ASPECTS OF NAD BIOSYNTHESIS IN CASTOR BEAN ENDOSPERM

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ABSTRACT

ASPECTS OF NICOTINAMIDE ADENINE DINUCLEOTIDE BIOSYNTHESIS IN CASTOR BEAN ENDOSPERM

By

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In addition to trigonelline and nicotinic acid, a third major product, C, was synthesized by an enzyme preparation from etiolated five-day-old castor bean endosperms in the presence of [7-^{14}C] nicotinamide, adenosine 5'-triphosphate, and Mg^{2+}. [7-^{14}C] nicotinic acid served equally well as a substrate for Product C formation. When [8-^{14}C] adenosine 5'-triphosphate and nonradioactive nicotinic acid were incubated with Mg^{2+} and a similar enzyme preparation, a radioactive product identical to Product C was obtained.

Product C was identified as nicotinamide adenine dinucleotide by its elution pattern from a Dowex 1-X2 formate column, by its R_f in three paper chromatographic systems, by its ultraviolet spectrum in the absence and presence of potassium cyanide and in the absence and presence of yeast alcohol dehydrogenase, and by its alkaline hydrolysis to radioactive nicotinamide and nicotinic acid.

Reaction characteristics consistent with this identification were dependence on adenosine 5'-triphosphate and Mg^{2+}, loss of activity after passage of the enzyme preparation through a Sephadex G-25 column,

inhibition by nicotinamide adenine dinucleotide, and stimulation by 5-phosphoribosyl-1-pyrophosphate.

The identification of nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide as reaction products supports the operation of the Preiss-Handler pathway in castor bean endosperm.

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By

Dorothy Boerner Mann

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1973

DEDICATED

to the memory of my father, Ray
to my mother, Rosalie
and to my husband and colleague, David

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Nam	nicotinamide
NA	nicotinic acid
QA	quinolinic acid
NaMN	nicotinic acid mononucleotide
NaAD	nicotinic acid adenine dinucleotide
NAD	nicotinamide adenine dinucleotide (oxidized)
NADP	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMn	nicotinamide mononucleoside
NMN	nicotinamide mononucleotide
PRPP	5-phosphoribosyl-1-pyrophosphate
ATP	adenosine 5'-triphosphate
ADPR	adenosine 5'-diphosphate ribose
FAD	flavin adenine dinucleotide
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
POPOP	1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
BBOT	2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene
Captan	N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide

ADH	yeast alcohol dehydrogenase
UV	ultraviolet
i.d.	internal diameter

LITERATURE REVIEW

Depending on the organism or tissue, the biosynthesis of NAD proceeds by one of three pathways, or by an integration of these pathways, as depicted in Figure 1. The elucidation of these pathways and their identification in various tissues, the preferential utilization of one pathway versus another, and the possible control mechanisms will be discussed.

The discovery by Kornberg (1) in 1948 of NMN adenyltransferase provided some indication of the route by which NAD was synthesized. This enzyme, purified from autolysates of ale yeast and extracts of hog liver acetone powder, catalyzed the reversible synthesis of NAD and PP_i from NMN and ATP; Mg^{2+} was required for activity (2). Since the cleavage of NAD by PP_i resembled phosphorolysis, Kornberg suggested that by analogy the term "pyrophosphorolysis" could be used to describe the reaction. Thus this enzyme was originally named NAD pyrophosphorylase. This terminology was adopted in the earlier papers for naming some of the enzymes involved in NAD biosynthesis, even though the direction of the reaction was not that of pyrophosphorolytic cleavage (3). Experiments with $^{32}P^{32}P_i$ provided evidence in support of the pyrophosphorolytic action of this enzyme: the radioactive ATP formed from NAD and $^{32}P^{32}P_i$ had the same specific activity as the $^{32}P^{32}P_i$; the NAD and NMN recovered were free of radioactivity; and the radioactivity in the ATP was located exclusively in the two terminal

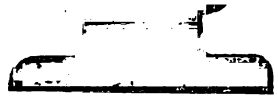
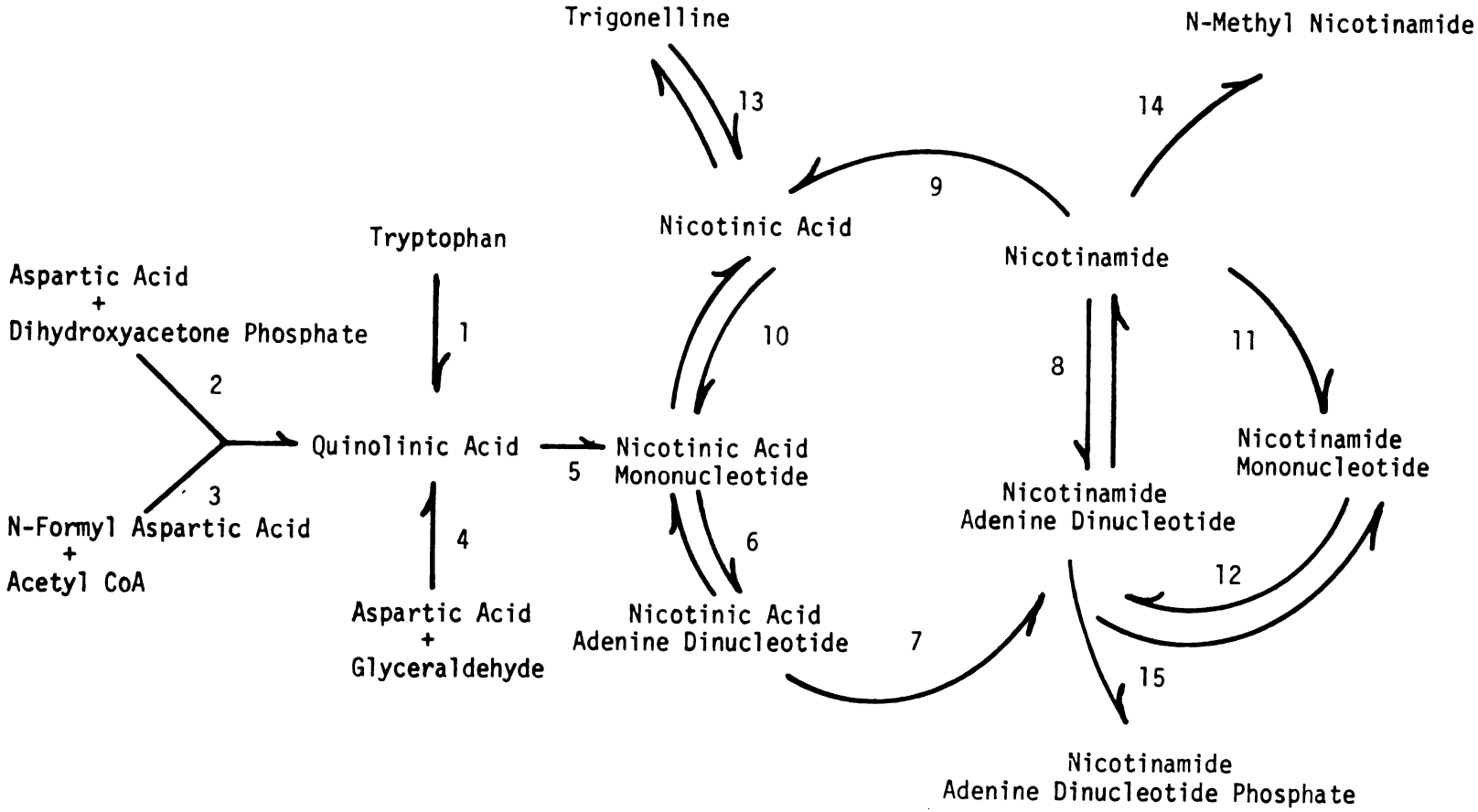


Figure 1. NAD Biosynthesis. Reactions 1, 2, 3, and 4 represent the various de novo routes. Reaction 5 is the common point of entry into the pyridine nucleotide cycle, which includes Reactions 6, 7, 8, 9, and 10. The Preiss-Handler pathway consists of Reactions 10, 6, and 7, whereas the Dietrich pathway involves Reactions 11 and 12.


Trigonelline
N-Methyl Nicotinamide
13
14
9
Nicotinic Acid
Tryptophan
Nicotinamide
Aspartic Acid
+
Dihydroxyacetone Phosphate
1
10
8
11
2
Quinolinic Acid
5
Nicotinic Acid
Mononucleotide
Nicotinamide
Mononucleotide
3
Nicotinamide
Adenine Dinucleotide
N-Formyl Aspartic Acid
+
Acetyl CoA
4
6
12
Aspartic Acid
+
Glyceraldehyde
Nicotinic Acid
Adenine Dinucleotide
7
15
Nicotinamide
Adenine Dinucleotide Phosphate

phosphates (4). Differential centrifugation of mouse liver homogenates resulted in the recovery of 69 to 101 percent of NMN adenyltransferase activity in the nucleus (5). The lower recoveries were obtained under conditions known to damage nuclei. When nuclei were intentionally disrupted by sonic oscillation, most of the enzyme activity remained unsedimented, even after centrifugation at 130,000xg.

Using hemolysates and acetone powder extracts of human erythrocytes, Preiss and Handler (6) established that Nam and PRPP reacted in the presence of Mg^{2+} to form NMN and PP_i; however, the high K_m for Nam, in the order of 0.1 M, temporarily excluded this enzyme, Nam phosphoribosyltransferase (NMN pyrophosphorylase), from the realm of physiological significance. It was later detected in acetone powder extracts of Ehrlich ascites cells from mice, but a high concentration of Nam, 0.1 M, was again required for full activity (7). An enzyme catalyzing the same reaction but with a specific requirement for ATP was isolated by Dietrich et al. (8) from Ehrlich-Lettre ascites cells grown in mice. These workers reported a K_m for Nam of 1.6×10^{-5} M. Attempts to isolate this enzyme with a similar high affinity for Nam from rat liver failed until it was realized that the enzyme was in an inhibited state in the crude homogenate (9). A protamine sufate precipitation step in the purification procedure resulted in a large increase both in specific activity and percent recovery; it was assumed that the protamine sulfate had removed an inhibitor of Nam phosphoribosyltransferase. The purified enzyme exhibited a specific requirement of ATP; its K_m for Nam was 2.96×10^{-6} M. In contrast to NMN adenyltransferase, this enzyme appeared to be restricted to the cytoplasm. Thus one biosynthetic pathway from Nam → NMN → NAD had

been established (Figure 1). To date, detection of the Dietrich pathway has been limited to mammalian systems and bacteria (21,15).

Although Preiss and Handler (6) had observed Nam phosphoribosyltransferase activity in human erythrocytes, there were several indications that a pathway other than the one described above was operative. They had shown that at low concentrations of NA, but not of Nam, isolated erythrocytes could synthesize appreciable amounts of NAD, even though the cells were freely permeable to both substrates (10). The previously mentioned high K_m for Nam exhibited by Nam phosphoribosyltransferase and the very low NMN adenyltransferase activity found in human erythrocytes further suggested an alternate pathway (6). After incubation of human erythrocytes with [^{14}C] NA, glutamine, and glucose, Preiss and Handler (11) were able to detect two NA derivatives besides NAD. In two classic papers they described the identification of these intermediates (12) and the characterization of the enzymatic reactions involved (13). They were able to detect the two intermediates both with defibrinated human erythrocytes and with acetone powder extracts. These compounds, I and II, were identified as NaMN and NaAD respectively by paper electrophoresis, acid and alkaline hydrolysis, as well as a determination of the molar ratio of NA, ribose, and phosphate. In addition, compound II was converted by purified NAD synthetase to NAD at the same rate as synthetic NaAD. Both compounds, I and II, were inactive with alcohol dehydrogenase. An in vivo time course showed that NaAD was formed before NAD in the rat. They concluded that NAD synthesis had proceeded via NA → NaMN → NaAD → NAD (Figure 1).

The enzyme responsible for the first step, NA phosphoribosyltransferase (NaMN pyrophosphorylase) was partially purified from erythrocyte acetone powders and yeast autolysates. It catalyzed the reaction of NA and PRPP to form NaMN and PP_i. The yeast enzyme exhibited an absolute requirement for ATP, even in the presence of PRPP, whereas the erythrocyte enzyme did not. Synthesis of NaAD and PP_i from NaMN and ATP by NaMN adenyltransferase (NaAD pyrophosphorylase) was observed with partially purified enzymes from erythrocyte acetone powders, yeast autolysates, and hog liver. The data indicated that NA phosphoribosyltransferase was an enzyme entirely distinct from Nam phosphoribosyltransferase; however, the close association of NaMN adenyltransferase and NMN adenyltransferase activities during the purification from hog liver as well as similar distribution of the two enzymes in subcellular fractions of rat liver suggested that they were one and the same enzyme catalyzing two analogous reactions. This latter conclusion was confirmed in yeast and E. coli by Dahmen et al. (14), although adenyltransferases specific for NMN or NaMN were later found in Lactobacilli (15). The last step in the scheme proposed by Preiss and Handler was the reaction catalyzed by NAD synthetase, which they partially purified from rat liver and yeast autolysates. Stoichiometric relationships indicated that NaAD, ATP, and glutamine had reacted to form NAD, AMP, PP_i, and glutamate. The Preiss-Handler pathway has been demonstrated in microorganisms (16,17), plants (18), and other mammalian tissues (20-22).

It had been shown earlier by Porcellati (23) that homogenates of rat kidney, liver, brain, heart, intestine, and muscle could synthesize Nam from NA in the presence of ATP. Preiss and Handler (12), too,

obtained [^{14}C] Nam from [^{14}C] NA in their *in vivo* studies with rats; the labeled Nam did not appear, however, until two hours after the [^{14}C] NA administration. They felt that the Nam had originated from NAD degradation. Further light on the mechanism involved was shed by the work of Langan *et al.* (24), who isolated and identified [^{14}C] NaAD from the livers of mice given injections of [^{14}C] Nam. These results suggested that Nam had been hydrolyzed to NA before it became available for NAD synthesis. A cyclic scheme for the degradation and resynthesis of NAD was first proposed by Sarma *et al.* (19). Their scheme incorporated two possible routes of NAD degradation. The one involved breakdown of NAD to NMN and then to free Nam, followed by deamidation to NA, which could then be reconverted to NAD. The other degradative pathway was implicated by their demonstration of the enzymatic deamidation of NMN by mouse liver homogenates which did not possess any Nam deamidase activity. Thus NaMN could be formed directly from NMN, a process involving one enzyme reaction instead of three. This latter more economical cycle was also very recently proposed by Friedmann and Garstki (25), who partially purified a specific NMN deamidase from *Propionibacterium shermanii*. Joshi and Handler (26) are also credited with having proposed a salvage pathway for reutilization of Nam which arose from NAD. The degradative route in their scheme was the action of NAD glycohydrolase to yield Nam directly from NAD.

