SOME ASPECTS OF NICOTINAMIDE METABOLISM

IN THE LABORATORY RAT

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in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1973

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ACKNOWLEDGMENTS

The author wishes to express his gratitude to his major adviser, Dr. Robert K. Gholson, for making these years an enriching personal and educational experience.

Thanks are extended to Dr. K. E. Ebner, Dr. E. J. Eisenbraun, Dr. E. T. Gaudy, and Dr. R. E. Koeppe for serving as members of the author's advisory committee, to Mr. Steve Wash for his conscientious technical assistance, and to Mrs. Emily Fuller for her preparation of the final manuscript.

The author is indebted to the Department of Biochemistry for providing the facilities for this study and to the people of the United States and, in particular, of Oklahoma who through various agencies supplied the financial support which made this portion of the author's education possible.

Special gratitude is extended to my wife, Linda, for her assistance in the preparation of this thesis and for her fortitude and understanding during its stormy creation, to my parents for their lifelong support and encouragement, and to the Faculty and Staff of the Department of Biochemistry and their families who in so many ways made the work easier and life in Stillwater enjoyable and rewarding.

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NA	Nicotinic acid
NAm	Nicotinamide
NMNAm	N^1-Methynicotinamide
NAmNO	Nicotinamide-N^1-oxide
2-pyridone	N^1-Methyl-2-pyridone-5-carboxamide
4-pyridone	N^1-Methyl-4-pyridone-3-carboxamide
6-OHNA	6-Hydroxynicotinic acid
6-OHNAm	6-Hydroxynicotinamide
NAgly	Nicotinuric acid
5-MNAm	5-Methylnicotinamide
QA	Quinolinic acid
PRPP	5-Phosphorylribose-1-pyrophosphate
DHAP	Dihydroxyacetone phosphate
NAMN	Nicotinic acid mononucleotide
NMN	Nicotinamide mononucleotide
dNAD	desamido-NAD
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
FAD	Flavin adenine dinucleotide
SAM	S-Adenosyl-L-methionine
QARTase	QA phosphoribosyltransferase
NARTase	NA phosphoribosyltransferase
NAmRTase	NAm phosphoribosyltransferase
NMTase	NAm N^1-methyltransferase
MATase	Methionine adenosyltransferase
HIOMTase	Hydroxyindole O-methyltransferase
GSH	Growth stimulating hormone
ACTH	Adrenocorticotrophic hormone
HCHS	Hydrocortisone hemisuccinate
T	Triamcinolone
BSA	Bovine serum albumin

CHAPTER I

INTRODUCTION

The discovery that nicotinamide was a component of coenzyme I (NAD) and coenzyme II (NADP) (1,2) and the demonstration that nicotinamide and nicotinic acid were effective in the prevention and cure of canine black tongue (3,4) and human pellegra (5,6) led to an extensive study of the metabolism and physiological effects of this vitamin.

The biosynthetic pathways for the formation of NAD from nicotinic acid and nicotinamide have been well established (7), although the relative significance of the various pathways is still in dispute.

Kaplan et al. (8,9) showed that hepatic NAD was markedly elevated by nicotinamide challenge (500 mg/Kg). Greengard et al. (10) reported that hypophysectomy increased both the degree and duration of this elevation. Further studies established that the pituitary gland may function in the regulation of NAD biosynthesis primarily through its control of the thyroid and adrenal glands (11). Other work has implicated enzyme inhibition by substrates and end products in the control of NAD(P) synthesis (12-16).

In addition to the unchanged vitamers, seven metabolites of nicotinic acid and nicotinamide have been isolated and identified in the urine of various mammalian species. These metabolites are: N^1-methylnicotinamide (17), nicotinuric acid (17), nicotinamide-N-oxide (18), N^1-methyl-2-pyridone-5-carboxamide (19), N^1-methyl-4-pyridone-3-

carboxamide (20), 6-hydroxynicotinamide (21), and 6-hydroxynicotinic acid (21).

Previous studies in these laboratories demonstrated that marked differences existed between the metabolite excretion patterns observed in normal and hypophysectomized rats when either low (5 mg/Kg) or challenge doses of nicotinamide were administered (22). The largest of these differences occurs in the excretion of N-methylnicotinamide where hypophysectomized rats were found to excrete approximately one-quarter of the amount observed in the urines of normal animals.

The latter observation suggested that lower levels of nicotinamide-N-methyltransferase (E.C.2.1.1.1) might exist in the hypophysectomized rat as a result of endocrinectomy. However, preliminary investigation (23) showed the basal levels of enzyme activity present in hypophysectomized rat liver to be higher than in the liver of the normal rat. Of all treatments tested, only the administration of challenge doses of nicotinamide appeared to effect any change in the enzyme activity observed. This treatment resulted in approximately a two-fold increase in the enzyme activity in both types of rat.

