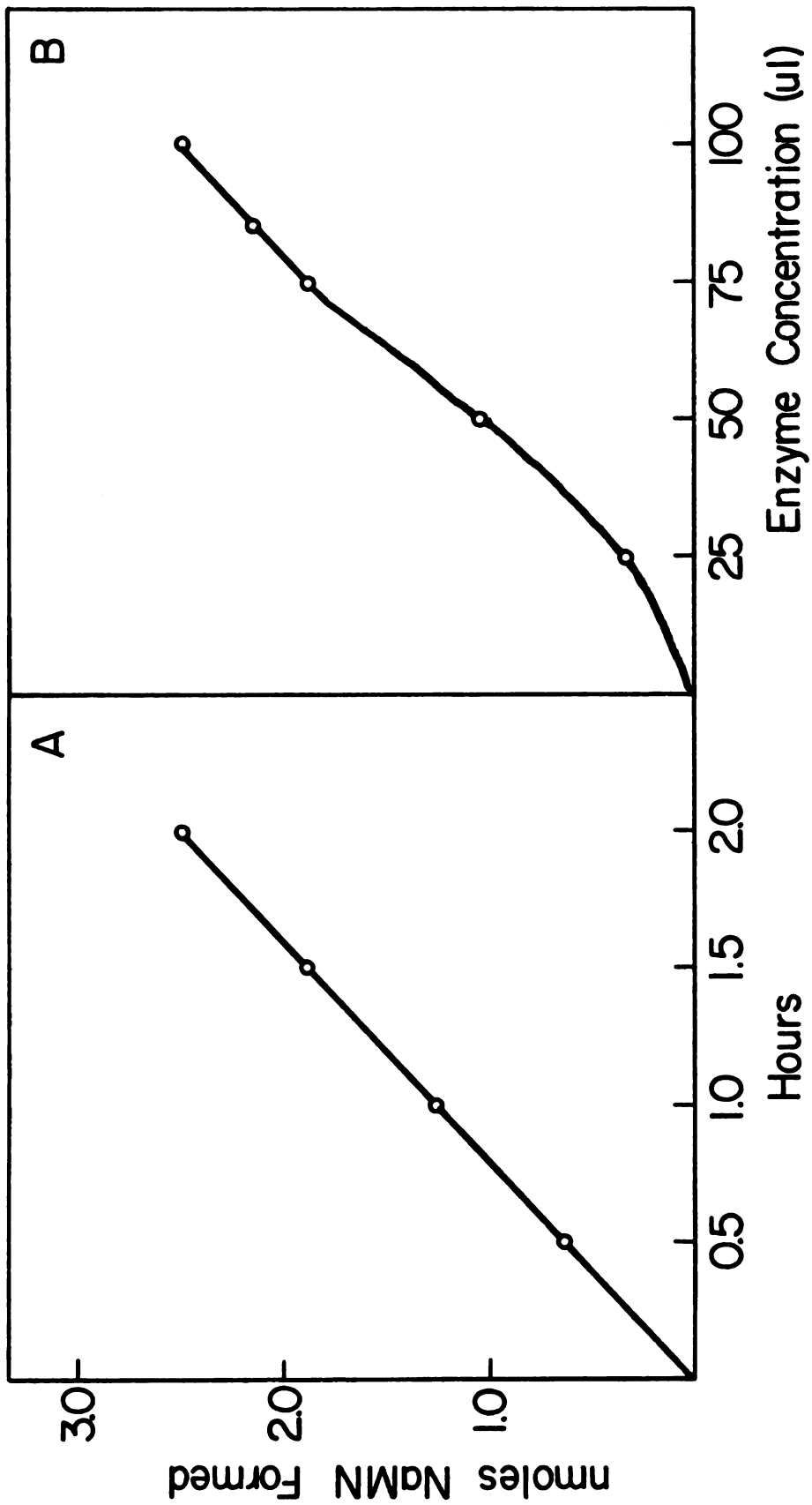
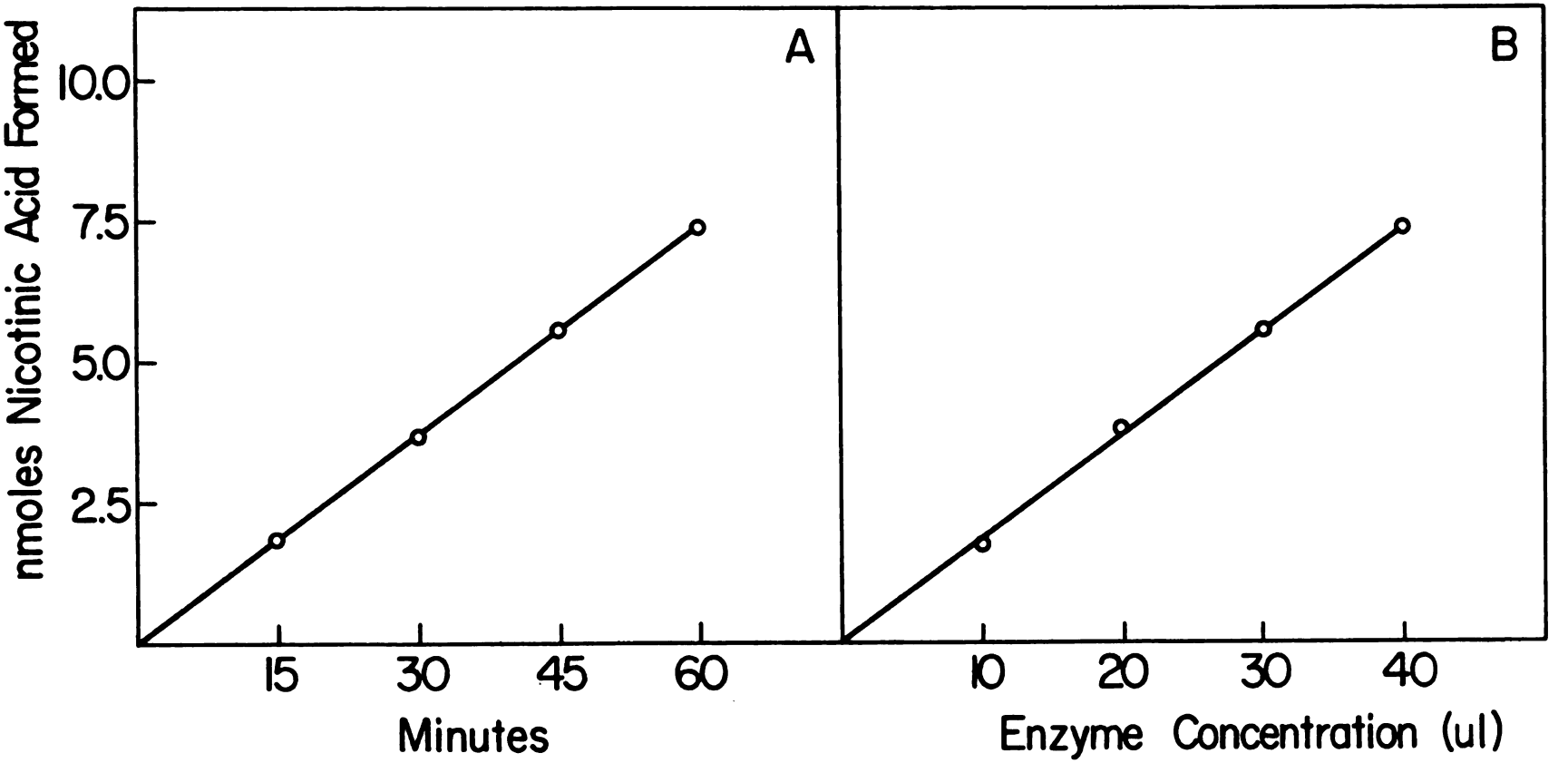