The enzymatic reactions responsible for the two types of degradation to Nam had been detected much earlier. Direct degradation to Nam was first observed in 1942 by Handler and Klein (27), who recovered Nam quantitatively from NAD destruction in brain, liver,

kidney, and muscle preparations from rabbits, rats, and dogs. Kornberg and Lindberg (28) later observed the direct degradation to Nam as well as degradation to Nam via NMN. They cited the cleavage of NAD at the pyrophosphate bond by NAD pyrophosphatase as the predominant mechanism in rabbit kidney; however, they attributed the splitting of NAD in rabbit brain to the activity of NAD glycohydrolase, which cleaves at the glycosidic bond between Nam and ADPR. Both the glycohydrolase and the pyrophosphatase have also been detected in plants (29,30) and microorganisms (31,32).

The enzyme that completed (or initiated) the proposed pyridine nucleotide cycle, Nam deamidase, was first observed by Hughes and Williamson (33) in Lactobacillus arabinosus. Investigators were able to demonstrate Nam deamidase activity in other microorganisms (26,34), plants (35,36), and birds (19), but not in mammals. In addition, Nam served as a better precursor of NAD in mouse liver than NA (37) and it appeared to be the primary dietary and circulatory form of the vitamin (38,39). These objections to the Preiss-Handler pathway were eventually overcome to some degree by Petrack et al. (40), who were able to detect a rat liver microsomal Nam deamidase which, they believed, had sufficient activity to account for the calculated maximum rate of in vivo NAD synthesis. It must be pointed out that the K_m for Nam was still relatively high (7×10^{-2} M), even after the removal of a lipid inhibitor during the purification (41,42). Kirchner et al. (43) found that in rabbits most of the Nam deamidase activity resided in the liver. In fact, the second most active tissue, small intestine, was only one-fifth as active as liver. The K_m reported for their deamidase was also high (4×10^{-2} M). In a

comparative *in vivo* study of the utilization of Nam and NA as precursors of NAD in mouse liver, Ijichi *et al.* (44) found that NA, if administered in small doses, was the better precursor; if large doses were used, however, Nam served as the more efficient precursor. A time course in which a large dose of [^{14}C] Nam was administered revealed that radioactivity in the liver disappeared rapidly and did not begin to increase until one to two hours after injection. The decrease in radioactivity indicated excretion of [^{14}C] Nam from the liver. The eventual increase in radioactivity in the liver paralleled the increase of [^{14}C] NAD. These workers explained their data by the assumption that deamidation had occurred in the gastrointestianl tract before reabsorption by the liver. By investigating Nam deamidase in the stomachs of normal rats and germ-free rats, Tanigawa *et al.* (45) demonstrated Nam deamidase activity in the pars preventricularis of normal rats and isolated the responsible bacillus; no activity was detected in the germ-free rats. Since they had also shown that NA was absorbed by the small intestine, they felt it very probable that Nam in the diet might be deamidated by microorganisms in the stomach and then be absorbed by the small intestine to serve as a precursor in the Preiss-Handler pathway in various tissues.

The importance of the Preiss-Handler pathway in the liver was eventually realized; evidence accumulated, however, that extrahepatic tissues might not employ this route of NAD biosynthesis. The data of Ikeda *et al.* (46) indicated high activity for NA phosphoribosyltransferase in rat liver, moderate activity in the kidney, and low activity in eight other tissues examined. As previously mentioned, an alternate pathway, described by Dietrich *et al.* (8), had been

demonstrated in a variety of tissues. Further evidence in favor of this latter pathway came from Greenbaum and Pinder (47), who, working with rat mammary gland extracts, reported K_m values which were low for both Nam and NMN (3.5×10^{-5} M and 1.5×10^{-4} M respectively). They also showed that there was a time lag in the in vivo incorporation of Nam into liver NAD; no such lag occurred, however, in mammary gland.

The in vivo study undertaken by Collins and Chaykin (21) on the management of Nam and NA in the mouse did much to clarify the integration of the Preiss-Handler and Dietrich pathways. After [^{14}C] NA injections it became evident that the Preiss-Handler pathway was operating in the liver. But the dramatic aspect of the results in liver was an extensive accumulation of [^{14}C] Nam following [^{14}C] NA injection. The time course data indicated that the labeled Nam was released by NAD which had been synthesized from NA. Similar results were obtained in the intestine. In the spleen, NAD synthesis correlated with Nam rather than NaAD availability; nevertheless, high levels of NaAD accumulated and were maintained. This phenomenon was even more exaggerated in skeletal muscle. Essentially no NAD synthesis occurred during the time in which NaAD levels were high. Furthermore, once the NaAD level decreased, only twenty-five percent of the decrease could be accounted for. It was clear that radioactivity had left the muscle, and it was assumed that it had exited as NA. These workers hypothesized that the infusion of NA from the peripheral tissues was responsible for the prolonged formation of Nam which had occurred in the liver. Other tissues examined (kidney, ovary, lung, heart, and brain) contained more radioactive NAD after Nam injections than after NA injection. A lag was also observed in the appearance of label

in NAD in these organs following injection of NA but not Nam. These observations were compatible with the idea that the role of the liver was to convert NA to Nam so that it could be used in the peripheral tissues for NAD synthesis via the Dietrich pathway. It is difficult to correlate with this hypothesis the data of Deguchi et al. (20), which indicated preference for the Preiss-Handler pathway in rat brain, and that of Lin and Henderson (22) which dubbed NA the more effective precursor of NAD in perfused rat kidney; the latter group did obtain compatible data for rat testis (22), liver (48), and blood (49), however.

In the experiments of Collins and Chaykin (21) just described, physiological levels of Nam were administered, yet no evidence for deamidation appeared. Previous studies of Ijichi et al. (44) and Langan et al. (24) which indicated deamidation in vivo, had been carried out with nonphysiological levels of Nam. By increasing the Nam dosage 100-fold, Collins and Chaykin (21) were able to show that some deamidation did occur in the intestine, possibly due to bacterial action, and that deamidation also occurred in the liver. This was reasonable, since after administration of such a large dose of Nam, the Nam concentration in the liver could approach the K_m of the deamidase.

The plant picture is not so complicated, perhaps because the research is not yet as extensive. No one has demonstrated the Dietrich pathway in plants. It seems that the Preiss-Handler pathway is of greater importance, a fact which would correlate with the active Nam deamidase in plants. Although they did not identify any Preiss-Handler intermediates, Waller et al. (50) did demonstrate in vivo

conversion of Nam to NA, *in vitro* and *in vivo* conversion of NA to NAD, and *in vivo* conversion of NAD to Nam in castor bean seedlings. A complete pyridine nucleotide cycle via the Preiss-Handler pathway was later demonstrated *in vivo* by Godavari and Waygood (18) in detached wheat leaves and by Ryrie and Scott (35) in detached barley leaves. These latter workers confirmed Nam deamidase activity *in vitro*.

A logical question at this point is the origin of the Nam which is utilized in the Dietrich pathway or which is converted to NA for utilization in the Preiss-Handler pathway. The answer to that question lies in either the diet or one of the *de novo* pathways. The only well-defined *de novo* pathway is that from tryptophan (Figure 1). Its discovery dates to the early treatment of pellagra in humans and black tongue in dogs, both now established as vitamin deficiency diseases (51). In 1921 Tanner (52) treated one of his pellagrous patients with tryptophan and observed: ... the improvement in this patient's skin condition has surpassed anything I have ever seen in a case of pellagra in an equal period of time. The biosynthetic relationship between tryptophan and NA became apparent when Krehl (53) demonstrated that NA could be replaced by tryptophan in the diet of the rat. Kinurenine, 3-hydroxykinurenine, and 3-hydroxyanthranilic acid were eventually established as intermediates in the tryptophan pathway (54); however, conversion of the latter compound to NA remained obscure. *In vitro* studies by Priest *et al.* (55) had shown that 3-hydroxyanthranilic acid could be converted to QA. Picolinic acid was the only other product that could be derived enzymatically from 3-hydroxyanthranilic acid (56). But neither of these two acids served as an *in vitro* precursor of NA. The mechanism was finally

elucidated by Nishizuka and Hayaishi (57), who demonstrated that enzyme preparations from rat liver and kidney catalyzed the conversion of 3-hydroxyanthranilic acid to NaMN in a PRPP dependent reaction. Prior investigations had failed because microorganisms used in the bioassay for NA were unable to use the actual product, NaMN, as a growth factor (56).

Besides mammals, the tryptophan pathway was demonstrated in Neurospora (58) and yeast (59). The latter organism was unique in that it could utilize the tryptophan pathway under aerobic conditions, but under anaerobic conditions it exhibited a pathway similar to that found in bacteria, as described below.

No isotopic evidence could be obtained for the operation of the tryptophan pathway in plants (60) or bacteria (61), except for the aerobe Xanthomonas pruni (62). Alternate routes were sought by radioactive feeding techniques and eventually by in vitro studies with radioactive precursors. The first in vivo evidence for the identity of NA precursors in E. coli came from the work of Ortega and Brown (63), who demonstrated that the carbon chains of [^{14}C] glycerol and [^{14}C] succinic acid were efficiently incorporated into NA. Because of the labeling patterns obtained, they concluded that the carbon skeleton of NA was probably derived from a three-carbon compound, such as glycerol, and a four-carbon dicarboxylic acid, so that one of the carboxyl groups of the dicarboxylic acid would become the carboxyl group of NA. The results of in vivo experiments with the aerobes Mycobacterium tuberculosis (64) and Serratia marcescens (65) were consistent with this hypothesis. Crude extracts of the anaerobe Clostridium butylicum were able to use glycerol and aspartic acid as precursors

of NA; after fractionation of the extract, however, aspartate, acetyl CoA, and formate were required (66). More recent work demonstrated that N-formyl aspartate was probably a more immediate precursor (67). Consistent data were obtained with anaerobically grown E. coli, which incorporated [1,3-^{14}C] glycerol into NA in a tetrahydrofolate dependent mechanism (68)(Figure 1).

An E. coli mutant which lacked QA phosphoribosyltransferase was conveniently used by Chandler and Gholson (69-72) to obtain an in vitro QA synthetase system. They showed that glycerol could be incorporated into QA when ATP was present, but in the absence of ATP, glycerol was only one sixth to one-third as effective a precursor as fructose-1,6-diphosphate or glyceraldehyde-3-phosphate (71). It was later found that dihydroxyacetone phosphate was two to three times more efficient than glyceraldehyde-3-phosphate (72)(Figure 1). FAD was a required cofactor, which was reasonable in view of the possible intermediates which had been proposed by Fleeker and Byerrum (73).

The plant work took advantage of the alkaloids which contained a pyridine or pyridone ring derived from NA or QA (74,75). It had already been established that the conversion of QA to NaMN occurred in microorganisms (76) and plants (74) which did not utilize tryptophan. Thus if a three- or four-carbon compound could be incorporated into a pyridine alkaloid such as ricinine or nicotine, then that compound could most likely be considered a precursor of QA and therefore of NAD. Jackanicz and Byerrum (77) demonstrated that carbon 3 of aspartic acid could form positions 2 and 3 of the pyridine ring of nicotine in Nicotiana rustica. Further experiments by Flecker and Byerrum (73,78) indicated that positions 4, 5, and 6 were

derived from glycerol or glyceraldehyde. They proposed that a non-symmetrical three-carbon compound condensed with aspartic acid to form QA (73). These results were consistent with those of Yang and Waller (79), who studied the biosynthesis of the pyridone ring of ricinine in Ricinus communis (Figure 1).

Chaykin (56) has summarized the integration of these three major routes of NAD biosynthesis. In microorganisms it seems likely that Nam or NA would be the preferred precursor of NAD. If they are absent and if tryptophan is present in sufficient amounts, then the de novo pathway via tryptophan would be utilized. If neither Nam nor NA is available, and if the microorganism is one in which the tryptophan pathway is inoperative, an alternate de novo pathway would be resorted to. This theory was supported by experiments which indicated a repression of the de novo pathway in the presence of excess NA (80).

He feels further that the principle of conservation of biosynthetic activity would apply in mammals as well. Thus the tryptophan pathway would operate only if the amount of Nam or NA were insufficient to maintain adequate pyridine nucleotide levels in the cell.

The de novo pathway is regarded by Chaykin as the probable ultimate source of NAD in plants, since plants do not exhibit nutritional requirements for complex organic molecules. The pyridine nucleotide cycle via the Preiss-Handler pathway would then operate as a salvage cycle for reutilization of Nam from nucleotide breakdown.

As would be expected, NAD biosynthesis is subject to control. Feedback inhibition, in which the end product of a metabolic sequence inhibits the enzyme catalyzing the first committed step in its formation, has been documented for NAD biosynthesis via the pyridine

nucleotide cycle (Preiss-Handler pathway), the Dietrich pathway, and the de novo pathways from aspartic acid-glycerol and tryptophan. Calbreath and Joshi (81) showed that yeast Nam deamidase, the enzyme catalyzing the first committed step in the pyridine nucleotide cycle, was inhibited by NAD. The inhibition was competitive, with an apparent K_i of 7×10^{-4} M. They demonstrated that NAD was an effective inhibitor of Nam deamidase in several other microorganisms as well.

Both NAD and NADP were shown by Dietrich and Muniz (82) to inhibit the activity of purified preparations of rat liver Nam phosphoribosyltransferase. Fifty percent inhibition occurred at 5×10^{-4} M NAD or NADP. These workers later reported that the inhibition was non-competitive (83).

The QA synthetase system of Chandler and Gholson (71) was also blocked by NAD. At 3.33 mM NAD, 98 percent inhibition was obtained; however, slight inhibition could be achieved at 0.5 mM NAD.

Tryptophan pyrrolase, the first enzyme in the tryptophan pathway, converts tryptophan to formylkinurenine (84). Its inhibition by NAD was demonstrated both in vitro and in vivo. Wagner (85), using a partially purified preparation from rat liver, demonstrated that NAD was a competitive inhibitor of tryptophan pyrrolase; forty-two percent inhibition was attained with 5 mM NAD. Because this enzyme is under hormonal control, Yamaguchi et al. (86) found it necessary to use hypophysectomized animals in their in vivo experiments. Nam administration evoked increased tryptophan pyrrolase activity in normal rats, but not in the hypophysectomized ones. Furthermore, it had been reported that NAD levels reached in the livers of hypophysectomized rats in response to Nam injection were twice those of

normal rats (87). Thus they felt that if NAD did inhibit tryptophan pyrrolase *in vivo*, the effect would be very pronounced in hypophysectomized rats injected with Nam. The expected inhibition resulted. Measurement of actual hepatic NAD concentrations proved that the tryptophan pyrrolase activity was inversely related to NAD concentration, rather than to some other effect of Nam administration. Experiments by Cho-Chung and Pitot (88) with highly purified tryptophan pyrrolase resulted in data slightly different from that of Wagner (85). Using the same concentrations of tryptophan (0.4 mM) and NAD (5 MM), Wagner (85) had obtained 42 percent inhibition whereas these workers obtained 33 percent inhibition. They showed further that at this tryptophan concentration, NADPH was an extremely potent inhibitor. The percent inhibition ranged from 52 percent at 0.1 mM NAD to 95 percent at 1.0 mM NAD.