It was originally hypothesized that nicotinamide-N-methyltransferase might be lower in the hypophysectomized rat and would be elevated by glucocorticoid therapy as was found to be the case with tryptophan oxygenase (24). However glucocorticoids were found to have no influence on enzyme activity. The above experiments therefore failed to explain the differences in metabolite excretion which were previously observed and the two sets of observations appear to be paradoxical.

It was the purpose of this study to investigate the factor(s) responsible for the observed differences in the excretion of N-methyl-

nicotinamide and nicotinamide and for the elevation of nicotinamide-N-methyltransferase activity resulting from nicotinamide challenge.

CHAPTER II

LITERATURE REVIEW

It is not within the scope of this work to attempt an in depth review of the discovery and characterization of NAD, nicotinic acid, nicotinamide, and related compounds, nor to fully document the many physiological and pharmacological effects of these compounds in biological systems. For a broad current coverage of the area as it applies to mammalian systems, the reader is referred to the proceedings of a workshop on the metabolic effects of nicotinic acid and its derivatives (25) and its extensive general bibliography. Special reference should also be made to reviews by Chaykin (7), Colowick (26), Kaplan (27,28), and Sund (29).

Catabolism of Nicotinic Acid and Nicotinamide

Since the synthesis of nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C (30) many papers dealing with the metabolism of these compounds have been published. These investigations (18,20,21,31-35) have established N^1-methylnicotinamide (NMNAm), nicotinic acid (NA), nicotinamide (NAm), nicotinamide-N-oxide (NAmNO), N^1-methyl-2-pyridone-5-carboxamide, N^1-methyl-4-pyridone-5-carboxamide, 6-hydroxynicotinamide (6-OHNAm), 6-hydroxynicotinic acid (6-OHNA), and nicotinuric acid (NAgly) as urinary excretion products of nicotinic acid and nicotinamide in various mammalian species. The release of carbon dioxide-^{14}C has been

observed in studies using the vitamers labelled in the carbon-7 position (31,35-38).

The metabolic transformations involved in the conversion of NA and NAm to their corresponding catabolites include decarboxylation, conjugation, methylation, and oxidation processes.

Decarboxylation

Carbon dioxide-^{14}C has been shown to be present in the expired air of the rat, mouse, dog, and hamster following the administration of either NA-7-^{14}C or NAm-7-^{14}C. Lee et al. (35) showed that decarboxylation was a microbial process by demonstrating that no labelled CO_2 was liberated when the vitamers were administered to germ-free rats. The mechanism of this microbial decarboxylation is unknown. In rats, approximately twice as much CO_2 is released from NAm as from NA (27,36). It is possible that the evolution of CO_2 involves an oxidative deamidation thus requiring NA to be recycled through NAD to NAm before it can be subjected to the loss of C-7, or, perhaps, a single enzyme with a markedly different affinity for each of the vitamers is responsible. No studies have been carried out using the ring-labelled vitamer, therefore nothing is known about the fate of the pyridine ring of the vitamers in the mammalian gastrointestinal tract. Kung and Tsai (39) have shown a pathway for the degradation of NA to pyruvate via 6-OHNA which is believed responsible for the degradation of NA in Clostridium barkeri (40). In this system C-7 of NA becomes C-1 in pyruvate. If such a pathway was possessed by any of the microbial species common to the mammalian gastrointestinal system, this too could explain the mechanism of CO_2 release. If this were the case, the greater CO_2 release from NAm might be explained on the basis of its more effective

compartmentalization in the gastrointestinal tract where it can be readily deamidated to supply NA to such a microbial pathway. Support for such a compartmentalization can be found if one considers the free and reversible passage of the vitamers across the gut membranes in combination with the rapid removal of nicotinic acid from the system by the internal organs.

Conjugation

NAgly is the primary condensation product of NA found in mammalian urine. Traces of β-nicotinyl glucuronide have been observed in rat urine (41); however, NA administration fails to enhance its excretion and its formation appears to be of no quantitative importance.

NAgly, on the other hand, can be a quantitatively significant urinary metabolite. It is one of the major compounds observed in urine following the administration of large doses of NA. It arises from the condensation of glycine and NA. The synthesis occurs in both liver and kidney. The enzyme responsible for the formation of this product appears to be localized in the mitochondria (42,43). A nicotinyl-coenzyme-A intermediate has been implicated in NAgly biosynthesis. The basis for this conclusion was the demonstration that the addition of Coenzyme-A to purified enzyme preparations stimulated the observed activity and that pantothenic acid deficiency in rats decreased the excretion of this conjugation product following the administration of large doses of NA (44).