A
B
nmoles NaMN Formed
3.0
2.0
1.0
0.5
1.0
1.5
2.0
Hours
25
50
75
100
Enzyme Concentration (ul)

Figure 22.--Deamidation of nicotinamide by nicotinamidase as a function of time and crude castor bean homogenate. The enzyme was assayed as described in the methods section using a crude castor bean homogenate as the enzyme source. A. Each assay contained 40 µl enzyme preparation. The reaction was stopped by placing the reaction flask in a boiling water bath for two min at the appropriate time. B. The assays contained the enzyme preparation in the amounts indicated. After one hr the reaction was stopped by placing the reaction flask in a boiling water bath for two min.


nmoles Nicotinic Acid Formed
10.0
7.5
5.0
2.5
A
15
30
45
60
Minutes
B
10
20
30
40
Enzyme Concentration (ul)

nicotinamide (3.52 μCi/μmole), 4.8 mM ATP, 8 mM $MgCl_2$, 76 mM potassium phosphate (pH 7.0), and 8 mM 2-mercaptoethanol in a total volume of 3 ml. (It was later shown that Mg^{2+} and ATP were not necessary for nicotinamidase activity.) After 4 hr, the reaction was stopped by heating the reaction flask for 3 min in a boiling water bath. The deproteinized solution was applied to a Dowex 1X2 (200-400 mesh) formate column (1 cm i.d. x 40 cm) and eluted with the same gradient as described in the legend of Figure 12. The nicotinic acid was well separated from nicotinamide, which did not adhere to the column. The fractions containing nicotinic acid were concentrated on a rotary evaporator and an aliquot was co-chromatographed with authentic nicotinic acid in solvent C. The radioactive material isolated from the column migrated to a spot having an R_f identical to that of the authentic nicotinic acid. After adding non-radioactive nicotinic acid to the isolated product, it was recrystallized to constant specific activity as seen in Table 10.*

*The product was identified as nicotinic acid by Anne Bosch, a 1971 summer NSF student.

TABLE 10.--Recrystallization of nicotinic acid to constant specific activity. The isolated [7-^{14}C] nicotinic acid from the Dowex 1X2 formate column was added to 200 mg nonradioactive authentic nicotinic acid, dissolved in water, and recrystallized four times, as reported below. A few crystals were dissolved in water and diluted to 5 ml with water each time. Two 0.5 ml aliquots of this material were counted in scintillation fluid C; 0.5 ml aliquots were diluted to 10 ml with water and the absorbance was determined at 260 nm with a Hitachi spectrophotometer.

Crystallization	cpm per 0.5 ml	Absorbance$_{260\ nm}$ of a 1:20 Dilution	Ratio Abs/cpm
#1	105	.58	5.5×10^{-3}
#2	106	.61; .61[a]	5.7×10^{-3}
#3	162	.91; .92[a]	5.6×10^{-3}
#4	85	.48	5.6×10^{-3}

[a]A reading from a duplicate dilution.

The Level of Quinolinic Acid and Nicotinic Acid Phosphoribosyltransferases in Developing and Mature Castor Bean Plants

In Figure 23 is shown the level of quinolinic acid and nicotinic acid phosphoribosyltransferases in both the endosperm and the cotyledons of etiolated castor bean seedlings at different stages of development. The corresponding visible physiological changes which occurred during seedling development are depicted in Table 11. The specific activity of quinolinic acid phosphoribosyltransferase in the endosperm increased dramatically during days three and four with a gradual leveling off at day five. The rapid drop in activity on days six and seven probably reflected the general wasting away of the endosperm tissue, as shown in Figure 24. The peak level of quinolinic acid phosphoribosyltransferase in the endosperm was a little less than three-fold higher than the peak level of the enzyme in the cotyledons. The specific activity of nicotinic acid phosphoribosyltransferase in the endosperm remained approximately constant during days two, three, and four, but started to decrease on day five. The level of this enzyme in the endosperm was approximately equal to its level in the cotyledons. Quinolinic acid phosphoribosyltransferase activity was 100-fold higher and 12-fold higher than nicotinic acid

Figure 23.--The specific activity of nicotinic acid phosphoribosyltransferase (A) and quinolinic acid phosphoribosyltransferase (B) in the endosperm and cotyledons of germinating castor bean seedlings. The seedlings were selected for their physiological age rather than their chronological age. Any one flat contained a random population of seedlings, which, although they were planted at the same time, did not germinate at the same rate. Table 11 gives a descriptive daily characterization of the castor bean germination. The 27,000xg supernatant was obtained as described in the section on enzyme isolation and was used to assay these two enzymes. Three to four plants were used for each series of determinations. The cotyledons were not separated from the endosperm for the enzyme preparations made on days two and three.