Besides the pituitary gland, both the adrenal glands and the pancreas have been implicated in the regulation of tryptophan pyrrolase. The lowering of tryptophan pyrrolase activity by adrenalectomy was shown by Knox and Auerbach (89). They showed further that treatment with cortisone, a hormone secreted by the adrenal cortex, could increase tryptophan pyrrolase activity in normal as well as adrenalectomized rats. On the other hand, Frieden *et al.* (90) later demonstrated *in vitro* that epinephrine, a hormone secreted by the adrenal medulla, was a potent non-competitive inhibitor of tryptophan pyrrolase. At 3×10^{-5} M epinephrine, 71 percent inhibition was achieved. The previously mentioned lack of induction of tryptophan pyrrolase in hypophysectomized rats following Nam injection was also demonstrated in adrenalectomized rats (86,91). Schlor and Frieden (92)

showed that insulin increased tryptophan pyrrolase activity three- to four-fold in normal rats and four- to five-fold in rats fasted for 48 hours prior to injection.

In the discussion of rat liver Nam deamidase, it was mentioned that removal of a lipid inhibitor during purification lowered the K_m of the enzyme (41,42). This inhibition was subsequently related to hormonal action as follows. The increased NAD levels in response to Nam injection in hypophysectomized rats were attributed in part to higher Nam deamidase activity (93). That this effect was due to a reduction of the concentration of the inhibitory material was demonstrated by the fact that the activity of purified Nam deamidase was twice as great in the presence of crude liver homogenate from hypophysectomized rats as it was in the presence of homogenates from normal rats. Furthermore, the K_m for Nam of liver homogenates from hypophysectomized rats was shown to be one-fourth the K_m determined with normal rats. The inhibitor was eventually identified as a mixture of free fatty acids, which consisted primarily of arachidonic, linoleic, and oleic acids (42). This composition of the inhibitor correlated well with the decrease in free fatty acid content known to occur following hypophysectomy (42).

Chaykin (56) has integrated these two types of control for mammalian systems with the suggestion that the feedback inhibition could provide an intracellular control whereas the hormonal effects could serve as intercellular controls.

The interplay of hormones and NAD biosynthesis was also demonstrated in plants. Waygood *et al.* (94) showed that chlorosis in floating *Elodea* leaves was accelerated by NAD at 1×10^{-3} M; this effect was

overcome by 5 x 10^{-4} M benzimidazole, a synthetic analog of kinetin. NA at 5 x 10^{-3} M was also a potent chlorotic agent; its effect was overcome by 4 x 10^{-4} M benzimidazole as well as by 1 x 10^{-4} M kinetin. This acceleration of chlorosis by NAD and NA was shown to be light mediated. In wheat leaves, benzimidazole treatment increased the total Nam nucleotide content by 20 percent after one day (16 hour photoperiod); kinetin effected a 50 percent increase in the same period. Treatment with the two compounds together resulted in an 80 percent increase. These workers showed further that the incorporation of [^{14}C] NA and [^{14}C] Nam into NaAD and NADP was greater in leaves floated on benzimidazole solutions than in leaves floated on water. There was no apparent effect of benzimidazole on the incorporation of label into NAD.

Three branches (Figure 1) off the main route of the pyridine nucleotide cycle are relevant to this thesis. The first is the reaction of NAD kinase to yield NADP and ADP from NAD and ATP. NADP formation according to this reaction has been established in fungi (95), bacteria (96), plants (97), and avian (98) and mammalian (99) tissues.

The second and third offshoots from the cycle are two analogous reactions, the formation of N-methylnicotinamide from Nam and the formation of N-methylnicotinic acid (trigonelline) from NA. The former derivative has long been known as a urinary metabolite, apparently excreted by carnivores and omnivores, rather than by herbivores (100,101). In 1951 Cantoni (102) obtained a cell-free preparation from rat liver which catalyzed the synthesis of N-methylnicotinamide from methionine and Nam in the presence of Mg^{2+} and ATP. The ATP requirement is now understood in light of S-adenosylmethionine,

the actual methyl donor (103). N-methylnicotinamide formation was also reported in castor bean sedlings by Waller et al. (50) and in bean, wheat, and corn seedlings by Stul'nikova (104). It is difficult to reconcile these results with those obtained in this laboratory (105) and those reported by Joshi and Handler (106). It was our experience that in castor bean seedlings Nam was rapidly and completely deamidated to NA, which was then converted to appreciable amounts of trigonelline, not N-methylnicotinamide. Waller et al. (50) based their identification of N-methylnicotinamide only on paper chromatography and did not state the degree of separation of trigonelline and N-methylnicotinamide by the solvent systems employed. Joshi and Handler (106) reported that pea extracts were able to synthesize trigonelline, but not N-methylnicotinamide. They demonstrated that S-adenosylmethionine was also the active donor in this methylation process. Either technique of species difference (for example, in the deamidase activity) could account for the conflict of results presented here. The biosynthetic relationship between NA and trigonelline was further indicated by an in vivo time course performed in wheat by Godavari and Waygood (18). These workers ascribed to trigonelline the role of detoxification of NA. It has also been shown that trigonelline can be used as a source of NA for NAD biosynthesis in Torula cremoris (107). This methyl derivative, first detected in the herb Trigonella foenum-graecum (108), has been isolated from several plant species (109); it is not a metabolic product of NA in mammals (110).

It was of interest in this laboratory to investigate the synthesis of N-methylnicotinamide in plants. The person assigned to this project, Anne Bosch* (112) found that an enzyme preparation from etiolated four-day-old castor bean seedlings was able to synthesize labeled nicotinic acid, trigonelline, and an unidentified compound, Product C, when incubated with [^{14}C] Nam, ATP, and Mg^{2+}. The NA and trigonelline were identified by their pattern of elution from a Dower 1-X2 (formate) column and by paper chromatography; the former was also recrystallized with nonradioactive standard NA to constant specific activity. No N-methylnicotinamide formation could be detected, even when S-adenosylmethionine was added to the reaction mixture. The synthesis of Product C was dependent on ATP as well as on some other compound(s). This was indicated by the fact that passage of the enzyme preparation through a Sephadex G-25 column resulted in loss of activity. Nam was the only compound tested which could serve as a substrate in the reaction.

The research presented here was performed in order to establish the identity of Product C and to characterize the enzymatic reaction(s) involved. The problem was approached with the hope that Product C might be some heretofore unknown metabolite of Nam; however, related pyridine compounds known to occur naturally were also kept in mind.

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*Participant in the NSF undergraduate research program, Summer, 1971.

MATERIALS AND METHODS

Radioactive Chemicals

[7-^{14}C] Nam and [7-^{14}C] NA were purchased from New England Nuclear and [8-^{14}C] ATP and [6-^{14}C] QA were purchased from Amersham/Searle Corporation. [6-^{14}C] NaMN was synthesized enzymatically from [6-^{14}C] QA according to the procedure described by Mann (185). The purity of all radioactive chemicals was checked in at least two solvent systems.

Nonradioactive Chemicals

The nonradioactive chemicals used in this work were purchased as follows: Nam from Nutritional Biochemicals Corporation; NA from Aldrich Chemical Company; NMN and NADP from P-L Biochemicals, Inc.; PRPP (sodium salt), QA, dithioerythritol, and yeast alcohol dehydrogenase from Sigma Chemical Company; NAD and ATP (sodium salt) from the latter two companies; Dowex 1-X2 (200-400 mesh, chloride form) from Dow Chemical Company; Sephadex G-25 from Pharmacia Fine Chemicals, Inc.; POPOP, PPO, and BBOT from Packard Instrument Company, Inc.; and Orthocide, 50% Captan, from a local vendor. The author is grateful to Dr. Richard Hiles for the synthesis of NaAD and to Dr. Thomas Griffith for the synthesis of trigonelline·HCl. All other chemicals were reagent grade.

Scintillation Fluids

(I) Bray's scintillation fluid was prepared by adding 289.8 g naphthalene, 28.98 g PPO, and 0.7225 g POPOP to 3.0 kg p-dioxane. (II)

This scintillation fluid was prepared by adding 15.16 g BBOT to 3.79 l toluene.

Plants

Etiolated castor bean seedlings, Ricinus communis L., variety Hale, were used for the studies in this thesis. The seeds were the generous gift of Mr. Walter Domingo, Director of the Oil Seeds Production Division of the Baker Castor Oil Company, La Mesa, California. After being washed with distilled water, the seeds were treated with a 10% solution of Orthocide for 10 min, drained, and planted in moist vermiculite in aluminum pans, which were then covered tightly with aluminum foil. Distilled water was used to water the beans except for the age studies, when tap water was used. On Day 5 the seedlings were harvested, and only those seedlings which possessed the same physiological characteristics were used for a given experiment. In general, the endosperm was firm and weighed between 0.5 and 0.6 g, the cotyledons were yellow with no pink or only a trace of pink, and the secondary roots were slightly developed.

Enzyme Preparation

The endosperm of each seedling harvested was split longitudinally with a razor blade so that the cotyledons could be discarded. The endosperms were placed in an ice bucket, rinsed with distilled water, blotted dry with paper towels, and weighed. The remaining procedures were carried out at 4°C. The tissue was ground to a slurry in a mortar with 2 volumes of 0.05 M potassium phosphate, pH 7.0, containing 0.01 M dithioerythritol. This extract was then squeezed through 4 layers of cheesecloth and centrifuged at 27,000xg for 15 min. The

supernatant fraction was used for all the enzyme studies.

Enzyme Assays

The standard assay for Product C formation contained the following reagents at the final concentrations given in a total volume of 150 µl: 66.7 mM potassium phosphate, pH 7.0; 6.7 mM dithioerythritol; 4.0 mM ATP; 13.3 mM $MgCl_2$; 0.10 mM [7-^{14}C] Nam or [7-^{14}C] NA; and 100 µl (1.5 mg protein) enzyme preparation. There was some variation from this standard assay, in the earlier experiments. Such deviations as well as the specific activities of the substrates will be stated for each experiment in the results section. The reaction mixture was generally incubated with shaking for 3 hr at room temperature and the reaction was stopped by heating the test tube in a boiling water bath for 2 min. After removal of the protein by centrifugation in a clinical centrifuge, 10 µl was spotted on Whatman No. 1 paper and developed by descending paper chromatography, usually in Solvent System I.

NA phosphoribosyltransferase and QA phosphoribosyltransferase were assayed as described by Mann (105).

Paper Chromatography

Descending chromatography on Whatman No. 1 paper was performed with four solvent systems: (I) 1-butanol-glacial acetic acid-water (4-1-2, v/v); (II) 1 M ammonium acetate, pH 5.0, -95% (v/v) aq. ethanol (3-7, v/v); (III) 60% (v/v) aq. 1-propanol; and (IV) upper phase of 1-butanol-acetone-water (9-1-10, v/v). System IV was particularly useful for the separation of Nam and NA in the hydrolysis studies.

Product C Purification

Sheets of Whatman No. 1 paper, 46 x 57 cm, were washed for 3 days with 0.1 M sodium citrate and then rinsed for 3 days with deionized distilled water. The sheets were air dried and then cut in half lengthwise. Product C, which had been eluted from a Dowex 1 formate column and concentrated on a rotary evaporator, was streaked on this paper and developed in Solvent System II. The UV absorbing band corresponding to Product C was then eluted with deionized distilled water by means of a spin thimble purchased from the Reeve Angel Company. This procedure was repeated with Solvent System III. For the Product C spectral data, a second streaking and developing in Solvent System III was done. Appropriate volumes were attained after the spin thimble elutions by concentration on a rotary evaporator.

Product Determination on Paper Chromatograms

UV absorbing products and standards were detected by visual UV quenching with a Mineralight UVS·11 Lamp. Radioactive products and standards were detected on a Model 7201 Radiochromatogram Scanner. The areas under the peaks obtained by the latter method were cut from the chromatograms and placed in counting vials filled with Scintillation Fluid II and counted for 10 min in a Packard Model 3310 Tri-Carb Liquid Scintillation Spectrometer.

Dowex 1-X2 Formate Column Chromatography

Dowex 1-X2 chloride (200-400 mesh) was first converted to the hydroxyl form by eluting with 39 volumes 1N NaOH. Complete removal of chloride was checked by acidifying a sample of the eluant with conc. HNO_3 and adding 1% $AgNO_3$. The resin was then washed with deionized

distilled water until the pH of the eluant was that of the water. It was then converted to the formate form by elution with 8 volumes 1N HCOOH. Complete conversion was indicated when the pH became less than 2. Finally, the resin was again washed with deionized distilled water. A deproteinized reaction mixture was placed on a column of this resin and eluted with a differential HCOOH gradient according to the procedure of Ijichi et al. (44), as modified by Ryrie and Scott (35), with the following two exceptions: for convenience, a 25 ml burette (1.0 cm i.d.) was used and the last reservoir, 4 N $HCOONH_4$, was eliminated from the gradient.

Product Determination on a Dowex 1-X2 Formate Column

The fractions eluted from the Dowex 1 formate columns were assayed for UV absorbance at 260 nm on a Hitachi-Perkin-Elmer Model 139 Spectrophotometer. A Gilson transferator was used to expedite the measurements. Radioactivity of the fractions was determined by counting 0.5 ml aliquots in 5.0 ml Scintillation Fluid A.

Sephadex G-25 Column Chromatography

Sephadex G-25, medium grade (50-150 μ particle size) was allowed to swell overnight in 0.05 M potassium phosphate, pH 7.0, and then was poured to form a column 18 cm x 7 mm i.d. The column was eluted with approximately 100 ml of the same buffer and calibrated with a 49:1 (v/v) solution of 0.05 M FAD and 0.2% (w/v) blue dextran which had been filtered. This same column was then used to desalt a castor bean endosperm enzyme preparation.

Protein Determinations

The amount of protein in the enzyme preparations was determined by the method of Lowry et al. (111).

UV Spectra of Product C and NAD

The author is grateful to Dr. Fritz Rottman and Karen Friderici for the use of the Model 240 Gilford Spectrophotometer. The instrument was adjusted to zero absorbance against air and the samples were scanned from 200 to 400 nm.

The incubation mixture for the cyanide complex formation contained NAD and KCN at the final concentrations of 13.6 μM and 67 mM respectively. A Product C sample was likewise incubated with KCN. Spectra were taken before and 10 min after the addition of KCN.

The incubation mixture for the yeast alcohol dehydrogenase assay contained at the final concentrations given in a total volume of 1.5 ml: 0.55 M ethanol; 13.6 μM NAD; 53 mM Tris, pH 8.7; and 50 μg yeast alcohol dehydrogenase. The assay was repeated with Product C in place of NAD. Spectra were taken before and 10 min after the enzyme was added to the reaction mixture.

Hydrolysis Studies

The samples used in the hydrolysis studies were the concentrated Fractions #5, #7, and #9 from the Dowex 1 formate column in Figure 7 and authentic [6-^{14}C] NaMN, NaAD, and NAD. They were each adjusted to a final volume of 350 μl and were made 0.1N in NaOH, with the exception of Fraction #9. Although this fraction was radiochemically pure, some compound, possibly residual HCOOH, prevented the sample from becoming alkaline. The final volume of this sample was 800 μl

and the final concentration of NaOH added was 0.28 N. The samples were heated in a boiling water bath for 10 min and 60 min. Paper chromatography was carried out in all four systems; however, there was only enough of Sample #9 for spotting in three systems.