Methylation

NMNAm is a major excretion product of both NA and NAm. Its formation from NAm is catalyzed by a soluble enzyme which is localized in

the liver. This enzyme, nicotinamide-N-methyltransferase (NMTase), was first studied by Cantoni (45,46). The enzyme requires S-adenosyl-L-methionine (SAM) as a methyl donor.

NMTase activity is not found in all mammalian systems. It is essentially absent in sheep since neither the enzyme was detected (45) nor its product or related oxidation products found in any quantity in sheep urine (46). The enzyme does not catalyze the methylation of NA (47). Trigonelline (N^1-methylnicotinic acid) is not normally observed in mammalian urines unless it or a precursor is consumed in the diet (48-50). The only known dietary precursor of trigonelline is niacytin, a complex molecule representing the bound NA of cereal grains. The mechanism of formation of trigonelline from niacytin is not known but its formation appears to take place while NA is in the bound state. It has also been demonstrated that trigonelline is not utilized as a precursor for NA in mammalian systems (51,52). Indeed the only catabolite of either vitamer which mammalian systems have an apparent capacity to recover is NAmNO.

The excretion of NMNAm is depressed in rats suffering from a cobalamine (vitamin B_{12}) deficiency (53). This may be due to a lack of methionine required for the formation of SAM. It has been established that a cobamide enzyme is required for the conversion of homocysteine and N^5-methylpteroyltriglutamic acid to methionine in Escherichia coli. Such a requirement has not been established in mammalian systems (54). This effect of a cobalamine deficiency thus may be due to some other yet to be understood factor or it may indeed indicate the involvement of a cobamide enzyme in the conversion of homocysteine to methionine in mammalian systems. This is distinctly possible, since the utilization

of SAM as a methyl donor generates homocysteine which must be reconverted to methionine by the above pathway. Interruption of this cycle could induce a methionine (and thus a methyl donor) deficiency in animals receiving minimal dietary methionine. Before this finding can be properly understood, experiments involving B_{12} deficiency would have to be performed with careful consideration being given to the methionine/cysteine status of such deficient animals.

Oxidation

Several oxidation processes are involved in the catabolic transformation of NA and NAm.

Two minor components of rat urine, which are direct oxidation products of the respective vitamers (6-OHNA and 6-OHNAm) have recently been isolated and shown to be of mammalian origin (21). The mode of synthesis of these compounds is unknown.

Another direct oxidation product of NAm is NAmNO. This compound can be a quantitatively significant component of mammalian urine (18,32). The oxidation is catalyzed by a liver microsomal system which requires both NADP and molecular oxygen (55,56). A soluble enzyme system has been observed to have the capacity for reduction of NAmNO to NAm (57). This enzyme system was isolated from hog liver and the reduction was shown to be dependent upon NADH or a low molecular weight constituent found in boiled liver extract (58). It was also shown that the enzyme preparation had xanthine oxidase activity, and that all acceptable electron donors were known substrates for xanthine oxidase (59). It was further shown that xanthine oxidase catalyzed the incorporation of the oxygen of NAmNO into uric acid as well as the NADH dependent

reduction of NAmNO (60). These two reduction reactions are thus believed to be catalyzed by two independent sites on xanthine oxidase.

Finally, NMNAm can be oxidized to either the 2- or 4-pyridone (N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide, respectively). The formation of both of these compounds is apparently catalyzed by the enzyme N^1-methylnicotinamide oxidase. The incorporated oxygen has been shown to be derived from water (61,62). The ratio of 2-pyridone to 4-pyridone in the urine is found to be constant within a species but widely variable between species ranging from 100/1 in the rabbit to 1.2/1 in the rat. Using either the 2- or the 4-pyridone as a measure of enzyme activity Felsted and Chaykin (63) found a constant K_m for NMNAm and differing V_{max} values.

NMNAm oxidase has been shown to be under both hormonal and genetic control (64-66). The enzyme activity has been demonstrated to vary between sexes within a strain and between different strains of mice.