(o——o) Endosperm

(●——●) Cotyledon

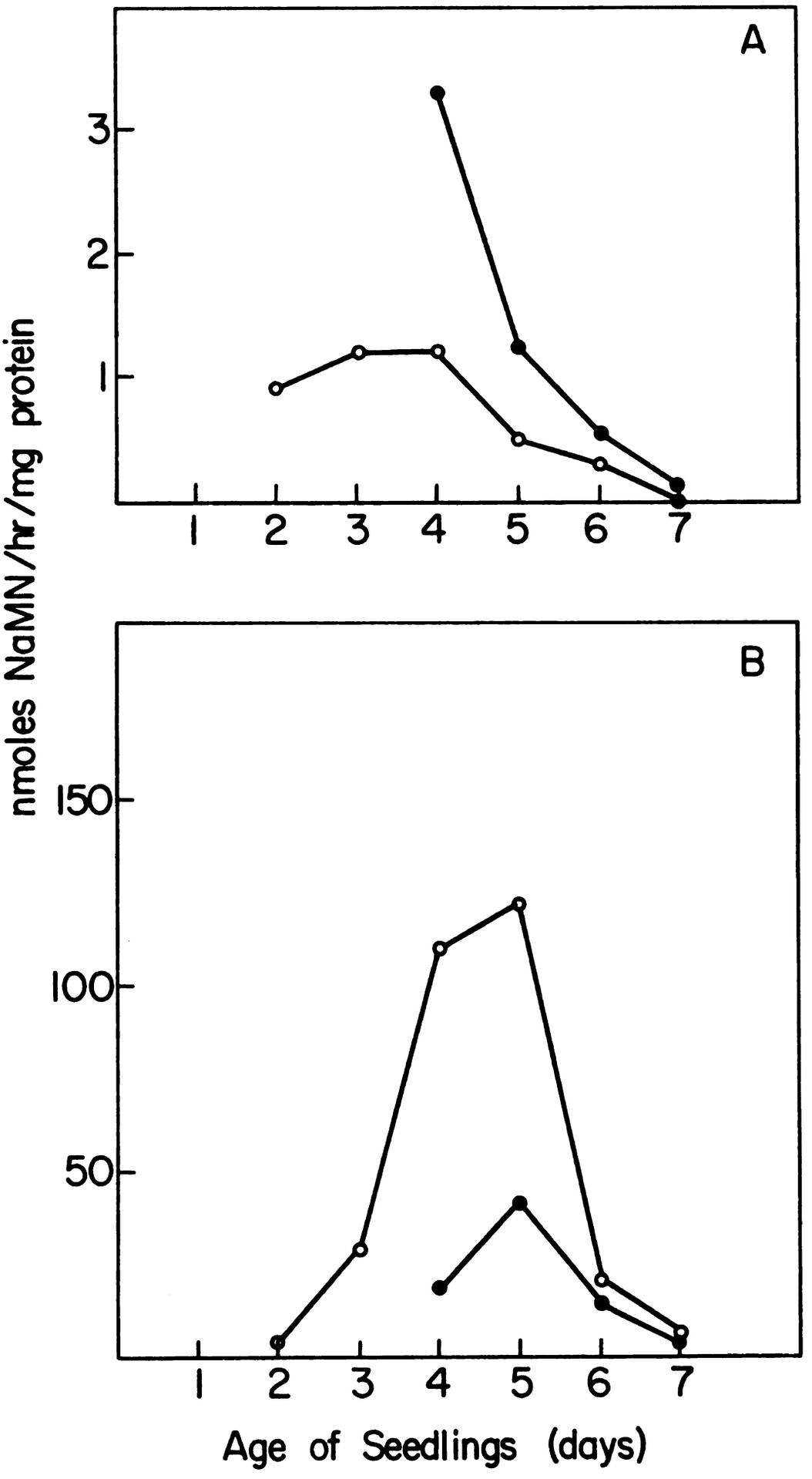

A
B
3
2
1
150
100
50
1
2
3
4
5
6
7
nmoles NaMN/hr/mg protein
Age of Seedlings (days)

TABLE 11.--Physiological development of the etiolated castor bean plant grown in moist vermiculite at 30°C. Moisture and temperature were very important factors in castor bean development. Castor beans germinated at 28°C instead of 30°C required two to three days longer to develop.

Day	Observations
2	Seed coat had just started to crack open.
3	The radicle was from 0.3 to 1.0 cm long.
4	The radicle was from 1.5 to 4.0 cm long with secondary roots.
5	The radicle was from 5.0 to 9.0 cm long and the hypocotyl was from 1.0 to 2.0 cm long. The secondary roots were well developed.
6	The hypocotyl was from 3.8 to 5.2 cm long with an average of 4.5 cm. At this point, the endosperm was starting to liquify.
7	The average length of the hypocotyl was 6.7 cm. The endosperm was transparent and slimy and had been nearly consumed.