Recrystallization to Constant Specific Activity

[7-^{14}C] NA and [8-^{14}C] trigonelline from typical incubations were isolated on a Dowex 1 formate column. The respective nonradioactive standards were added to each of the two samples and they were recrystallized to constant specific activity. After each recrystallization, a small amount of crystals was dissolved in deionized distilled water and diluted to volume. Since these compounds follow Beer's Law, the ratio of cpm to absorbance is a valid representation of specific activity.

RESULTS AND DISCUSSION

Initial experiments were performed to further establish the nature of Product C synthesis. Depending on the activity of the enzyme preparation, radiochemical yields of Product C as high as 11% (1.6 nmoles) could be obtained from a 3 hour incubation. On occasion, there appeared a fourth product, D, which never exceeded one-third the amount of Product C formed. A time course (Figure 2) indicated that Nam was being rapidly deamidated; Products C and D, however, continued to be synthesized even after the supply of Nam had been depleted. This suggested that NA might serve as the substrate for Products C and D, as it did for trigonelline. Original attempts to answer this question failed because of excessive streaking of NA in Solvent System I. This difficulty was overcome by using NA of higher specific activity so that less sample had to be spotted. Table 1 shows that NA was as effective as Nam as a precursor of Product C. In view of the rapid deamidation of Nam as well as the fact that the reversibility of this deamidation has never been reported, it was assumed that NA was the more immediate precursor of Product C.

Since 8 mM NAD had been reported to inhibit Nam deamidase in microorganisms (81), it was of interest to determine the effect of NAD on Product C formation. In a preliminary set of experiments, almost identical inhibition of Product C production resulted when either [7-^{14}C] Nam of [7-^{14}C] NA was used as the substrate. This indicated

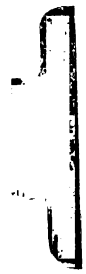


Figure 2. Time Course of Nam Utilization and NA Formation (A) and Product C, Product D, and Trigonelline Formation (B). A large scale incubation (2.25 ml) was performed as described in the methods section, except that the ATP concentration was 6.67 mM. [7-^{14}C] Nam (5.03 mC/mmole) at a concentration of 0.103 mM was used as the substrate. Aliquots (100 µl) were removed at the times indicated and the results were obtained from Solvent Systems I and IV.

(A) Nam (●—●)

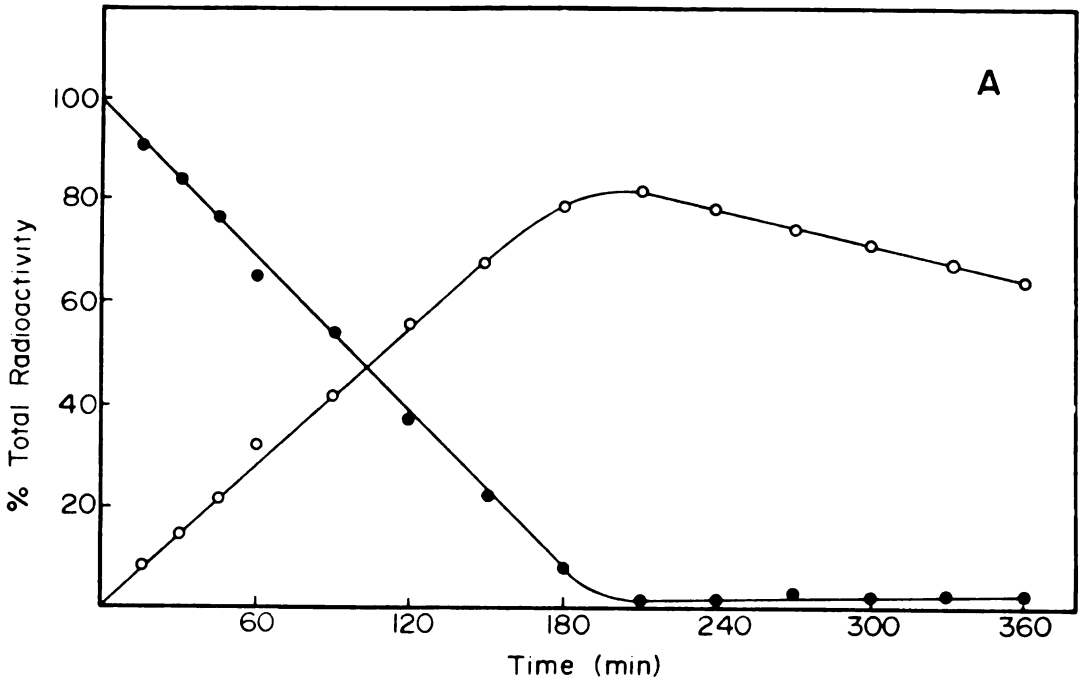
(A) NA (o—o)

(B) Product C (o—o)

(B) Product D (●—●)

(B) Porduct C + Product D (■—■)

(B) Trigonelline (▲—▲)


A
100
80
60
40
20
% Total Radioactivity
60
120
180
240
300
360
Time (min)

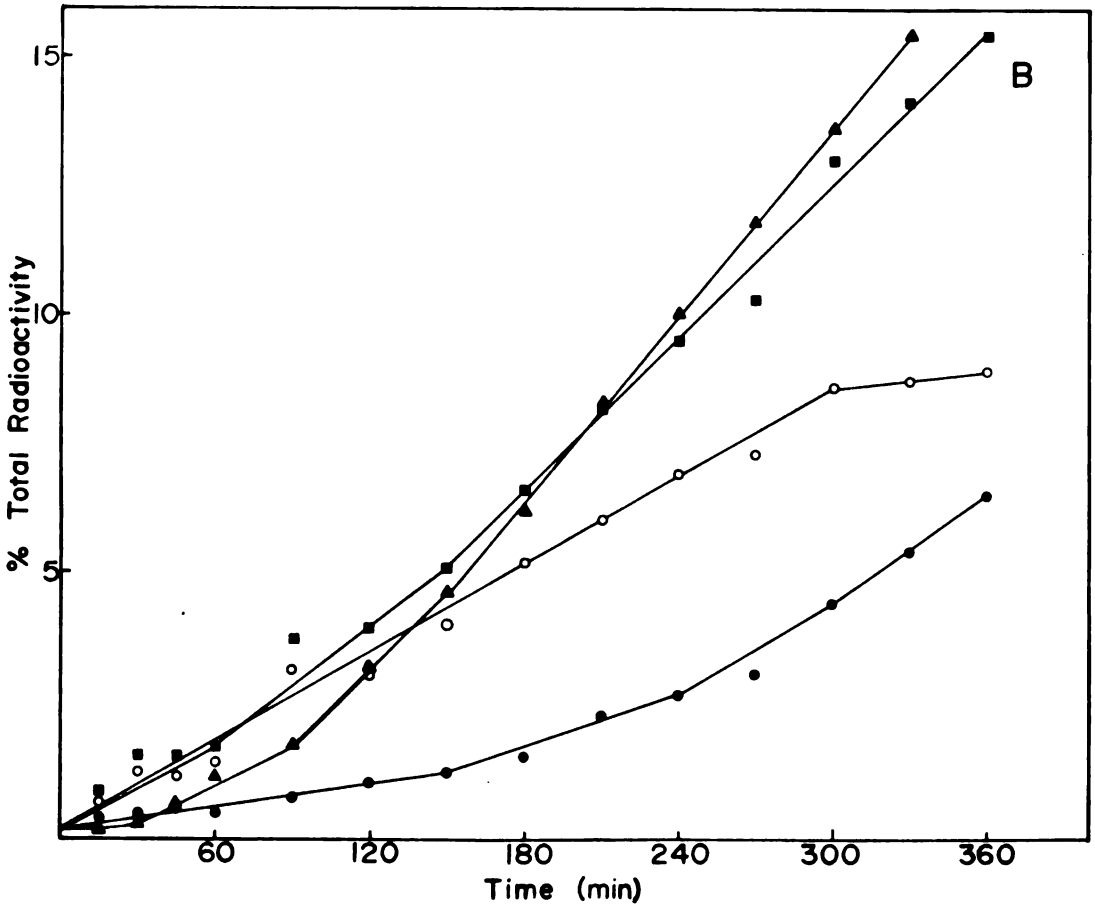

B
15
10
5
% Total Radioactivity
60
120
180
240
300
360
Time (min)

TABLE 1. [7-^{14}C] Nam and [7-^{14}C] NA as Precursors of Product C, Product D, and Trigonelline. The assay procedure for both Experiments I and II was that described in the methods section, except that the concentration of ATP was 6.67 mM. In Experiment I the concentration of [7-^{14}C] Nam (13.5 mC/mmole) was 0.098 mM and that of [7-^{14}C] NA (6.60 mC/mmole) was 0.094 mM. In Experiment II the concentration of [7-^{14}C] Nam (5.03 mC/mmole) was 0.103 mM and that of [7-^{14}C] NA (6.60 mC/mmole) was 0.102 mM. The incubation time was 2 hr for Experiment I and 4 hr for Experiment II. Solvent System II was used to obtain the results of Experiment I and Solvent System I was used in Experiment II.

Product	[7-^{14}C] Nam				[7-^{14}C] NA			
	Experiment I		Experiment II		Experiment I		Experiment II	
	nmoles Product	% Total Radioactivity	nmoles Product	% Total Radioactivity	nmoles Product	% Total Radioactivity	nmoles Product	% Total Radioactivity
Product C	1.26	8.6	1.17	7.6	1.09	7.7	1.16	7.6
Product D	-	-	0.45	2.9	-	-	0.40	2.6
Trigonelline	1.50	10.2	1.60	10.4	1.74	12.3	2.05	13.4

that the inhibition was not due solely to inhibition of the deamidase. (It was evident from the scans of the radiochromatograms that some inhibition of Nam deamidase had occurred, but it was not quantitated.) Table 2 presents the inhibition effected by NAD on Product C and trigonelline fromation when [7-^{14}C] NA was used as the substrate. At 1 mM NAD, Product C and trigonelline formation was inhibited by 86.6% and 22.3 % respectively. Synthesis of the latter compound was more effectively inhibited (70.9%) by 5 mM NAD. NAD had never been reported to inhibit trigonelline formation. Since funneling of NA into trigonelline is a detoxification mechanism (18), the inhibition by NAD was unexpected. Furthermore, since there is a constant level of NAD in plants at a particular stage of development (18), and since trigonelline can serve as a source of NA for NAD synthesis in Torula cremoris (107), high levels of NAD might be expected to inhibit the demethylation of trigonelline but not its synthesis. No methyl donor had been added to the reaction mixture; thus it is possible that NAD inhibited the synthesis of S-adenosylmethionine rather than the synthesis of trigonelline itself.

The requirements for the synthesis of Product C are summarized in Table 3. The reaction was completely dependent on ATP and less dependent on Mg^{2+}. Trigonelline formation exhibited a lesser requirement for ATP but hardly any requirement for Mg^{2+}. These results indicate the presence of endogenous Mg^{2+}, since methyl group transfers from methionine as well as other reactions involving ATP require the participation of Mg^{2+} (113). That a high level of ATP was required for the optimum formation of Product C is evident from Table 4. The highest yields were obtained at 2.67 or 4.00 mM ATP.

TABLE 2. NAD Inhibition of Product C and Trigonelline Synthesis. The assay procedure was that described in the methods section, except that the concentration of ATP was 5.33 mM. [7-^{14}C] NA (6.60 mC/mmole) at a concentration of 0.102 mM was used as the substrate. Solvent System I was used to obtain the results. The values reported are the average of the results from duplicate assays.

NAD Concentration (mM)	nmoles Product		% Total Radioactivity		% Inhibition	
	Product C	Trigonelline	Product C	Trigonelline	Product C	Trigonelline
0.0	1.30	1.58	8.5	10.3	-	-
0.1	1.57	1.38	10.2	9.0	-20.8	12.7
0.5	1.03	1.45	6.8	9.4	20.8	8.2
1.0	0.21	1.22	1.4	8.0	83.8	22.8
5.0	0.03	0.46	0.2	3.0	97.7	70.9
10.0	0.01	0.24	0.1	1.6	99.2	84.8

TABLE 3. Requirements for the Synthesis of Product C, Product D, and Trigonelline. The data in this table were compiled from several experiments. All activities are relative to the control of the particular experiment, which was given a value of 100. The assay procedure was that described in the methods section, except that in Experiment I, IV, and V the concentration of ATP was 6.67 mM and in Experiment II, an equivalent concentration of Tris, pH 7.0, was used in place of phosphate or in addition to phosphate. The substrates were [7-^{14}C] Nam (39.4 mC/mmole) at a concentration of 0.095 mM in Experiment I, [7-^{14}C] Nam (13.5 mC/mmole) at a concentration of 0.098 mM in Experiment II, and [7-^{14}C] NA (6.60 mC/mmole) at a concentration of 0.102 mM in the remaining experiments.

Modification of Assay	Relative Activity		
	Product C	Product D	Trigonelline
None	100	100	100
(I) - ATP	0	-	19
(I) - Mg^{2+}	44	-	81
(I) - ATP, - Mg^{2+}	0	-	19
(II) - Phosphate, + Tris	4	22	8
(II) - Phosphate, + Tris, + 4 mM ATP	0	0	0
(II) + Phosphate, + Tris	26	22	28
(III) Boiling of Enzyme	0	0	0
(IV) Sephadex G-25 Treatment of Enzyme	0	-	106
(V) + Supernatant of Boiled Enzyme	50	-	76

TABLE 4. Optimum ATP Concentration for Product C and Trigonelline Synthesis. The data in this table were compiled from two experiments. The assay procedure was that described in the methods section, except that the ATP concentration was varied as given in the table below. [7-^{14}C] NA (6.60 mC/mmole) at a concentration of 0.102 mM was used as the substrate. Solvent System I was used to obtain the results. The values for 1.33 - 8.00 mM ATP are the average of the results from duplicate assays.

ATP Concentration (mM)	Relative Activity	
	Product C	Trigonelline
0.05	13	34
0.10	24	43
0.20	37	48
1.33	79	96
2.67	100	96
4.00	100	100
5.33	93	99
6.67	93	96
8.00	91	84

Almost no activity for Product C formation was obtained in Tris buffer, although about one-fourth the control activity could be obtained in Tris buffer supplemented with the amount of phosphate in a regular reaction mixture (Table 3). Observation of the chromatograms under UV light revealed that the ATP was completely degraded in the assays containing Tris buffer, but hardly degraded at all in those containing phosphate buffer alone. A two-fold increase of ATP in the reaction mixture did not overcome this effect. Similar results were obtained for trigonelline formation, which is consistent with its requirement for ATP under these assay conditions. Product D was detected in the experiments with Tris and phosphate buffers. No stimulation of its synthesis resulted from the addition of phosphate to the reaction mixture containing Tris buffer and no activity at all was obtained in Tris buffer in an assay fortified with the extra ATP.

Boiling of the enzyme preparation for one minute resulted in complete loss of activity for Product C formation, as did passage of the enzyme preparation through a Sephadex G-25 column (Table 3). This latter treatment did not affect trigonelline formation, a result which is not understood in light of a probable requirement for endogenous methionine under these conditions. Since desalting of the enzyme preparation had prevented Product C synthesis, it was expected that addition of the low molecular weight fraction from the Sephadex G-25 column would restore activity to the desalted protein fraction. Addition of the supernatant from a boiled enzyme preparation would likewise be expected to stimulate the activity of an assay containing untreated enzyme. The former experiment was tried by Bosch (112), who found that activity could not be restored. The author of this thesis

found that the latter treatment did not stimulate activity either; in fact, both Product C and trigonelline formation appeared inhibited by 50% and 24% respectively.