Huff and Chaykin (64) demonstrated that mice of the C57BL/6 strain possess ten times the oxidase activity observed in the DBA/2 strain. This was credited at least in part to a two-fold change in K_m. These workers also showed a two- to four-fold greater activity in the adult males as compared to the adult females in each strain. It was suggested that this sex difference was a result of testosterone mediated induction since androgen was found to be essential to the initiation and maintenance of the increased enzyme activity. Testosterone administration was found to stimulate the liver oxidase in both intact females and immature males. The slight oxidase activity observed in lung tissue was found to be insensitive to androgenic influence (65). Other workers (66) reported similar results in comparing the t^3/t^3 strain with the

DBA/1 strain. They found the liver enzyme to be in the soluble fraction. In agreement with the findings of Huff and Chaykin (64,65), breeding experiments (66) indicated that the level of enzyme activity was controlled by a pair of autosomal alleles, one determining the high level and the other the low level of enzyme activity. A greater variation in the activity observed in males as compared to females was noted. The administration of estradiol was shown to depress enzyme activity in the adult male. Testosterone administration slightly stimulated the activity in the intact female. Dexamethasone (a potent synthetic glucocorticoid) was found to depress activity to half the normal levels observed in both sexes.

The Absorption and Excretion of Nicotinic Acid, Nicotinamide, and Related Catabolites

Early studies on NAm and NA metabolism and excretion suffered from an incomplete knowledge of the compounds involved and the necessity of utilizing relatively insensitive chemical methods for quantitation of the recognized metabolites. With the advent of radiotracer methodology and the application of chromatographic techniques, quantitative excretion studies became more feasible.

Relatively little is known about the dynamics of the distribution of this family of compounds in the intact system. The distributional kinetics of the unchanged vitamers with respect to dose is of particular importance to the understanding of their metabolic fate.

Excretion

The quantity and distribution of the catabolites excreted has been shown to vary with species, age, sex, diet, dosage and form of the

vitamin administered, and route of administration. The nutritional, hormonal, and disease statuses and the genetic makeup of the animals in question are also involved (22,31-35,44,45,53,63-69). The inconsistent mixing of all of these variables can and indeed has contributed to the variability in the excretion data available. Lack of recognition of many of these factors combined with a general penchant for utilizing hyperphysiological doses has contributed to the controversy surrounding the metabolism of NAm and NA.

The most complete data on excretion of the urinary catabolites of NAm and NA has been reported by Chaykin et al. (32) in mice and by Greengard and coworkers (33,34) and Lee, et al. (22,35) in rats. All of these studies have involved the administration of relatively high doses of NAm and NA (the lowest in the rat being 5 mg/Kg). The lowest of these doses approximates the expected daily intake of an animal being maintained on a normal commercial diet. These diets in themselves are fortified to levels in excess of both the nutritional requirements of the animal and those levels which would normally be attainable in a naturally selected diet. The above doses were administered to fully fed animals which had therefore already received more than the physiologically required amounts of vitamin in one form or another.

In order for the dietary vitamin to become available, it must be solubilized from an essentially dry feed stuff. This necessitates the total wetting of the diet and may also require its partial or complete digestion. The daily food consumption is spread over a period of many hours. Thus, the dietary vitamin would tend to be infused into the metabolit pools over an extended period. A single intraperitoneal or per os administration of readily available, water solubilized, vitamin

equivalent to the expected daily intake in addition to that already consumed does not therefore represent a true physiological dose. Further, since trace doses of nicotinic acid are so readily taken up by the tissues (liver in particular) and converted to NAD, one would expect its eventual and essentially complete conversion to NAm. Therefore, no particularly significant differences in the metabolite excretion pattern should be observed regardless of the dietary form of the vitamin supplied. Experimental support for the above argument is to be found in the work of Feigelson et al. (70). Rats were given NA in their drinking water at a level yielding an intake of approximately 50 mg/Kg/day. Administration of this quantity of the vitamer as a single dose will yield a maximum NA mediated elevation of hepatic NAD. After two weeks of NA supplementation at the above level, however, there was no change in hepatic pyridine nucleotide content.

Of the above cited excretion studies, probably the most reliable are the studies of Greengard and coworkers (33). Their work is based on relatively large groups of animals which were well matched for age, weight, sex, and nutritional status. If one considers the findings obtained from the injection of 5 mg/Kg of each vitamer reported by these workers, there is an apparently marked difference in the metabolite excretion patterns obtained for each of the vitamers when excretion is considered over a 24 hour period. However, if the data for the first 2 hours are eliminated the differences are drastically reduced and if the data for the first 4 hours are eliminated the differences disappear. Of the label excreted during the 4-24 hour period, 90% is in the form of NMNAm and the 2- and 4-pyridone regardless of the original form of the vitamer administered. It is noteworthy that 50% of the administered

label still remained to be excreted after 24 hours in both cases. It would thus appear that in the rat the predominant basal urinary catabolites of both NAm and NA are NMNAm and the two pyridones with NAm and NAmNO constituting the bulk of the remaining products. Only traces of NA and/or NAgly are excreted. This latter situation is indicative of the fact that any NA generated under physiological conditions will be very efficiently utilized in the synthesis of pyridine nucleotides.