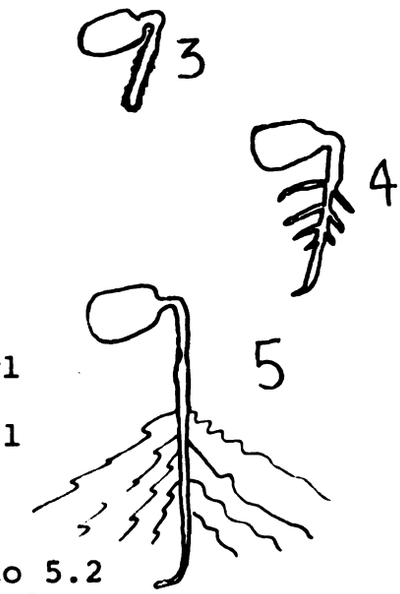
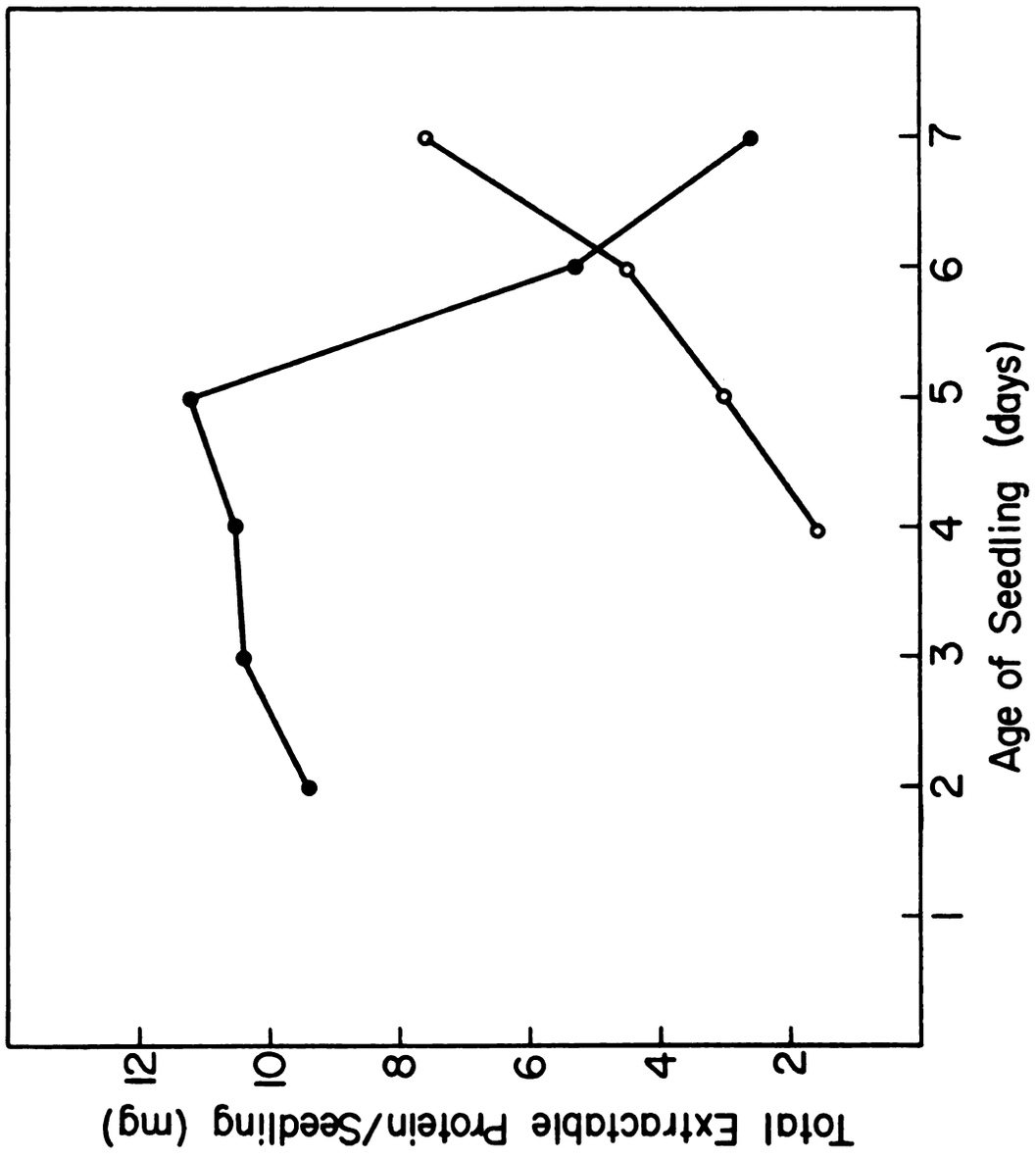


Figure 24.--Total soluble protein of the cotyledons and endosperm of castor bean seedlings. The protein was determined by the method of Lowry et al (76) and was based on the amount of protein present in the enzyme extract of either four endosperm (●——●) or four cotyledons (o——o) all at the same stage of development.


Total Extractable Protein/Seedling (mg)
2
4
6
8
10
12
1
2
3
4
5
6
7
Age of Seedling (days)

phosphoribosyltransferase activity in the endosperm and cotyledons respectively.

These data demonstrated the high specific activity of quinolinic acid phosphoribosyltransferase in the developing castor bean endosperm and cotyledons. The day-by-day increase in the activity of this enzyme did not directly correlate with the increase in ricinine biosynthesis observed. There was a lag period between the peak of quinolinic acid phosphoribosyltransferase activity in the endosperm and the commencement of rapid alkaloid biosynthesis. However, the enzyme activity in the cotyledons peaked at the onset of ricinine biosynthesis but dropped off while the alkaloid was still being produced.

The cotyledons must play an important role in the actual synthesis of ricinine, as the endosperm was almost completely degenerated by days six and seven, when the level of the alkaloid was increasing. Figure 24 depicts the decrease in protein in the endosperm with the concomitant increase in protein in the cotyledon just at the time of ricinine synthesis.

In the green plant the levels of quinolinic acid phosphoribosyltransferase were also consistently higher than the levels of nicotinic acid phosphoribosyltransferase (Table 12). However, the levels of quinolinic acid phosphoribosyltransferase were quite low in

TABLE 12.--The specific activity of quinolinic acid phosphoribosyltransferase, nicotinic acid phosphoribosyltransferase, and nicotinamidase in green castor bean plants. The plants were grown in the greenhouse using natural lighting. The age was based on the date at which the plants broke through the ground. All of the leaves from two plants were used to prepare the plant homogenate except in the case of the 12-week-old plants where only two leaves were used. The enzymes were assayed as described in the methods section.