At this point it was desired to obtain Product C in a quantity sufficient for identification; therefore, a large scale incubation was performed. Dowex 1 formate column chromatography of the reaction mixture revealed a radioactive peak with corresponding UV absorbance that was eluted after NA (Figure 3). The percentage of radioactivity in this peak was close to the amount converted to Product C, as calculated from the results of paper chromatography. The fractions belonging to this peak were pooled and concentrated on a rotary evaporator; the sample was then purified by paper chromatography in Solvent Systems II and III. The radioactivity ran with the UV absorbance both during and after the purification. Furthermore, the R_f of the purified product in all four solvent systems was that expected for Product C.

Alkaline hydrolysis of this sample provided one of the main clues leading to the identification of Product C: besides UV absorbing radioactive products, there were UV absorbing products without radioactivity. This raised the possibility that ATP might be serving as a substrate in the reaction. That this was indeed the case is illustrated in Figure 4. When nonradioactive NA and [8-^{14}C] ATP were incubated with an enzyme preparation, a product was formed which was eluted from a Dowex 1 formate column in the same position as Product C. In anticipation of the result, carrier NAD had been added to the reaction mixture placed on the column, since the enzyme preparation was not on a large enough scale for endogenous UV absorbing material

Figure 3. Separation of the Products from [7-^{14}C] Nam on a Dowex 1 Formate Column. A large scale incubation (30.0 ml) was performed for 4 hr as described in the methods section, except that the concentration of ATP was 6.67 mM. The [7-^{14}C] Nam (6.75 mC/mmole) was at a concentration of 0.098 mM. The products were eluted with a differential HCOOH gradient at a flow rate of 27 ml/hr, as described in the methods section. UV absorbance and radioactivity were determined on each fraction.

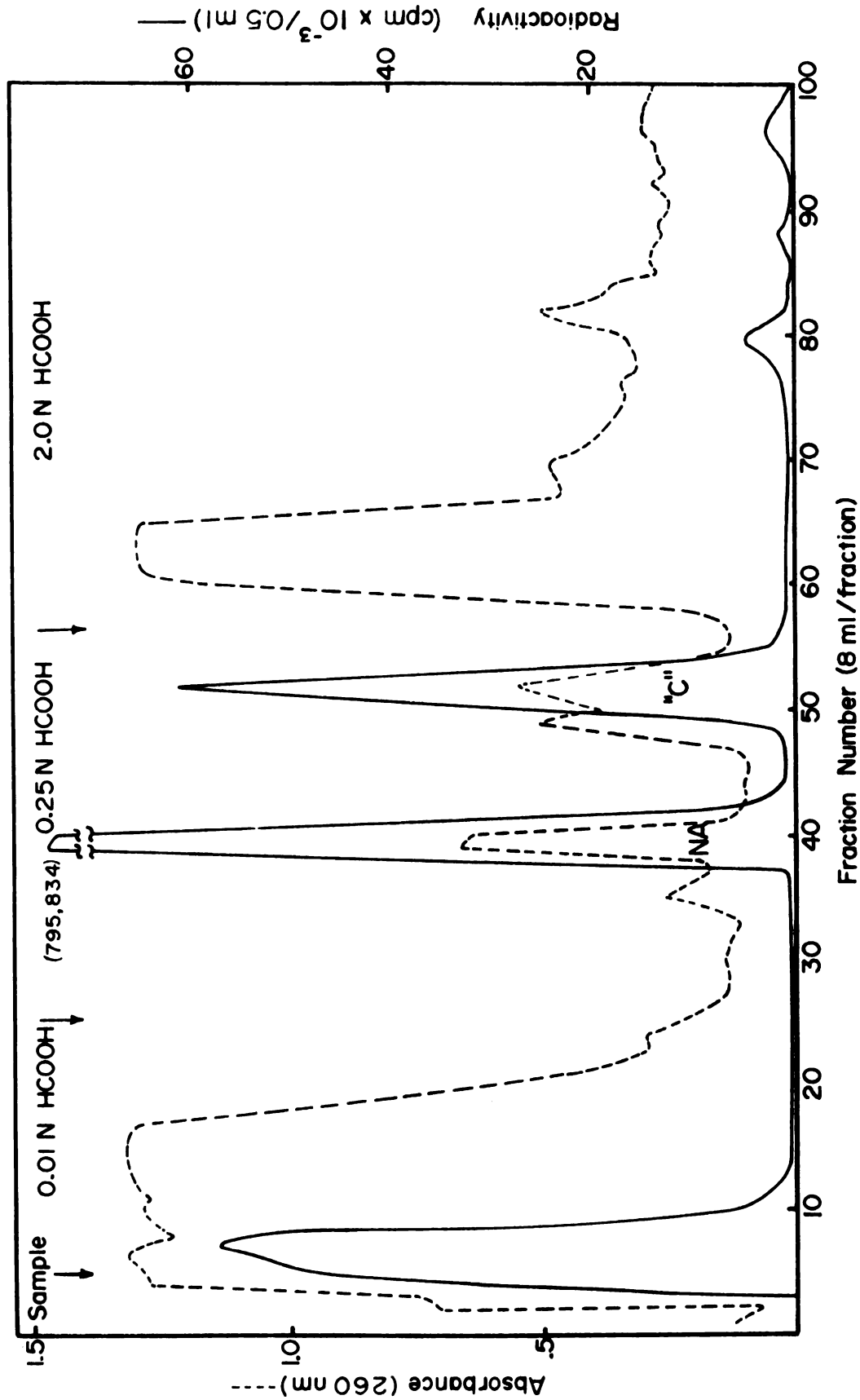
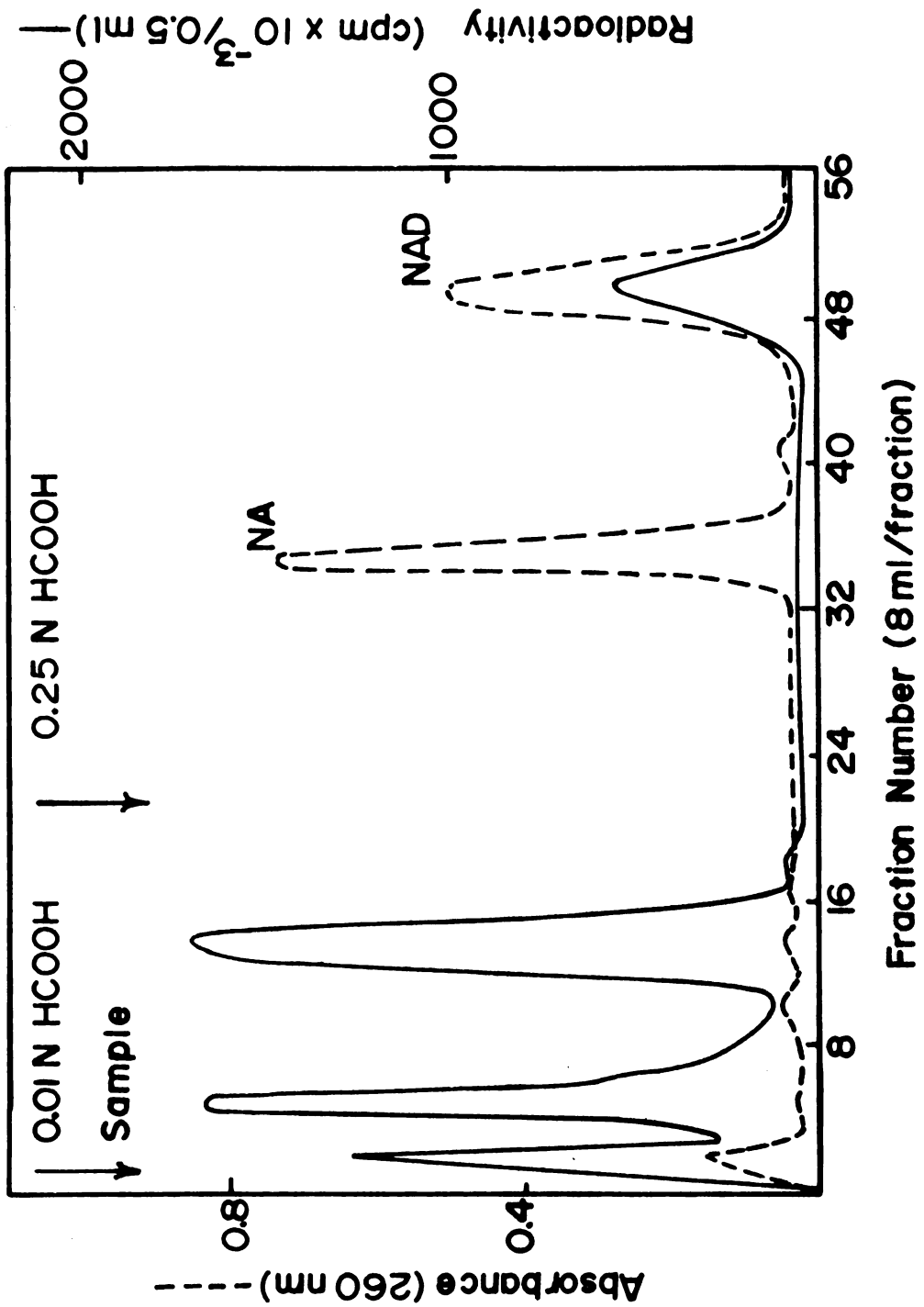

Absorbance (260 nm)----
Radioactivity (cpm x 10^{-3}/0.5 ml) ——
Fraction Number (8 ml/fraction)
Sample
0.01 N HCOOH
(795, 834)
0.25 N HCOOH
2.0 N HCOOH
"C"
NA

Figure 4. Separation of the Products from [8-^{14}C] ATP on a Dowex 1 Formate Column. A 4 hr incubation was performed as described in the methods section, except that [8-^{14}C] ATP (5.37 mC/mmole) at a concentration of 3.70 mM was used as the radioactive substrate. Nonradioactive NA was present at a concentration of 0.104 mM. Carrier NA (2.0 umoles) and NAD (0.7 umoles were added to the deproteinized reaction mixture placed on the column. The products were eluted with a differential HCOOH gradient at a flow rate of 38 ml/hr, as described in the methods section. UV absorbance and radioactivity were determined on each fraction.


Absorbance (260 nm) ----
0.8
0.4
0.01 N HCOOH
Sample
0.25 N HCOOH
NA
NAD
2000
1000
Radioactivity (cpm x 10⁻³/0.5 ml) —
8
16
24
32
40
48
56
Fraction Number (8 ml/fraction)

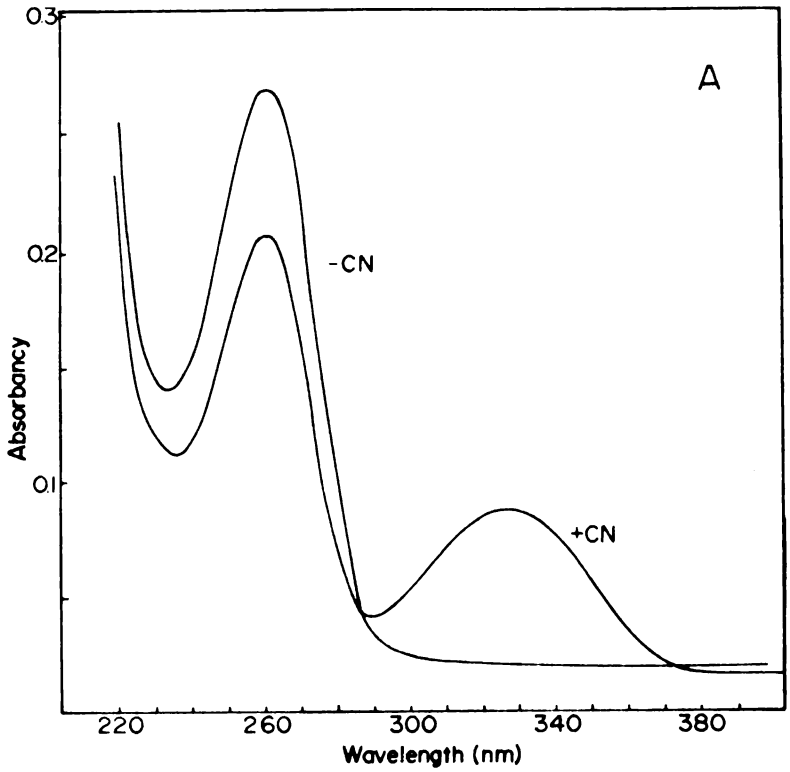
to be detected. The UV absorbance from the carrier NAD corresponded exactly to the radioactivity of the peak. The fractions belonging to this peak were pooled, concentrated on a rotary evaporator, and then purified by paper chromatography in Solvent Systems II and III. When this sample and a similarly purified sample of Product C synthesized enzymatically from [7-^{14}C] NA were each co-chromatographed with authentic NAD, there was perfect correspondence of radioactivity and UV absorbance of the NAD standard in Solvent Systems I, II, and III. The Product C from [8-^{14}C] ATP was then mixed with the Product C from [7-^{14}C] NA, and this sample was again co-chromatographed with the authentic NAD. The radioactive peaks were coincident with each other and with the UV absorbance in these three systems.

Further evidence that Product C was NAD was obtained from the UV spectrum of another sample of Product C which had been isolated from a Dowex 1 formate column and purified by paper chromatography in Solvent Systems II and III. Authentic NAD and Product C exhibited almost identical spectra, both before and after the formation of the cyanide addition product (Table 5 and Figure 5). The reaction with cyanide is not specific for NAD; NMn, NMN, NaAD, and NADP also form cyanide complexes with UV spectral characteristics similar to those of NAD (114). When Product C was reacted with yeast alcohol dehydrogenase, additional proof of its identity as NAD was obtained (Table 5, Figure 6). In contrast to the maximum at 327 nm resulting from the cyanide complex, a peak appeared at 340 nm, representing the reduced Nam moiety of NAD. Since both Nam and adenine contribute to the 260 nm maximum, a decrease, but not complete loss, in absorption at this wavelength was associated with the formation of the new maxima at

TABLE 5. Absorption Maxima and Minima of Authentic NAD and Product C in the Absence and Presence of KCN and in the Absence and Presence of Yeast Alcohol Dehydrogenase. The spectra were obtained as described in the methods section.

Sample	Absorption Maxima (nm)		Absorption Minima (nm)	
NAD - KCN	260		233	
Product C - KCN	260		234	
NAD + KCN	261	327	237	290
Product C + KCN	260	327	236	290
NAD - ADH	260		234	
Product C - ADH	260		234	
NAD + ADH	260	340	234	290
Product C + ADH	260	340	236	290

Figure 5. Absorption Spectra in the Absence and Presence of KCN. (A) NAD; (B) Product C. The spectra were obtained as described in the methods section.