The administration of hyperphysiological doses of a compound can be a useful means of delineating the feasible metabolic pathways involved in the utilization of that compound. However, as is all too frequently presumed in many areas of biological research, the results of such investigations cannot be taken to reflect the physiological norm. A case in point is the work of Chaykin et al. (32). These workers utilized a very complex experimental design aimed at determining the urinary excretion patterns of nicotinic acid and nicotinamide in mice maintained on various dietary regimens. It is not clear whether the imposition of a "niacin deficiency" was accompanied by a concurrent restriction of dietary tryptophan. Moreover, the duration of the imposed dietary restriction was not sufficient to permit the development of any clinical symptoms of such a deficiency even in the absence of all exogenous injections. Imposed on this design were marked changes in age (2-6 weeks) and weight (13-36 gms). There was one animal per dietary treatment to which multiple doses of either NAm or NA (1-500 mg/Kg) were administered during the experimental period by either the intraperitoneal or oral routes. Comparisons between the excretion patterns obtained for NA and NAm did not appear to be based on comparable doses. The best estimate is that the dose of nicotinic acid was

as much as twice that of nicotinamide. All comparisons were made on the bases of a complete 24 hour excretion period or were restricted to the first 4 hours following administration when, as previously shown in the rat, the entire and only observable differences are seen (at a 5 mg/Kg dose level). Under no circumstances was more than 23% of the injected dose accounted for in the short term studies and in most instances the interpretations were based on recoveries of from 1-10% of the injected dose.

The excretion of NA and NAgly derived from the administration of hyperphysiological doses of NA, or of NAm and related metabolites derived from a similar administration of NAm, cannot be taken as evidence which bears on the metabolic involvement of either of these vitamers in NAD metabolism. Nor can the use of such methods be expected to yield physiologically representative results. Yet based on this type of data derived from a weakly designed experiment the above authors (32) have reached many conclusions concerning the influence of a dietary niacin deficiency, the absorption of the two vitamers from the digestive tract, and the function of the pyridine nucleotide cycle. This work has been much quoted in subsequent years and it and similar reports with their associated weak and often unfounded presumptions have contributed significantly (in a nonconstructive manner) to the current controversy enveloping the area of pyridine nucleotide biosynthesis.

The work of Lee *et al.* (22,35) is open to the same criticism with respect to dose levels used. In addition the numbers of animals utilized was small and these were run in groups rather than individually thus eliminating any possible measure of interanimal variability which has in fact never been reported in any of the recent work. Also

comparisons were made between normal mature males and pre- or peri-pubertally hypophysectomized animals obtained from two different sources although the animals were originally derived from the same Sprague Dawley stock. A similar criticism applies to the work to be reported here although differences in age were eliminated and the animals were run individually. There is, however, a generally good agreement between the findings of Lee et al. (22) and those of Greengard et al. (34) in normal and hypophysectomized male (22) and female (34) rats receiving a NAm challenge. The data obtained by Lee (22) using 5 mg/Kg and 500 mg/Kg doses of NAm or NA in normal adult males are also in good agreement with the findings of Petrack et al. (33) in similarly dosed adult female rats. No comparable data are available for hypophysectomized animals receiving the lower dose.

The urinary excretion data discussed above are widely considered to reflect basal conditions. As should be apparent from the above discussion such a presumption is probably totally invalid. Thus, any conclusions concerning basal pyridine nucleotide biosynthesis based on these proclaimed differences in excretion are likely to be unfounded.

Absorption

Traces of NAm, NA, and their catabolites are secreted into the intestine via the bile (71,72). All of these compounds, regardless of the means of entry into the gastrointestinal tract (bile, diet, or reabsorption) appear to be very efficiently absorbed from at least the intestine since little or no radioisotope is detectible in the feces of animals which have been injected with the labelled vitamers (73).

Whether the vitamers taken *per os* can be absorbed directly from the stomach or must pass into the intestine prior to being absorbed has not yet been definitively demonstrated.

Classically, the stomach has been considered as a nonabsorptive organ. However, the rapidity with which the labelled vitamers are detected in the liver and blood following *per os* administration suggests the possibility that one or both of the vitamers might be absorbed directly from the stomach. On the other hand, the method normally utilized in oral administration may merely cause a significant disturbance of the stomach contents so as to give the administered vitamin almost immediate access to the small intestine.

Rerat *et al.* (74) found that NA was absorbed from the abomasum but not the rumen of sheep and that this absorptive capacity continued throughout the small intestine. On the other hand, Tanigawa *et al.* (75) claim that NA is not absorbed by rat stomach but report no supporting evidence for this claim. On the basis of relatively indirect evidence, Shimoyama *et al.* (76) argue that NA must pass into the small intestine of the rat prior to being absorbed. These workers also claim that NAm is deamidated in the stomach by microorganisms prior to its entry into the small intestine and have convincingly demonstrated the capacity of the rat stomach to perform this function (75,76).