Enzyme	Enzyme Activity nmoles product/hr/mg protein				
	Week				
	1	2	3	4	12
Quinolinic Acid Phosphoribosyltransferase[a]	1.9	1.5	0.7	0.8	0.4
Nicotinic Acid Phosphoribosyltransferase[a]	1.1	1.0	0.1	0.1	0.1
Nicotinamidase[b]	26.5	16.8	40.5	32.0	24.2

[a]Values reported are for nicotinic acid mononucleotide.

[b]Values reported are for nicotinic acid.

comparison to the peak levels in the etiolated seedlings. There did not appear to be any inhibitor of quinolinic acid phosphoribosyltransferase in the green castor plant, since no inhibition of enzyme activity resulted when extracts from the green plant and seedling were mixed. Those values obtained for nicotinic acid phosphoribosyltransferase in the young green plant were about the same as found in the seedling. After a small initial drop in enzyme activity, the level of the two enzymes remained fairly constant during a three-month period. The level of nicotinamidase was nearly constant for the entire three-month period. These results correlated well with those of Waller and Henderson (57), who found that nicotinic acid and nicotinamide incorporation into ricinine was more efficient in younger plants.

The levels of quinolinic acid and nicotinic acid phosphoribosyltransferases as well as nicotinamidase in the levels of soybeans and tobacco are given in Table 13. These levels seemed typical in comparison with other plants not reported here. Corn, spinach, pigweed, sunflower, and wheat were surveyed for quinolinic acid phosphoribosyltransferase using the less sensitive $[^{14}C]$ carbon dioxide assay. The $[^{14}C]$ values obtained were not more than twice the background values. This meant that less than 3 to 4 nmoles nicotinic acid

TABLE 13.--The specific activity of quinolinic acid phosphoribosyltransferase, nicotinic acid phosphoribosyltransferase, and nicotinamidase in soybeans and tobacco. The plant material was ground with two volumes of 0.05 M potassium phosphate (pH 7.0) containing 0.01 M dithioerythritol and strained through 4 layers of cheese cloth. The 27,000xg supernatant was used as the enzyme source in the assays described in the methods section.

Enzyme	Enzyme Activity nmoles product/hr/mg protein	
	Soybeans	Tobacco
Quinolinic Acid Phosphoribosyltransferase[b]	0.3	0.6[a]
Nicotinic Acid Phosphoribosyltransferase[b]	3.1	0.4
Nicotinamidase[c]	5.2	2.7

[a]This value may have been depressed from what is normally found *in vivo* (see text).

[b]Values reported are for nicotinic acid mononucleotide.

[c]Values reported are for nicotinic acid.

mononucleotide were formed per hr, a rate which was 150-fold less than that observed in castor bean seedlings.

Since the de novo pathway for NAD biosynthesis was found to be more active in the castor bean plant, especially at the point when ricinine is being synthesized, one might also expect the de novo pathway to be more active in other plant tissues which synthesize excess pyridine derivatives. To test this hypothesis, the tobacco plant, Nicotiana rustica L., which synthesizes the pyridine alkaloid nicotine, was examined. Dawson et al (98) demonstrated that nicotine was synthesized in the roots and constituted up to 5% of the dry weight of Nicotiana rustica. The peak period of nicotine synthesis was at or just prior to flowering.

The leaves and regenerated roots of tobacco plants were assayed for quinolinic acid and nicotinic acid phosphoribosyltransferases (Table 14). The roots were regenerated by the method of Byerrum et al (43) and the leaves were taken from potted tobacco plants. In the roots the level of quinolinic acid phosphoribosyltransferase was approximately 40 times higher than nicotinic acid phosphoribosyltransferase.

To determine whether the enzyme in the leaf was being inactivated by the large amounts of chlorogenic acid present in tobacco or by some other

TABLE 14.--The specific activity of quinolinic acid phosphoribosyltransferase, nicotinic acid phosphoribosyltransferase, and nicotinamidase in leaves and roots of tobacco plants. The plant material was ground with two volumes of 0.05 M potassium phosphate (pH 7.0) containing 0.01 M dithioerythritol and strained through 4 layers of cheese cloth. The 27,000xg supernatant was used as the enzyme source in the assays described in the methods section.

Enzyme	Enzyme Activity nmoles product/hr/mg protein		
	Root	Leaf	Root and Leaf
Quinolinic Acid Phosphoribosyltransferase[a]	23.9	0.6	7.4
Nicotinic Acid Phosphoribosyltransferase[a]	0.6	0.4	0.5
Nicotinamidase[b]	13.5	2.7	13.3

[a]Values reported are for nicotinic acid mononucleotide.

[b]Values reported are for nicotinic acid.

inhibitors, tobacco leaves were ground in a mortar using the 27,000xg supernatant of the root extract as the grinding buffer. Table 14 shows that nicotinamidase and nicotinic acid phosphoribosyltransferase were not inhibited at all, whereas the quinolinic acid phosphoribosyltransferase was inhibited 70% when the extracts were mixed in this manner. Nevertheless, these results did show that the de novo pyridine nucleotide pathway as represented by quinolinic acid phosphoribosyltransferase was more active in tobacco than in other plants tested.

There are two other plants which should be investigated. One of the plants, located in the southwestern United States, is Aplopappus hartwegi, which contains free pyridine as 2% of its dry weight; the other, located in Australia, is Duboisia hopwoodi, which contains nicotine as 10% of its dry weight.