A
0.3
0.2
0.1
Absorbancy
-CN
+CN
220
260
300
340
380
Wavelength (nm)

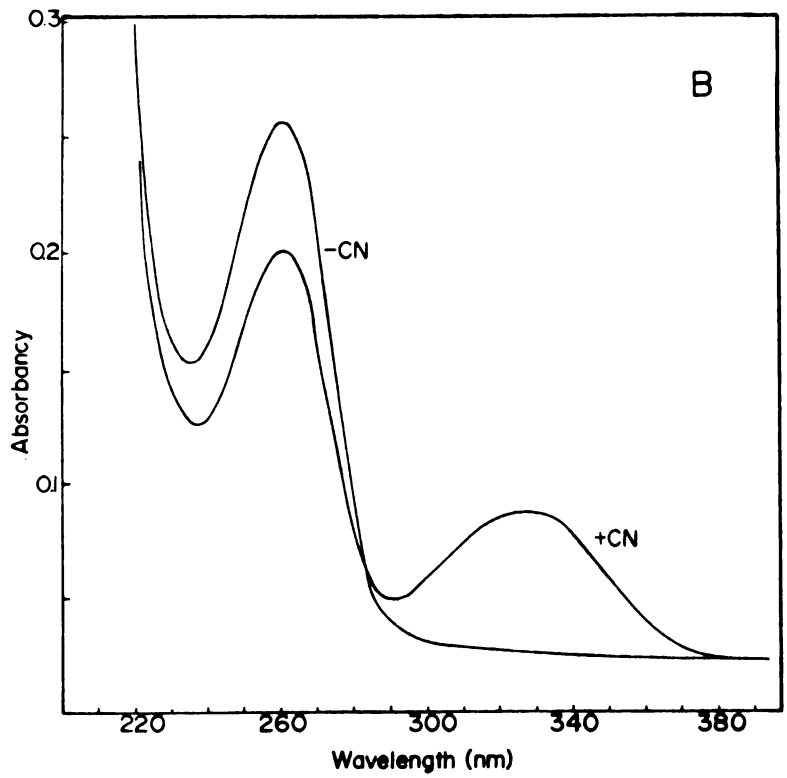
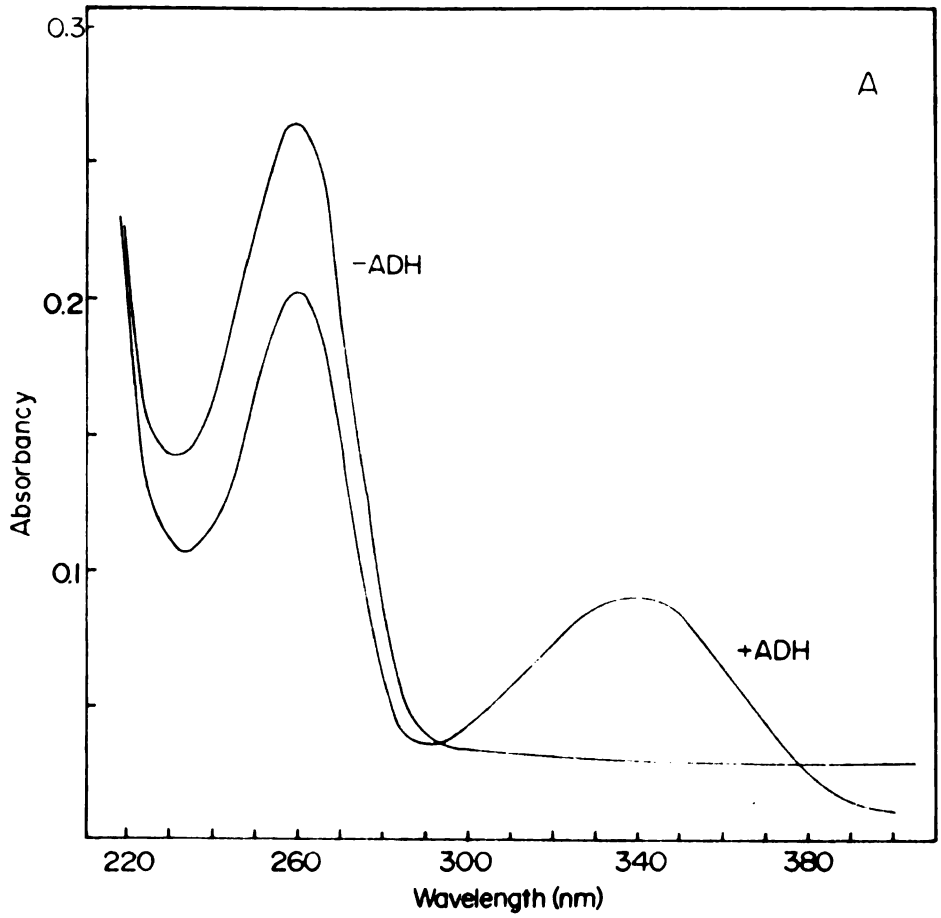
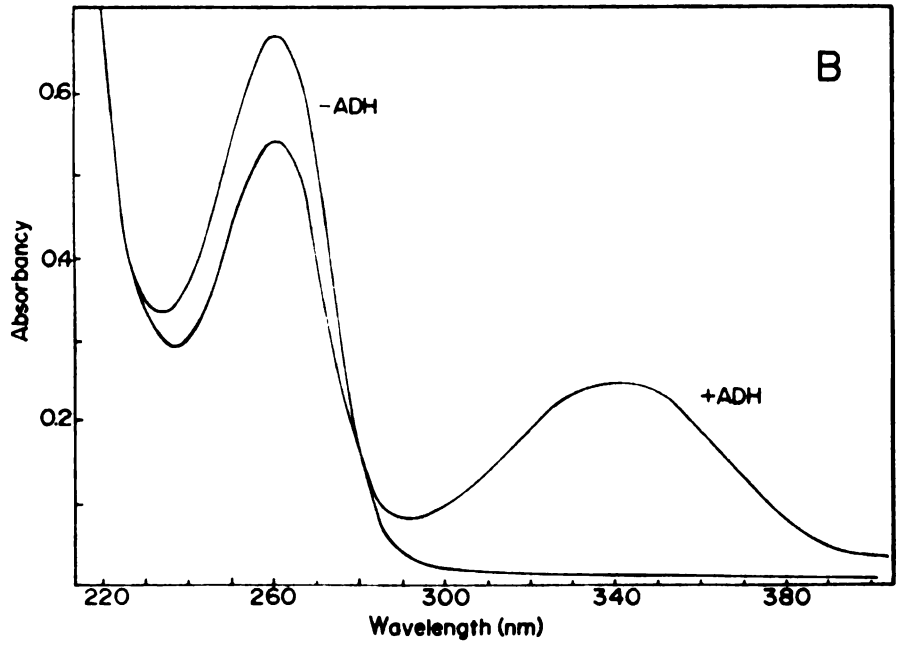

B
0.3
0.2
0.1
Absorbancy
-CN
+CN
220
260
300
340
380
Wavelength (nm)

Figure 6. Absorption Spectra in the Absence and Presence of Yeast Alcohol Dehydrogenase. (A) NAD; (B) Product C. The spectra were obtained as described in the methods section.


A
0.3
0.2
0.1
Absorbancy
-ADH
+ADH
220
260
300
340
380
Wavelength (nm)


B
0.6
0.4
0.2
Absorbancy
-ADH
+ADH
220
260
300
340
380
Wavelength (nm)

327 nm and 340 nm. Yeast alcohol dehydrogenase is NAD dependent, although slow rates have been reported for NADP (115,116). The spectral data taken together with the column and paper chromatographic data to be presented later preclude the identity of Product C as any one of the nucleotides mentioned here other than NAD which also form cyanide complexes or react with yeast alcohol dehydrogenase.

It seemed likely that the NAD was being formed via the Preiss-Handler pathway, since NA was able to serve as the substrate. Accordingly, standard assays for NA phosphoribosyltransferase and QA phosphoribosyltransferase were performed in order to compare the products with those formed in a Product C assay. Scans of radiochromatograms developed in Solvent Systems I and II from assays of the former enzyme resulted in a pattern essentially identical to that obtained when assaying for Product C. Radioactive peaks corresponding to both Products C and D were evident. The product of QA phosphoribosyltransferase also ran at the R_f of Product D in these two systems. These two enzymes both form the same product, NaMN (Figure 1). Since the basic difference between the assay for NA phosphoribosyltransferase and Product C synthetase was the presence of PRPP in the former, it appeared that ATP was serving to promote the synthesis of PRPP. The loss of activity after passage of the enzyme preparation through a Sephadex G-25 column could be partially explained by the loss of the other substrate for PRPP synthesis, i.e., ribose-5-phosphate. The assay conditions were probably favorable for PRPP synthesis, since it has been shown to require Mg^{2+} in Salmonella typhimurium (117) as well as P_i in human erythrocytes (118). This could correlate, too, with the lack of activity in Tris buffer in the absence of phosphate.

Furthermore, the PRPP synthesizing capacity would be expected to be high in castor bean endosperm extracts, since PRPP is also a required substrate for the rapid RNA and DNA synthesis which occurs in developing tissues (119).

Since NA was being converted to NAD in the absence of PRPP, one would expect the same of QA. This phenomenon, however, was not observed by Mann (105), who was using castor bean seedlings which were slightly older physiologically. When seedlings at the same growth stage as those used for Product C synthesis were assayed for QA phosphoribosyltransferase activity in the presence and absence of PRPP, the results in Table 6 were obtained. Essentially no enzymatic activity occurred in the absence of both PRPP and ATP. PRPP alone was sufficient to permit almost complete conversion of QA to NaMN. In the absence of PRPP, ATP was able to support some synthesis of NaMN and other nucleotides, but not to the extent that occurred when PRPP was present either alone or with ATP. Of the products obtained in the absence of PRPP, 62% ran at the R_f of NAD in Solvent System I; no attempt was made to determine if other nucleotides which run at this R_f were formed.

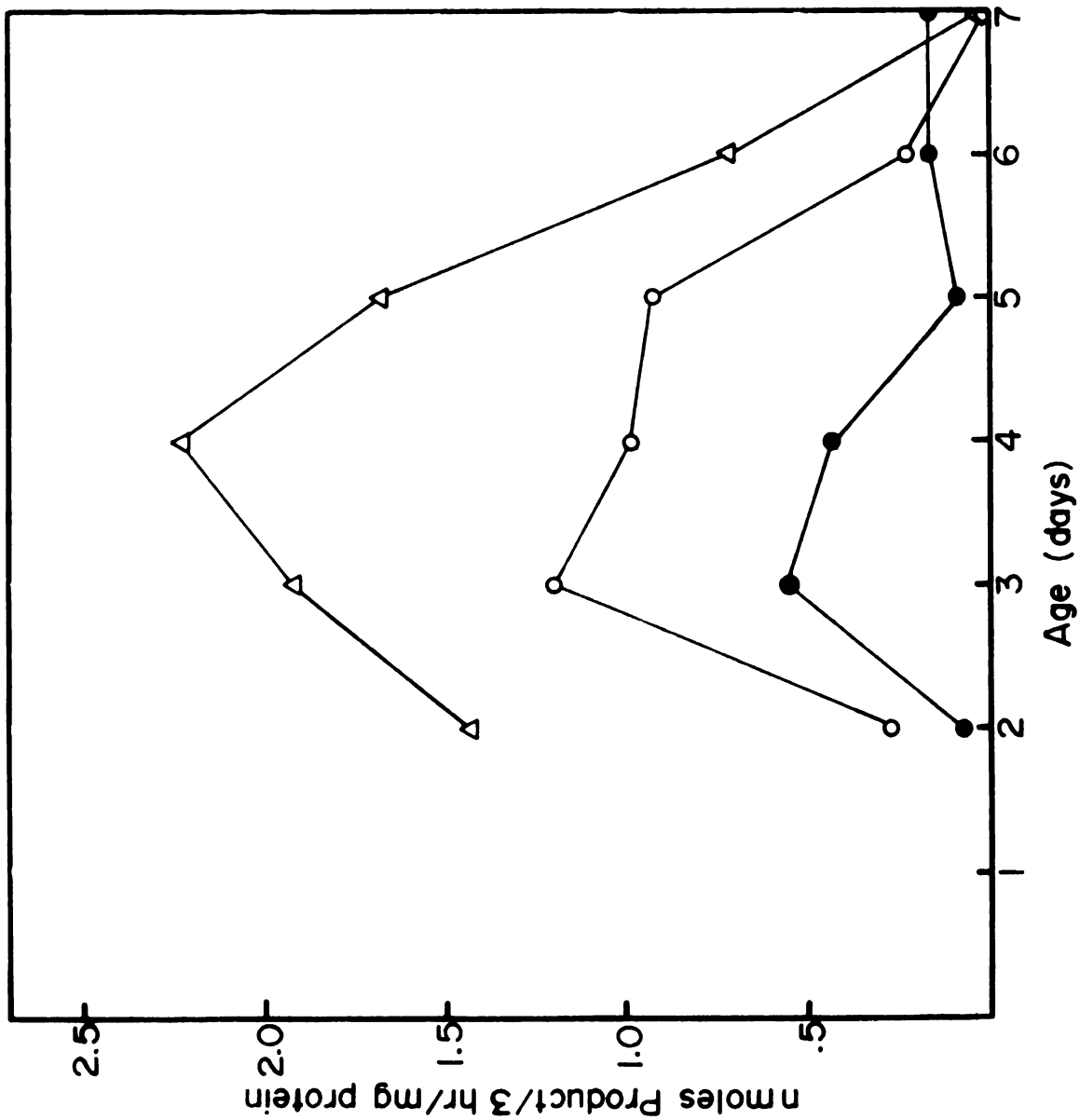
Since it had been noticed throughout this study that the size of the seedlings was very critical to the formation of Product C under these conditions, the enzyme activity was measured over a seven-day germination period. As can be seen in Figure 7, optimum synthesis of Product C occurred on Day 3 and good activity was maintained through Day 5. Product D showed the same optimum, but its formation dropped off a day earlier than that of Product C. Trigonelline synthesis peaked on Day 4, although good activity was also obtained on

TABLE 6. PRPP Requirement of QA Phosphoribosyltransferase. The assays were performed according to the procedure of Mann (105), except that ATP was present at a concentration of 6.0 mM. The incubation time was 3 hr.

Omission	nmoles Product*	% Total Radioactivity
None	59.3	97.2
- PRPP	11.1	18.2
- ATP	53.9	88.4
- PRPP, - ATP	0.1	0.2

*NaMN and NaAD and/or NAD

Figure 7. Formation of Product C, Product D, and Trigonelline during Germination. The incubations were performed as described in the methods section. [7-^{14}C] NA (6.60 mC/mmole) at a concentration of 0.102 mM was used as the radioactive substrate. Solvent System I was used to obtain the results. Each point is the average of the results from duplicate assays. On days 2 and 3 the cotyledons were not removed from the endosperm. Product C (o—o); Product D (●—●); and Trigonelline (△—△).


n moles Product/3 hr/mg protein
2.5
2.0
1.5
1.0
.5
1
2
3
4
5
6
7
Age (days)

Days 2, 3, and 5. In the course of this research, the synthesis of trigonelline always accompanied the synthesis of Product C. This association is evident from the results presented here. By Day 7, NA was no longer being converted to Product C or trigonelline; a minimal amount of Product D was still being formed though. A single attempt to obtain Product C with an extract from mature green castor bean leaf tissue failed. It must be stressed that the seedlings used in the age study were selected according to physiological as well as chronological age. The seedlings harvested on any one day were approximately the same size and exhibited the same physiological characteristics (Table 7). These results, representing in vitro enzymatic activities, do not necessarily represent in vivo NAD synthesis, where substrate and cofactor availability may be controlling factors (115).