Once in the small intestine NA is readily absorbed. This vitamer has been shown to diffuse passively across the intestinal wall with equal facility in either direction (77,78). NAm is apparently capable of passing through the intestinal wall in the same manner (79).

As indicated above, once in the body both vitamers can easily reenter the small intestine by reabsorption or to a limited extent via

the bile. Ijichi et al. (73) clearly demonstrated the reentry of label into the stomachs of mice following the administration of either vitamer. This result was also observed by Petrack et al. (33) following the administration of NAm to rats. The mechanism of this entry into the stomach has not been elucidated. This subject will be discussed further below with respect to its potential significance in NAD biosynthesis.

One further point of interest is that dietary pyridine nucleotides or those administered directly into the gastrointestinal system cannot enter the body without first being degraded to the vitamer level (78). Further it has been demonstrated that NAD(H) cannot pass from the circulation into liver cells (80). This is in contrast to a report by Everse et al. (81). The conclusions reached in the latter report are based on the gross misinterpretation of radioisotope studies which cannot or do not yield any meaningful information concerning the passage of these nucleotides across membrane barriers. On the other hand there is strong indirect evidence in the latter work to support the findings of Turner and Hughes (78).

The Biosynthesis of Nicotinic Acid and Nicotinamide

NA and NAm arise directly or indirectly from the degradation of various pyridine nucleotides. These nucleotides must first be derived from the "de novo" pathways for pyridine nucleotide biosynthesis. No biosynthetic sequence has yet been demonstrated which gives rise to either vitamer without the prior formation of a pyridine nucleotide excepting the direct conversion of NAm to NA. Thus, although the vitamers are significant contributors to the biosynthesis and maintenance of normal pyridine nucleotide levels, they are intermediates in

salvage pathways only. The significance of these salvage pathways should not be belittled, however, for they are the only routes for NAD biosynthesis available to tissues other than liver and possibly kidney.

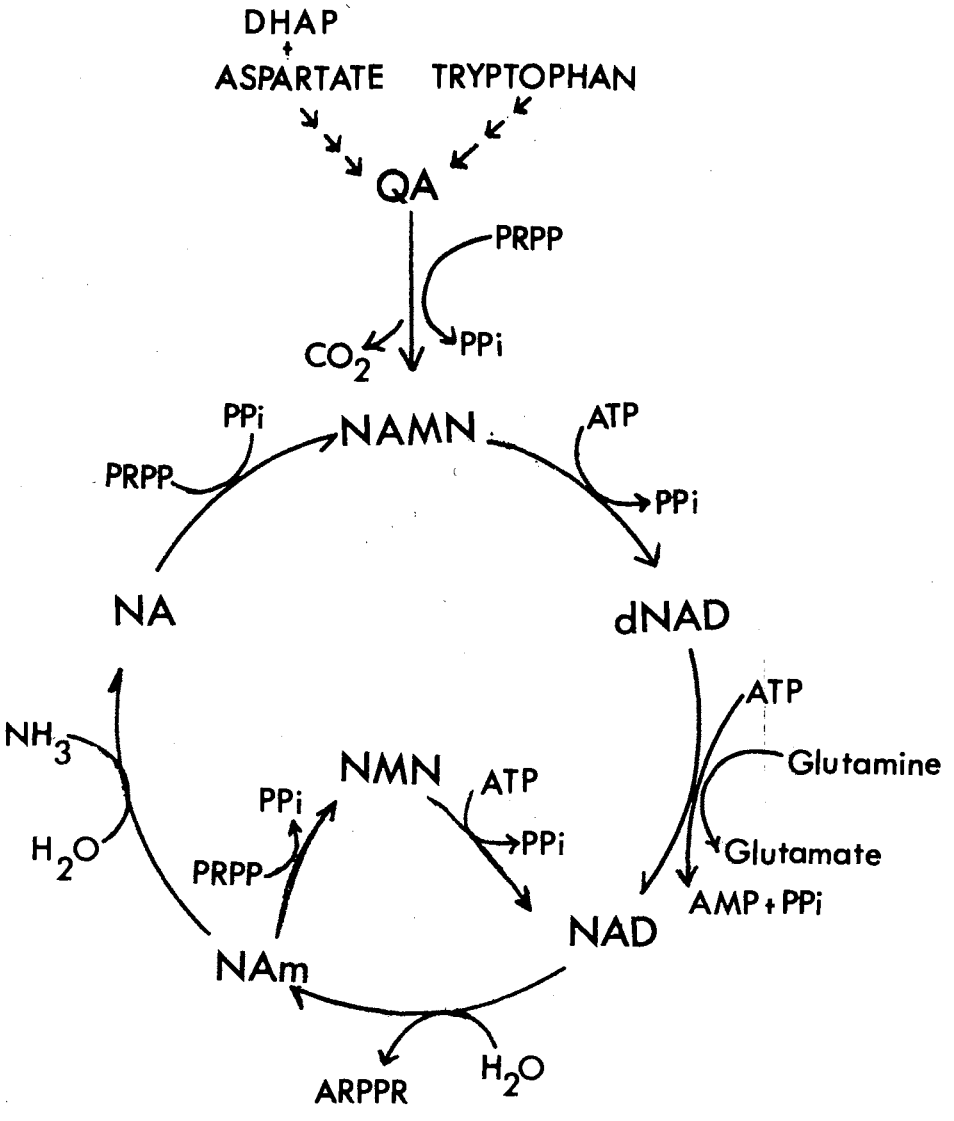
The de Novo Biosynthesis of Pyridine Nucleotides