DISCUSSION

Quinolinic acid phosphoribosyltransferase is noted for its low level of activity found in the various organisms which have been studied. Gholson et al (47) abandoned their first attempts at purification of the enzyme from rat liver in favor of beef liver, which had approximately three times as much activity in the crude homogenate (0.83 nmoles/hr/mg protein). The enzyme was also of limited distribution, being found only in the liver and kidney of the rat and mouse (40). Packman and Jakoby (49) sought to avoid these problems and isolated, by enrichment culture technique, a pseudomonad that used quinolinic acid as its sole carbon source. The pseudomonad enzyme had an initial specific activity of 4.8 μmoles/hr/mg protein, which was 5,800 fold higher than that found in bovine liver. The pseudomonad enzyme was purified eight-fold to give a crystalline enzyme.

Plants in general have much lower levels of quinolinic acid phosphoribosyltransferase than animals. Etiolated castor bean endosperm was unique, since it

had relatively high levels of this enzyme. The crude extract had an initial specific activity of 1.5 nmoles/hr/mg protein, which is two fold higher than the initial specific activity found in bovine liver. In comparison to other plants, the level of quinolinic acid phosphoribosyltransferase in castor bean endosperm was shown to be approximately 150 times greater than the level recorded in most plants. It is paradoxical that plants have such low levels of this enzyme, since they depend upon a de novo supply of NAD rather than on the vitamin form. On a gram-per-gram basis, the NAD levels of plants are in the nmolar range whereas they are in the μmolar range in mammalian livers (62, 99). In addition to the fact that no previous study has been done on the plant enzyme, the plant enzyme is also of interest because of the possible role of the de novo pathway in NAD biosynthesis and pyridine alkaloid biosynthesis.

Quinolinic acid phosphoribosyltransferase was purified approximately 500-fold from etiolated castor bean endosperm. The final specific activity of the enzyme is compared below to the specific activity of the enzyme from a pseudomonad and from bovine liver.

The enzyme isolated from castor bean endosperm appeared to be fairly stable when stored in the presence of 50% sucrose (w/v) and 10 mM dithioerythritol at

Source	Fold Purification	Specific Activity[a]	Conditions
Castor Bean Endosperm	500	0.75	pH 7.0, P_i[b], 30°C
Pseudomonad (50)	8	0.88	pH 7.0, P_i, 23°C
Bovine Liver (47)	1500	0.073	pH 6.0, P_i, 37°C

[a] μmoles Product/min/mg Protein

[b] Potassium Phosphate Buffer

-90°C and at 4°C. The enzyme lost part of its activity when the dithioerythritol was omitted. One-third of this activity lost over a period of one month, could be restored by the addition of 10 mM dithioerythritol. Sucrose apparently stabilizes the protein conformation since 50% sucrose protected the enzyme from thermal inactivation (i.e., high as well as low temperatures). When the pseudomonad enzyme was stored for 9 months at -100°C in 0.05 M potassium phosphate (pH 7.0) containing 5 mM glutathione, it retained 90% of its activity. Hayaishi et al (40) stated that the bovine liver enzyme was stable when stored at -20°C for a month. Neither group working with the bovine liver enzyme reported having used a sulfhydral protective agent.

Purity

The enzyme was judged to be pure on the basis of SDS-disk gel electrophoresis. However, disk gel

electrophoresis of the native enzyme resulted in three closely spaced bands, at least two of which appeared to have enzymatic activity. It is not known whether the three bands of protein observed on analytical disk gels were an artifact of the purification procedure or whether the three forms possibly existed in vivo. Several possible explanations for multiple forms of the enzyme are explored here.

A portion of the enzyme might tightly bind some of the quinolinic acid which was used in the first step of the purification scheme. Since the enzyme was shown to have two subunits and therefore possibly two active sites per molecule, it might be that band 1 (slowest migration) was devoid of substrate, band 2 (intermediate migration) contained 1 mole of quinolinic acid per mole of enzyme, and band 3 (fastest migration) contained 2 moles of quinolinic acid per mole of enzyme (Figure 6 B). Such substrate binding could be checked by using $[^{14}C]$ labeled quinolinic acid as the protective agent during the heating step. A normal enzyme preparation usually yielded 1 mg protein, which represented approximately 14 nmoles enzyme, based on an assumed molecular weight of 70,000. Therefore, quinolinic acid of very high specific activity would have to be used.

The possibility of the enzyme's existence in dimer and trimer states of aggregation was eliminated

by the results of the Ferguson plots (87). If the enzyme were present in different aggregated states, the slopes of the curves obtained by plotting the log of the R_f of the different protein bands versus the percentage acrylamide would be different. This was not the case. The slopes of the Ferguson plots were parallel, which meant that the three protein species were of identical molecular weight and differed only in charge. These results confirmed those obtained with the SDS-disk gel electrophoresis, which indicated that the three proteins were composed of two subunits of identical molecular weight.

The observed difference in charge might be attributed to subunits differing in charge which dissociated and then reassociated during the purification to give the three possible combinations. The difference in charge must have been very small, as it was not possible to separate the multiple forms of the enzyme with the isoelectric focusing column, even when the narrow range of ampholytes was used.

The possibility also exists that the enzyme was present in three forms (isoenzymes) <u>in vivo</u>. Although it is in the realm of speculation, isoenzymes could possibly account for the abnormally high levels of quinolinic acid phosphoribosyltransferase present in etiolated seedlings. However, we have no data to support this possibility.

Biochemical Properties

Nicotinic acid mononucleotide formation was dependent upon Mg^{2+} as well as quinolinic acid and PRPP. Equimolar (12.5 μM) Mn^{2+} could partially replace the Mg^{2+} requirement (28% of normal), however Ca^{2+} and Ba^{2+} were completely inefficient. When Mn^{2+} was added along with Mg^{2+} the reaction was inhibited 41% and 67% at 10^{-3} and 10^{-2} M Mn^{2+}, respectively. The Ca^{2+} and Ba^{2+} salts had no effect. Hayaishi et al (40) reported that the bovine liver enzyme was completely inhibited at 10^{-3} M by Ca^{2+}, Ba^{2+}, and Mn^{2+}.