In the earlier large scale incubations it was noticed that minor radioactive products were formed. These compounds, which could be eluted from a Dowex 1 formate column, were apparently being masked in paper chromatography by the major products formed. In order to obtain a more complete profile of the reaction products, another large scale incubation of [7-^{14}C] NA of higher specific activity was carried out. Figure 8 shows that twelve radioactive peaks were eluted from a Dowex 1 formate column. The fractions belonging to each of these peaks were pooled, concentrated on a rotary evaporator, and adjusted to a volume appropriate for detection of the radioactive compound(s) by paper chromatography. Chromatography of all twelve fractions was performed in Solvent Systems I, II, and III, and the results were compared to those of standards chromatographed in the same three systems. After this preliminary identification, the

TABLE 7. Physiological Characteristics of Etiolated Castor Bean Seedlings Germinated in Moist Vermiculite at 30°C.

Day	Characteristics
2	The seed coat had just cracked open and the radicle was not over 1.0 cm long. The average fresh weight was 0.4 g/endosperm.
3	The average length of the radicle was 1.35 cm and the average fresh weight was 0.5 g/endosperm.
4	The average length of the radicle was 3.4 cm and the average fresh weight was 0.5 g/endosperm. Secondary roots were present but not well developed.
5	The average length of the hypocotyl was 0.7 cm and the average fresh weight was 0.65 g/endosperm. The endosperm was still firm and there was good secondary root development.
6	The average length of the hypocotyl was 2.7 cm and the average fresh weight was 0.8 g/endosperm. The endosperm was soft.
7	The average length of the hypocotyl was 4.4 cm and the average fresh weight was 0.7 g/endosperm. The endosperm was mushy and nearly consumed.

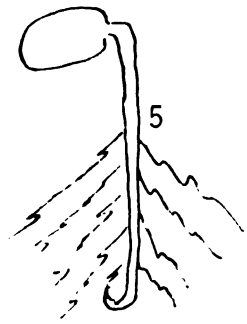
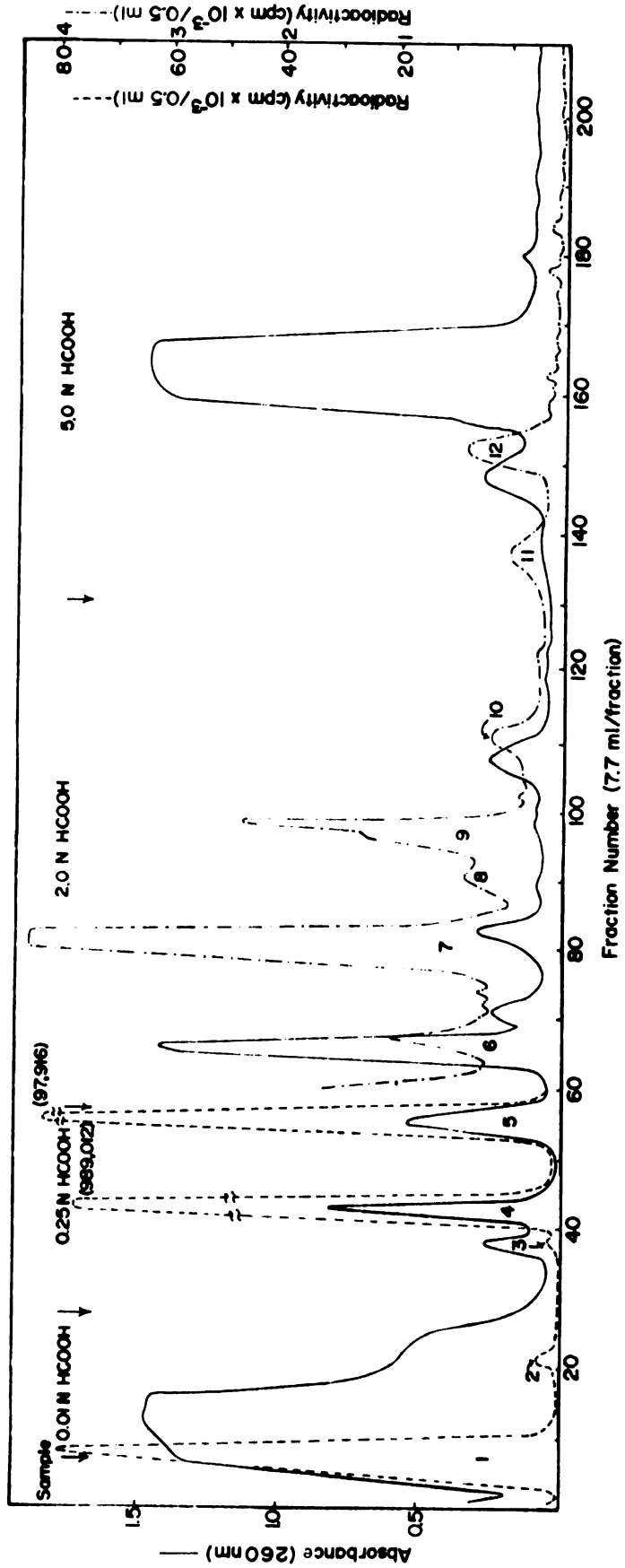


Figure 8. Separation of the Products from [7-^{14}C] NA on a Dowex 1 Formate Column. A large scale incubation (30.0 ml) was performed at 30°C for 4 hr. The [7-^{14}C] NA (6.60 mC/mmole) was at a concentration of 0.098 mM. The products were eluted with a differential HCOOH gradient at a flow rate of 37 ml/hr, as described in the methods section. UV absorbance and radioactivity were determined on each fraction.


Absorbance (260 nm)
Radioactivity (cpm x 10^{-3}/0.5 ml)
Fraction Number (7.7 ml/fraction)
Sample
0.01 N HCOOH
0.25 N HCOOH
(97,945)
(989,012)
2.0 N HCOOH
5.0 N HCOOH
1
2
3
4
5
6
7
8
9
10
11
12

samples were co-chromatographed with the respective standards in these three systems. Six of the twelve fractions from the column were identified by this method (Table 8). Fraction #1 contained 16.7% NMN and 83.3% trigonelline, whereas the other five fractions identified contained a single radioactive compound: NA, NAD, NaMN, NaAD, and NADP. The radioactivity of the fractions migrated coincident with the UV absorbance of the standards for all the samples. In the case of Fraction #7 where a radioactive standard was employed, the two peaks were coincident. The elution pattern of these compounds from a Dowex 1 formate column is consistent with the column data reported by Ijichi et al. (44) and thus supports their identity.

In this particular incubation, paper chromatographic analysis in Solvent System I had indicated four peaks with the following percentages of total radioactivity: Product C, 11.9%; Product D, 4.8%; trigonelline, 17.9%; and NA, 65.4%. Since the R_f on paper was now known for each of the twelve radioactive fractions eluted from the column, it was possible to calculate the percentage composition of the above four radioactive peaks. The peak on paper considered Product C was comprised of 95.8% NAD, 3.4% NaAD, and 0.8% NADP. What was being measured as Product D included 27.8% NaMN and 72.2% NMN. The presence of NMN was not realized until the very end of this study; its identification was therefore limited to its elution pattern from the Dowex 1 formate column and its R_f on paper, as just described. The identification of NADP was also restricted to these two methods, since such a small quantity of it was formed. Trigonelline and NA accounted for 97.7% and 99.3% of the radioactivity on paper which had been attributed to them. The remaining radioactivity in these two

TABLE 8. Co-chromatography of Fractions from the Dowex 1 Formate Column in Figure 7 with Standards. The compounds were detected as described in the methods section.

Sample	R_f of Sample in Solvent Systems		
	I	II	III
#1 + Trigonelline	.27	.62	.50
#1 + NMN	.09	.25	.29
#4 + NA	.69	.73	.67
#5 + NAD	.04	.16	.17, .29*
#7 + [6-^{14}C] NaMN	.10	.21	.32
#9 + NaAD	.04	.13	.31
#11 + NADP	.02	.04	.26

*Two peaks because of streaking; the intensity of the UV absorbance corresponded to the intensity of the radioactivity.

peaks was due to unidentified compounds.

Since [7-^{14}C] NA was the precursor used in this research, it might be expected that it would have lost radioactivity in the form of $^{14}CO_2$ and that the above percentages would thus be invalid. This was checked by incubating the reaction mixture in a vessel equipped for CO_2 collection, as described by Mann (105). Not more than 1.0% of the initial radioactivity was released as $^{14}CO_2$.

Recrystallization to constant specific activity confirmed the identity of the NA and trigonelline eluted from the Dowex 1 formate column (Tables 9 and 10). Since the Product C fraction from the column was now known to be NAD, its alkaline hydrolysis was repeated along with that of authentic NAD. The fractions believed to be NaMN and NaAD were also hydrolyzed and the results compared to those of standards (Table 11). Fraction #5 from the column yielded both Nam and NA, and the former was seen to decrease and the latter to increase from 10 to 60 minutes. The authentic NAD sample behaved similarly, i.e., both Nam and NA could be detected under UV light; the NA spot, which was faint at 10 minutes, became darker by 60 minutes. On the other hand, when Fraction #7 from the column and authentic [7-^{14}C] NaMN were hydrolyzed, no Nam could be detected and NA accounted for the only radioactive peak from both the sample and the standard at 10 minutes and at 60 minutes. Likewise only one radioactive peak and only one UV spot corresponding to NA resulted from the hydrolysis of Fraction #9 from the column and NaAD respectively. A slight complication with Fraction #9 occurred in one solvent system, IV, where there was a small radioactive peak at the origin in addition to the peak for NA. Standard NaAD exhibited the same behavior in this solvent system.

TABLE 9. Recrystallization of [7-^{14}C] NA to Constant Specific Activity. A fraction equivalent to Fraction #4 of the Dowex 1 formate column in Figure 7 was combined with authentic NA and recrystallized from hot water. Specific activities were determined as described in the methods section.*

Crystallization	Absorbance$_{260\ nm}$ (1:20 dilution)	cpm/0.5 ml	Ratio of cpm: absorbance
#1	.58	105	181
#2	.61, .61	106	174
#3	.91, .92	162	178, 176
#4	.48	85	177

*These results were obtained by Anne Bosch (112).

TABLE 10. Recrystallization of [8-^{14}C] Trigonelline to Constant Specific Activity. A fraction equivalent to Fraction #1 of the Dowex 1 formate column in Figure 7 was combined with authentic trigonelline and recrystallized from hot absolute ethanol. Specific activities were determined as described in the methods section.

Crystallization	Absorbance$_{260\ nm}$		cpm/0.5 ml	Ratio of cpm: absorbance	
	1:50 dilution	1:100 dilution		1:50 dilution	1:100 dilution
#1	.770	.385	542	704	1408
#2	.150	.075	104	693	1387
#3	.225, .225	.120	157	698	1308
#4	.162	.084	118	728	1405

TABLE 11. Base Hydrolysis of Standards and of Fractions from the Dowex 1 Formate Column of Figure 7. The samples were hydrolyzed as described in the methods section.

Sample	Product			
	10 min		60 min	
	Nam	NA	Nam	NA
NAD	+	+	+	+
Fraction #5	+ (35%)	+ (65%)	+ (95%)	+ (91%)
[6-^{14}C] NaMN	-	+ (100%)	-	+ (100%)
Fraction #7	-	+ (100%)	-	+ (100%)
NaAD	-	+	-	+
Fraction #9	-	+ (>96%*; 81%‡)	-	+ (>96%*; 82%‡)

*Solvent Systems II and III

‡Solvent System IV

The important point here is that no Nam was detected and NA was.

In a final characterization of the reaction, PRPP and glutamine were added to the reaction mixture to see if an increase in Product C formation could be obtained. As can be seen in Table 12, 40% more Product C was formed when PRPP was added to the incubation mixture. Addition of glutamine was, however, without significant effect. When both PRPP and glutamine were added, the effect was similar to that of PRPP alone. Both glutamine and NH_4Cl have been found to serve as amide donors for the NAD synthetase reaction in yeast (120). The effect of NH_4Cl was not tested in this study.

As stated previously, the ATP present in the incubation mixture could be serving as a PRPP generator. The ATP requirement also encompasses, of course, the role of actual substrate in the synthesis of NaAD. Beyond this are its implicit roles in the NA phosphoribosyltransferase and NAD synthetase reactions. Mann (121) has shown that if ATP was not present, almost no activity of the former enzyme could be obtained in the presence of PRPP with a castor bean endosperm enzyme preparation which had been passed through a Sephadex G-25 column to remove endogenous substrates. A requirement of NA phosphoribosyltransferase for ATP in addition to PRPP has also been demonstrated in yeast (13,118) and bovine liver (123,124). Exceptions are the NA phosphoribosyltransferase found in human erythrocytes (13) and Astasia longa (125). Stoichiometric utilization of ATP in both the NA phosphoribosyltransferase and NAD synthetase reactions has been established in yeast (120,126).

In conclusion, the results presented here indicate the operation of the Preiss-Handler pathway in etiolated castor bean endosperm for

TABLE 12. Effect of PRPP and Glutamine on Product C Synthesis. The assays were performed as described in the methods section, except that the volume was increased by 10 ul; the reagent concentrations therefore were: 62.4 mM potassium phosphate, 6.2 mM dithioerythritol, 3.8 mM ATP, 12.5 mM $MgCl_2$, and .096 mM [7-^{14}C] NA (6.60 mC/mmole).

Addition	nmoles Product C	% Total Radioactivity
None	1.62	10.6
PRPP	2.26	14.8
Glutamine	1.56	10.2
PRPP, Glutamine	2.08	13.6

the following reasons: (1) synthesis of NaMN and NaAD, the two Preiss-Handler intermediates, along with NAD was observed; (2) NA as well as Nam could serve as the substrate in the biosynthetic pathway; and (3) an active Nam deamidase was apparent (Figure 2A). The fact that 3.5% of the total radioactivity (10.1% of the products) corresponded to NMN does not rule out the operation of the Preiss-Handler pathway. Prior conversion of NA to Nam would have had to occur, and this has never been reported. Furthermore, an active nucleotide pyrophosphatase, which could account for the breakdown of NAD to NMN, has been demonstrated in plants (29,127).

All the reaction products identified in this investigation were characterized by paper chromatography in three solvent systems and by their pattern of elution from a Dowex 1 formate column. In addition, NA and trigonelline were recrystallized to constant specific activity and NaMN, NaAD, and NAD were subjected to alkaline hydrolysis. NAD was further identified by its UV spectrum in the absence and presence of KCN and in the absence and presence of yeast alcohol dehydrogenase.

The formation of NAD from Nam or NA under these conditions was dependent on ATP, Mg^{2+}, and another compound(s) which was removed by Sephadex G-25 treatment of the enzyme preparation. The latter may be explained in light of the PRPP requirement of NA phosphoribosyl-transferase and the amide donor requirement of NAD synthetase. NAD was also shown to inhibit its own biosynthesis.