At least three de novo pathways for the biosynthesis of pyridine nucleotides have been identified (82,83). One of these, the aspartate pathway, involves the formation of quinolinic acid (QA) from aspartic acid and a three carbon precursor. This pathway is restricted to higher plants and microorganisms. Recently it has been shown that, in E. coli at least, the three carbon precursor is dihydroxyacetone phosphate (DHAP), and that the conversion of DHAP and aspartic acid to QA involves at least two proteins and requires FAD (84). A second pathway involving N-formyl-L-aspartate as an intermediate in the formation of QA has been identified in Cl. butylicum (85,86). The last and most well defined pathway is that which involves the conversion of tryptophan to QA (7). The tryptophan pathway is the only one to be found in mammalian and avian species. It is also present in Neurospora crassa, Fusarium oxysporum, and Xanthomonas pruni (82,83). The yeast Saccharomyces cereviseae is capable of utilizing both the tryptophan and aspartate pathways, the former when grown under aerobic and the latter under anaerobic conditions (87).

All of the above pathways have QA as a common intermediate (Figure 1). This compound is converted to NAD via a series of reactions which are common to all living systems so far investigated.

Nishizuka and Hayaishi (88) isolated from rat liver a system capable of converting 3-hydroxyanthranilic acid to nicotinic acid

Figure 1. NAD Biosynthesis from Tryptophan, NAm, and NA.


DHAP
ASPARTATE
TRYPTOPHAN
QA
PRPP
CO_2
PPi
PPi
PRPP
NAMN
ATP
PPi
NA
dNAD
ATP
Glutamine
Glutamate
AMP + PPi
NH_3
H_2O
NMN
ATP
PPi
PPi
PRPP
NAD
NAm
ARPPR
H_2O

mononucleotide (NAMN) in the presence of PRPP. Subsequently, the PRPP dependent formation of NAMN was shown to occur in microorganisms, higher plants, and fungi (89-91). Gholson et al. (92) isolated an enzyme capable of converting QA to NAMN. This enzyme, QA phosphoribosyltransferase (decarboxylating) (QARTase), requires PRPP and was demonstrated to be different from NA phosphoribosyltransferase (NARTase) (E.C.2.4.2.1). This latter enzyme also requires PRPP, converts NA to the same product, and its activity is stimulated by ATP (93). NAMN is converted to desamido-NAD (dNAD) by an ATP-requiring enzyme, NAMN adenylyltransferase (E.C.2.7.7a). dNAD is then converted to NAD by NAD synthetase (E.C.6.3.5.1) in the presence of glutamine and ATP. The reaction sequence from tryptophan to NAD constitutes the tryptophan pathway for the "de novo" biosynthesis of NAD.

Salvage Pathways for NAD Synthesis

The Pyridine Nucleotide Cycle

The reaction sequence from NA to NAD is known as the Preiss-Handler pathway (94,95). NAD can be hydrolyzed at the NAm-ribose linkage to yield NAm and ADPR. This reaction is catalyzed by NAD glycohydrolase (E.C.3.2.2.5). NAm can then be deamidated by NAm deamidase to form NA. These two reactions in combination with the Preiss-Handler pathway constitute the pyridine cycle (96), one of the two potential salvage pathways for the conservation of NA and NAm.

There is much experimental evidence which supports the existence of such a cycle for the reutilization of NAm. The first strong evidence was provided by Langan et al. (97) who demonstrated the ability of NA to interfere with the incorporation of NAm into hepatic NAD.