Many of the heavy divalent metal ions very characteristically inhibited quinolinic acid phosphoribosyltransferase activity. The bovine liver enzyme purified by Hayaishi et al (40) was inhibited 40% to 70% by Zn^{2+} or Fe^{2+} at 10^{-2} M, whereas the enzyme from castor bean seedlings was completely inhibited at 10^{-3} M by these two metals. In general the castor bean enzyme seemed to be much more sensitive to heavy metals than was the case for the bovine liver enzyme (40).

Quinolinic acid phosphoribosyltransferase isolated from castor bean endosperm was just as active in tris·HCl buffer as it was in potassium phosphate buffer. The enzyme was not at all affected by the addition of K^+, Li^+, Na^+, or NH_4^+. Gholson et al (47) reported that the bovine liver enzyme retained only 20% of its normal

activity in tris·HCl buffer compared to its activity in potassium phosphate buffer. They also found that part of the activity, as given in the parenthesis, could be recovered by adding back K^+ (25%), Li^+ (19%), and NH_4^+ (40%) at 2×10^{-2} M. Packman and Jakoby (50) found that the pseudomonad enzyme irreversibly lost all its activity when the 0.05 M potassium phosphate (pH 7.1) containing 5 mM glutathione was replaced with a similar tris·HCl buffer; the activity was not regained when the phosphate buffer was added back. They did not state, however, the reason for the loss of activity, i.e., whether it was due to the missing phosphate, potassium, or a combination thereof.

pH Optimum

The pH optimum of the castor bean enzyme was rather broad, with 50% of maximal activity reached at pH 5.5 and 9.4. This was in contrast to the sharp optima reported for the other two enzymes: 6.2 for the bovine liver enzyme (47) and 7.1 for the pseudomonad enzyme (50). Nicotinic acid phosphoribosyltransferase and NAD synthetase, both from yeast (95, 96), had pH profiles similar to that of the castor bean quinolinic acid phosphoribosyltransferase.

Protection Against Heat Inactivation

The enzyme from castor beans was protected from heat inactivation by just one of its substrates,

quinolinic acid. This fact was used to advantage in the purification scheme. It is believed that the protection was due to the binding of the quinolinic acid to the enzyme, although it could not be definitely said if the binding were at the kinetically active site. It is not known why PRPP failed to protect the enzyme from heat inactivation. However, this might reflect the enzyme's preferential binding of quinolinic acid compared to PRPP.

Inhibitor Studies

Inhibitor studies with a series of quinolinic acid analogues revealed several compounds which might prove useful in physiological studies where it is of advantage to block the de novo pathway for NAD biosynthesis. These studies also provide some clues regarding the degree of recognition by the enzyme for certain substituents on the pyridine ring. Because the activity of quinolinic acid phosphoribosyltransferase was not inhibited by compounds possessing a substituent (-COOH, $-CONH_2$, -CN) on the 3 position of the pyridine ring yet was inhibited by analogues having an additional carboxyl group at the 2 position, it was suggested that a carboxyl group at the 2 position might play a role in binding at the active site. This proposition was supported by two other lines of evidence. If the carboxyl group in the 2 position of the above mentioned compounds was methylated, then their effectiveness as inhibitors was

reduced four- to eight-fold at 10^{-2} M. Picolinic acid, which is the 2 carboxylic acid of pyridine, was one of the most effective inhibitors tried. The enzyme seemed to prefer a carboxyl group over a carboxyamide group over a cyano group at position 3. From this data it would appear that the enzyme recognized both carboxyl groups of quinolinic acid, but that the carboxyl group at the 2 position was more important in the recognition process.

Several reported inhibitors of quinolinic acid phosphoribosyltransferase in other systems proved to be ineffective with the purified enzyme from castor bean endosperm. The enzyme would appear to be a potential control point in NAD biosynthesis as it forms the same product as nicotinic acid phosphoribosyltransferase, one of the enzymes of the salvage cycle. The bovine liver enzyme reported by Gholson et al (47) was inhibited by ATP at 1×10^{-3} M; however, no inhibition was observed with the castor bean enzyme. In fact, the report of ATP inhibition was of dubious significance, as such inhibition could be merely a chelation of Mg^{+2} (2×10^{-4}) by ATP. No attempt to see if higher levels of Mg^{2+} could overcome the inhibition was reported. NAD was found to be slightly inhibitory (19% at 10^{-3} M) but not to the extent that it could function as an in vivo regulator of the activity of the castor bean

endosperm quinolinic acid phosphoribosyltransferase activity. Ricinine, which was reported to inhibit the pyridine nucleotide cycle and its own biosynthesis (69), had no effect on the quinolinic acid phosphoribosyltransferase activity.

Kinetics

The non-parallel character of the double reciprocal plots of the initial velocity of the enzyme reaction indicated that a ternary complex among enzyme, quinolinic acid, and PRPP was formed. This was in agreement with those results obtained by Packman and Jakoby (50) with the enzyme from the pseudomonad. This initial data set quinolinic acid phosphoribosyltransferase apart from those phosphoribosyltransferases such as adenine phosphoribosyltransferase which has the so-called "ping-pong" mechanism (100).

Physical Characteristics

The molecular weight of castor bean endosperm quinolinic acid phosphoribosyltransferase was estimated by three methods with generally good agreement. The molecular weight estimates are listed below.

Method	Molecular Weight Estimate
Sucrose Density Centrifugation	72,000
Sephadex G-200 Chromatography	68,000
SDS-disk Gel Electrophoresis	35,000

The results of the SDS-disk gel electrophoresis experiment suggested that the enzyme was composed of two subunits of identical molecular weight. These results were very different from those obtained with the crystalline pseudomonad enzyme. After high-speed equilibrium centrifugation studies, the molecular weight of that enzyme was calculated to be 165,000 which was more than twice the molecular weight estimated for the enzyme from castor bean endosperm. After the pseudomonad enzyme was dissociated with 4.8 M guanidine hydrochloride at pH 7.0, the molecular weight of the subunits was estimated at 54,000 by low-speed equilibrium centrifugation studies. Thus it apparently differed in subunit size and composition from castor bean quinolinic acid phosphoribosyltransferase. The molecular weight of the bovine liver enzyme was not determined.