REFERENCES

REFERENCES

1. Kornberg, A., J. Biol. Chem., 176, 1475 (1948).

2. Kornberg, A., J. Biol. Chem., 182, 779 (1950).

3. Imsande, J. and Handler, P., in The Enzymes, (ed. by P. D. Boyer, H. Lardy, and K. Myrbäck), 5, 281, (Academic Press, New York, 1961).

4. Kornberg, A. and Pricer, W. E., Jr., J. Biol. Chem., 191, 535 (1951).

5. Hogeboom, G. H. and Schneider, W. C., J. Biol. Chem., 197, 611 (1952).

6. Preiss, J. and Handler, P., J. Biol. Chem., 225, 759 (1957).

7. Dold, U., Mielsch, M., and Holzer, H., Biochem. Z., 338 349 (1963).

8. Dietrich, L. S., Fuller, L., Yero, I. L., and Martinez, L., Nature, 208, 347 (1965).

9. Dietrich, L. S., Fuller, L., Yero, I. L., and Martinez, L., J. Biol. Chem., 241, 188 (1966).

10. Preiss, J. and Handler, P., J. Amer. Chem. Soc., 79, 1514 (1957).

11. Preiss, J. and Handler, P., J. Amer. Chem. Soc., 79, 4246 (1957).

12. Preiss, J. and Handler, P., J. Biol. Chem., 233, 488 (1958).

13. Preiss, J. and Handler, P., J. Biol. Chem., 233, 493 (1958).

14. Dahmen, W., Webb, B., and Preiss, J., Arch. Biochem. Biophys., 120, 440 (1967).

15. Ohtsu, E., Ichiyama, A., Nishizuka, Y., and Hayaishi, O., Biochem. Biophys. Res. Comm., 29, 635 (1967).

16. Imsande, J., J. Biol. Chem., 236, 1494 (1961).

17. Kahn, V. and Blum, J. J., J. Biol. Chem., 243, 1448 (1968).

18. Godavari, H. R. and Waygood, E. R., Can. J. Bot., 48, 2267 (1970).

19. Sarma, D. S. R., Rajalakshmi, S., and Sarma, P. S., Biochem. Biophys. Res. Comm., 6, 389 (1961).

20. Deguchi, T., Ichiyama, A., Nishizuka, Y., and Hayaishi, O., Biochim. Biophys. Acta, 158, 382 (1968).

21. Collins, P. B., and Chaykin, S., J. Biol. Chem., 247, 778 (1972).

22. Lin, L-F. H., and Henderson, L. M., J. Biol. Chem., 247, 8023 (1972).

23. Porcellati, G., Giorn. Biochem., 3, 231 (1954).

24. Langan, T. A., Jr., Kaplan, N. O., and Shuster, L., J. Biol. Chem., 234, 2161 (1959).

25. Friedmann, H. C. and Garstki, C., Biochem. Biophys. Res. Comm., 50, 54 (1973).

26. Joshi, J. G. and Handler, P., J. Biol. Chem., 237, 929 (1962).

27. Handler, P. and Klein, J. R., J. Biol. Chem., 143, 49 (1942).

28. Kornberg, A. and Landberg, O., J. Biol. Chem., 176, 665 (1948).

29. Roberts, D. W. A., J. Biol. Chem., 234, 655 (1959).

30. Bevilacqua, L. R., Atti Accad. Nazl. Lincei Rend. Classe Sci. Fis. Mat. e Nat., 16, 107 (1954).

31. Kaplan, N. O., Colowick, S. P., and Nason, A., J. Biol. Chem., 191, 473 (1951).

32. Villela, G. G., Nature, 213, 79 (1967).

33. Hughes, D. E. and Williamson, D. H., Biochem. J., 51, 330 (1952).

34. Sarma, D. S. R., Rajalakshmi, S., and Sarma, P. S., Biochim. Biophys. Acta, 81, 311 (1964).

35. Ryrie, I. J. and Scott, K. J., Biochem. J., 115, 679 (1969).

36. Newell, J. A. and Waller, G. R., Proc. Oklahoma Acad. Sci., 45, 143 (1965).

37. Kaplan, N. O., Goldin, A., Humphreys, S. R., and Stolzenbach, F. E., J. Biol. Chem., 226, 365 (1957).

38. Chaykin, S., Dagani, M., Johnson, L., and Samli, M., J. Biol. Chem., 240, 932 (1965).

39. Chaykin, S., Dagani, M., Johnson, L., Samli, M., and Battaile, J., Biochim. Biophys. Acta, 100, 351 (1965).

40. Petrack, B., Greengard, P., Craston, A., and Kalinsky, H. J., Biochem. Biophys. Res. Comm., 13, 472 (1963).

41. Petrack, R., Greengard, P., Craston, A., and Sheppy, F., J. Biol. Chem. 240, 1725 (1965).

42. Greengard, P., Petrack, B., and Kalinsky, H. J., Biochim. Biophys. Acta, 184, 148 (1969).

43. Kirchner, J., Watson, J. G., and Chaykin, S., J. Biol. Chem., 241, 953 (1966).

44. Ijichi, H., Ichiyama, A., and Hayaishi, O., J. Biol. Chem., 241, 3701 (1966).

45. Tanigawa, Y., Shimoyama, M., Murashima, R., Ito, T., Yamaguchi, K., and Ueda, I., Biochim. Biophys. Acta, 201, 394 (1970).

46. Ikeda, M., Tsuji, H., Nakamura, S., Ichiyama, A., Nishizuka, Y., and Hayaishi, O., J. Biol. Chem., 240, 1395 (1965).

47. Greenbaum, A. L. and Pinder, S., Biochem. J., 107, 55 (1968).

48. Hagino, Y., Lan, S. J., Ng, C. Y., and Henderson, L. M., J. Biol. Chem., 243, 4980 (1968).

49. Lin, L-F. H., Lan, S. J., Richardson, A. H., and Henderson, L. M., J. Biol. Chem., 241, 8016 (1972).

50. Waller, G. R., Yang, K. S., Gholson, R. K., and Hadwiger, L. A., J. Biol. Chem., 241, 4411 (1966).

51. Hundley, J. M., in The Vitamins, (ed. by W. H. Sebrell, Jr., and R. S. Harris), 2, 565, (Academic Press, New York, 1954).

52. Quoted by J. M. Hundley, in The Vitamins, (ed. by W. H. Sebrell, Jr., and R. S. Harris), 2, 553, (Academic Press, New York, 1954).

53. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., Science, 101, 489 (1945).

54. Meister, A., Biochemistry of the Amino Acids, 2nd ed. 2, 849, (Academic Press, New York, 1965).

55. Priest, R. E., Bokman, A. H., and Schweigert, B. S., Proc. Soc. Exptl. Biol. Med., 78, 477 (1951).

56. Chaykin, S., Ann. Rev. Biochem., 36, Pt. 1, 149 (1967).

57. Nishizuka, Y., and Hayaishi, O., J. Biol. Chem., 238, 3369 (1963).

58. Partridge, C. W. H., Bonner, D. M., and Yanofsky, C., J. Biol. Chem., 194, 269 (1952).

59. Ahmad, F. and Moat, A. G., J. Biol. Chem., 241, 775 (1966).

60. Henderson, L. M., Someroski, J. F., Rao, D. R., Wu, P-H. L., Griffith, T., and Byerrum, R. U., J. Biol. Chem., 234, 93 (1959).

61. Yanofsky, C., J. Bacteriol., 68, 577 (1954).

62. Wilson, R. G. and Henderson, L. M., J. Bacteriol., 85, 221 (1963).

63. Ortega, M. V. and Brown, G. M., J. Biol. Chem., 235, 2939 (1960).

64. Gross, D., Feige, A., Stecher, R., Zureck, A., and Schütte, H. R., Z. Naturforschg., 20, 1116 (1965).

65. Scott, T. A. and Hussey, H., Biochem. J., 96, 9C (1965).

66. Isquith, A. H. and Moat, A. G., Biochem. Biophys. Res. Comm., 22, 565 (1966).

67. Scott, T. A., Bellion, E., and Mattey, M., Eur. J. Biochem., 10, 318 (1969).

68. Mattey, M. and Robbie, L. J., Biochem. J., 119, 36P (1970).

69. Ogasawara, N., Chandler, J. L. R., Gholson, R. K., Rosser, R. J., and Andreoli, A. J., Biochim, Biophys. Acta, 141, 199 (1967).

70. Chandler, J. L. R., Gholson, R. K., and Scott, T. A., Biochim. Biophys. Acta, 222, 523 (1970).

71. Chandler, J. L. R. and Gholson, R. K., Biochim. Biophys. Acta, 264, 311 (1972).

72. Suzuki, N., Griffith, G., and Gholson, R. K., Fed. Proc., 30, Pt. 2, 1252, Abs. #1167, (1971).

73. Fleeker, J. and Byerrum, R. U., J. Biol. Chem., 242, 3042 (1967).

74. Hadwiger, L. A., Badei, S. E., Waller, G. R., and Gholson, R. K., Biochem. Biophys. Res. Comm., 13, 466 (1963).

75. Dawson, R. F., Christman, D. R., D'Adamo, A., Solt, M. L., and Wolf, A. P., J. Amer. Chem. Soc., 82, 2628 (1960).

76. Andreoli, A. J., Ikeda, M., Nishizuka, Y., and Hayaishi, O., Biochem. Biophys. Res. Comm., 12, 92 (1963).

77. Jackanicz, T. M. and Byerrum, R. U., J. Biol. Chem., 241, 1296 (1966).

78. Fleeker, J. and Byerrum, R. U., J. Biol. Chem., 240, 4099 (1965).

79. Yang, K. S. and Waller, G. R., Phytochem., 4, 881 (1965).

80. Saxton, R. E., Rocha, V., Rosser, R. J., Andreoli, A. J., Shimoyama, M., Kosaka, A., Chandler, J. L. R., and Gholson, R. K., Biochim. Biophys. Acta, 156, 77 (1968).

81. Calbreath, D. F. and Joshi, J. G., J. Biol. Chem., 246, 4334 (1971).

82. Dietrich, L. S. and Muniz, O., Fed. Proc., 25, 747, Abs. #3152, (1966).

83. Dietrich, L. S. and Muniz, O., Biochem., 11, 1691 (1972).

84. Meister, A., Biochemistry of the Amino Acids, 2nd ed., 2, 850, (Academic Press, New York, 1965).

85. Wagner, C., Biochem. Biophys. Res. Comm., 17, 668 (1964).

86. Yamaguchi, K., Shimoyama, M., and Gholson, R. K., Biochim. Biophys. Acta, 146, 102 (1967).

87. Greengard, P., Guinn, G. P., and Reid, M. B., J. Biol. Chem., 239, 1887 (1964).

88. Cho-Chung, Y. S. and Pitot, H. C., J. Biol. Chem., 242, 1192 (1967).

89. Knox, W. E. and Auerbach, V. H., J. Biol. Chem., 214, 307 (1955).

90. Frieden, E., Westmark, G. W., and Schor, J. M., Arch. Biochem. Biophys., 92, 176 (1961).

91. Pallini, V., Vasconetto, G., and Picsi, C., Giorn. Biochim., 14, 439 (1965).

92. Schor, J. M. and Frieden, E., J. Biol. Chem., 233, 612 (1958).

93. Greengard, P., Petrack, B., Craston, A., and Kalinsky, H. J., Biochem. Biophys. Res. Comm., 13, 478 (1963).

94. Waygood, E. R., Mishra, D., and Godavari, H. R., in Biochemistry and Physiology of Plant Growth Substances, (ed. by F. Wrightman and G. Setterfield), 1495, (Runge Press, Ottawa, Canada, 1968).

95. Kornberg, A., J. Biol. Chem., 182, 805 (1950).

96. Imsande, J. and Pardee, A. B., J. Biol. Chem., 237, 1305 (1962).

97. Yamamoto, Y., Plant Physiol., 41, 523 (1966).

98. Wang, T. P., Kaplan, N. O., and Stolzenbach, F. E., J. Biol. Chem., 211, 465 (1954).

99. Katchman, B., Betheil, J. J., Schepartz, A. I., and Sanadi, D. R., Arch. Biochem. Biophys., 34, 437 (1951).

100. Meister, A., Biochemistry of the Amino Acids, 2nd ed., 2, 861, (Academic Press, New York, 1965).

101. Hundley, J. M., in The Vitamins, (ed. by W. H. Sebrell, Jr., and R. S. Harris), 2, 515, (Academic Press, New York, 1954).

102. Cantoni, G. L., J. Biol. Chem., 189, 203 (1951).

103. Cantoni, G. L., J. Biol. Chem., 204, 403 (1953).

104. Stul'nikova, R. I., Nauk. Zapiski Chernivetsk Uni., 23, 102 (1956).

105. Mann, D. F., Ph. D. Thesis, Michigan State Univ., (1972).

106. Joshi, J. G. and Handler, P., J. Biol. Chem., 235, 2981 (1960).

107. Joshi, J. G. and Handler, P., J. Biol. Chem., 237, 3185 (1962).

108. Jahns, E., Ber. Deut. Chem. Ges., 18, 2518 (1885).

109. Willaman, J. J. and Li, H. L., Alkaloid Bearing Plants and Their Contained Alkaloids, Supplement to Lloydia, 33, 280 (1970).

110. Hundley, J. M., in The Vitamins, (ed. by W. H. Sebrell, Jr., and R. S. Harris), 2, 537, (Academic Press, New York, 1954).

111. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

112. Bosch, A., Unpublished Results.

113. Mahler, H. R. and Cordes, E. H., Biological Chemistry, 248, (Harper and Row, New York, c. 1966).

114. Kaplan, N. O., in The Enzymes, (ed. by P. D. Boyer, H. Lardy, and K. Myrbäck), 2nd ed., 3, Pt. B, 130 (Academic Press, New York, 1960).

115. Pfleiderer, G., Sann, E., and Ortanderl, F., Biochim. Biophys. Acta, 73, 39 (1963).

116. Fawcett, C. P. and Kaplan, N. O., J. Biol. Chem., 237, 1709 (1962).

117. Switzer, R. L. and Sogin, D. C., J. Biol. Chem., 248, 1063 (1973).

118. Hershko, A., Razin, A., and Mager, J., Biochim. Biophys. Acta, 184, 64 (1969).

119. Ferris, G. M. and Clark, J. B., Biochem. J., 121, 655 (1971).

120. Yu, C. K. and Dietrich, L. S., J. Biol. Chem., 247, 4794 (1972).

121. Mann, D. F., Unpublished Results.

122. Kosaka, A., Spivey, H. O., and Gholson, R. K., J. Biol. Chem., 246, 3277 (1971).

123. Imsande, J., and Handler, P., J. Biol. Chem., 236, 525 (1961).

124. Smith, L. D. and Gholson, R. K., J. Biol. Chem., 244, 68 (1969).

125. Kahn, V. and Blum, J. J., Biochim. Biophys. Acta, 146, 305 (1967).

126. Honjo, T., Nakamura, S., Nishizuka, Y., and Hayaishi, O., Biochem. Biophys. Res. Comm., 25, 199 (1966).

127. Kornberg, A. and Pricer, W. E., Jr., J. Biol. Chem., 182, 763 (1950).