Physiological Characteristics

To date the accumulated evidence associating the pyridine nucleotide cycle with pyridine alkaloid biosynthesis is based on *in vivo* feeding experiments using $[^{14}C]$ labeled quinolinic acid, nicotinic acid, and nicotinamide (57, 14). A definite connection between the pyridine nucleotide cycle and ricinine and nicotine biosynthesis awaits the day when their exact biosynthetic pathways are elucidated. The research presented here

points to a connection between an elevated enzymatic activity for de novo synthesis of NAD and pyridine alkaloid biosynthesis.

A similar phenomenon has been observed with human tubercle bacilli which excrete large amounts of nicotinic acid in culture. Konno, Oizumi, and Oka (97) demonstrated that the amount of nicotinic acid produced in the culture filtrate of this and eight other species of mycobacteria was proportional to the activity of the quinolinic acid phosphoribosyltransferase found in the bacteria.

Using quinolinic acid phosphoribosyltransferase as an indicator of the level of the de novo pathway for NAD biosynthesis in castor bean plants, it appears that the level of the de novo pathway is elevated at a point in development which corresponds fairly well with a large increase in ricinine biosynthesis. This, of course, is not proof of a relationship between the pyridine nucleotide cycle and ricinine biosynthesis. However, it could account for the large rapid formation of ricinine observed in the seedling. If quinolinic acid phosphoribosyltransferase were operating at half maximal velocity on days 3, 4, and 5 of plant germination it would form approximately 3, 13, and 14 μmoles of product respectively. This rate of synthesis would be sufficient to supply the 20 μmoles of precursor

needed to form ricinine in the three-day period beginning on the fifth day in these experiments. The in vivo levels of quinolinic acid are not known in the castor bean; however, unpublished data from our laboratory indicated that the levels of PRPP were sufficient to account for the speculated rate of nicotinic acid mononucleotide biosynthesis.

In the case of the castor bean plants used in these experiments, there was a lag between the highest levels of de novo activity and the peak production of ricinine. De novo activity started to increase on day 3 whereas ricinine biosynthesis did not start to increase until two days later. The lag period in ricinine biosynthesis may be an artifact induced by a nutrient deficiency. Waller (91) obtained rapid ricinine biosynthesis on day 3 when he used tap water to germinate the castor seeds. The use of nutrient solution or distilled water gave the same results as reported here. If ricinine formation started on day 3 under field conditions, it would correspond to the rise in quinolinic acid phosphoribosyltransferase activity.

Certain questions must be answered before it can be said that the de novo pathway for NAD biosynthesis is actually the source of precursors for ricinine. In what form is the precursor from NAD biosynthesis stored during the lag period observed here? Are the levels of

the pyridine nucleotides affected by the dramatic fluctuation in the de novo formation of the pyridine nucleus? The level of NAD in 5-day-old castor bean endosperm was found to be similar to that in other seedlings (62). However, NAD levels should be monitored daily during seedling development. Is the observed decrease in the activity of the salvage cycle significant in ricinine biosynthesis? Since the decrease in activity paralleled the increase in ricinine biosynthesis, it would be of interest to see if there was a corresponding build-up of nicotinamide and nicotinic acid. One might also ask if there is any synthesis of ricinine in the endosperm or is all of it synthesized in the cotyledons? In either case, in what form is the pyridine nucleus transported from one tissue to another? Answers to these questions await further investigation and will help put the pyridine nucleotide cycle and ricinine biosynthesis in proper perspective.

Ricinine is formed throughout the life cycle of the castor plant, not just during seedling development. The rate of ricinine synthesis in the adult stages of growth is much lower than in the germinating seedling, however (91, 92). The level of nicotinic acid phosphoribosyltransferase in the seedling and the young green plant were about the same although the levels did decrease later as the plant matured. The quinolinic acid

phosphoribosyltransferase activity was much lower in the green plant than the etiolated seedling. However, it was always greater than the activity of the nicotinic acid phosphoribosyltransferase. These observations may help explain the results of Waller and Henderson (57), who found that nicotinamide, and nicotinic acid were more easily incorporated into ricinine with young seedlings and that the percentage of incorporation decreased with the age of the plant. Waller et al (14) later found that quinolinic acid was twice as efficient as nicotinic acid or nicotinamide as a precursor of ricinine in castor bean seedlings. The level of incorporation of these various precursors (nicotinic acid, nicotinamide, and quinolinic acid) into ricinine correlated well with the level of the enzymes responsible for the first step in their metabolism. Therefore, an assessment of the level of quinolinic acid and nicotinic acid phosphoribosyltransferase at the time of the in vivo feeding experiment would predict the relative degree of incorporation of the respective precursor into ricinine.

It is apparent from the studies with tobacco roots that this pyridine alkaloid-producing plant also had high levels of quinolinic acid phosphoribosyltransferase. This correlation also lends support to the hypothesis that the pyridine nucleotide cycle is involved in the synthesis of nicotine. Such a

proposition is not that surprising because if the pyridine nucleotide cycle intermediates are used in alkaloid biosynthesis, then one would expect that more pyridine nucleotides would have to be synthesized in order to replace the intermediates removed from the cycle.

Since the activity of the de novo pathway for NAD biosynthesis was elevated during pyridine alkaloid biosynthesis, one wonders what originally caused the elevation. Alkaloid formation may merely depress pyridine nucleotide levels causing a needed increase in pyridine nucleotide biosynthesis. If, however, the rate of NAD biosynthesis increased and consequently, if the levels of nicotinamide or nicotinic acid were elevated, one might invoke alkaloid formation as a means of detoxification. Trigonelline has been postulated as such a detoxification compound (52). Still, an alkaloid such as ricinine appears quite elaborate in structure to be merely a detoxification form of the pyridine ring.

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