Subsequently, Narrod et al. (98), utilizing NAm-^{14}C and -^{15}N demonstrated the marked dilution of ^{15}N relative to that of ^{14}C in the resulting NAD. Petrack et al. (99) then demonstrated the existence of a NAm deamidase activity in rat liver which was sufficiently active to account for the NAD synthesis which was observed under the challenge conditions in the above experiments. Finally, Ijichi et al. (73) clearly demonstrated that NAm-^{14}C gave rise to all the intermediates of the Preiss-Handler pathway and showed the inhibitory influence of NA on their production. Unfortunately, all of this research was carried out using large doses of the vitamers. Though the concept of the pyridine nucleotide cycle is sound in so far as it pertains to NAm metabolism under challenge conditions, the significance of this pathway in the mammalian system at physiological levels of NAm has come under strong attack. Regardless of the importance of this pathway in mammals, it is the only one available to other organisms lacking the NMN pathway and is, therefore, still a pathway of biological import.

The Nicotinamide Mononucleotide (NMN) Pathway

A second possible salvage pathway is via the NMN pathway. In this pathway, NAm is converted to NMN by the enzyme, NAm phosphoribosyltransferase (NAmRTase) (E.C.2.4.2.12). This enzyme requires PRPP and its activity is modified by ATP (100,101). NMN is then converted to NAD by NMN-adenylyltransferase (E.C.2.7.7.1), which again requires ATP. There is a possibility that this enzyme is identical to NAMN-adenylyltransferase. The evidence for this is relatively weak, being based entirely on the copurification of the two activities which has been shown in both red blood cells (95) and Erlich ascites cells (102).

Further support for this idea is to be found in the observation that the only pyridine nucleotide cycle enzyme which is apparently active in rat mammary gland is NAMN adenylyltransferase (103).

A Critical Evaluation of the Pathways Available for the Synthesis of NAD in Mammalian Tissues

The contribution of the various pathways of NAD biosynthesis to the maintenance of the normal levels of this and related coenzymes is the subject of great controversy, in particular when one considers the metabolism of NAm for this purpose. It is generally agreed that hepatic NAD synthesis from NAm, under challenge conditions, is predominantly, if not exclusively, via the pyridine nucleotide cycle. In vitro evidence suggests, however, that mammalian tissues have insufficient NAm deamidase activity for the pyridine nucleotide cycle to function at physiological NAm concentrations.

In the face of this objection, one school has suggested that the gastrointestinal microflora carry out the deamidation step of the cycle. Some evidence supporting this position has been obtained although the necessity for such a systemic cycle has not been demonstrated.

A second school has presented evidence indicating that NAm can be incorporated into hepatic NAD without deamidation via the NMN pathway. These workers have concluded, on the basis of sometimes strong but never definitive evidence, that the hepatic pyridine nucleotide cycle is not functional at physiological levels of NAm. They have further drawn the unsubstantiated conclusion that the suggested systemic pyridine nucleotide cycle is of no significance.

Based on the literature to 1968, Dietrich (104) proposed two metabolic schemes for the utilization of NAm and NA in the synthesis of NAD in mammals, one for hepatic and the other for extrahepatic tissues. The hepatic system differs from the extrahepatic in two ways. First, it has the capacity to incorporate QA into NAMN via QARTase, which links tryptophan to NAD synthesis (88,92). Second, a tentative capability for the deamidation of NAm to NA, the closing link in the pyridine nucleotide cycle (92), is considered.

Extrahepatic tissues lack any demonstrable NAm deamidase or QARTase activities (105,106). Dietrich (100) found that NAmRTase in various tissues is capable of functioning at physiological levels of NAm in vitro. Further, a comparative study of the distribution of NAmRTase and NARTase showed that tissues appeared to fall into three classes based on their expected ability to utilize NAm or NA via these two enzymes (104). Based on these findings, Dietrich (107) further suggested that NAm formation from hepatic NAD served as the primary source of NAm for non-NA utilizing extrahepatic tissues.

At that time, the function of the hepatic pyridine cycle was being questioned since the high apparent K_m of NAm deamidase made its function at physiological levels of NAm seem untenable. Demonstration of the synthesis of NAD from NAm via NMN in ascites cells (102) and later in rat mammary gland (103) further supported the contention that the hepatic pyridine nucleotide cycle may not be functional under physiological conditions. Recent reports by Grunicke et al. (71,72) and Streffer and Benes (108) extend this argument by demonstrating the apparently direct synthesis of hepatic NAD from NAm via NMN at physiological NAm levels. These authors have concluded that the pyridine

nucleotide cycle is physiologically insignificant in perfused liver and *in vivo*.

Though they may prove to be correct with respect to the significance of the hepatic cycle, the dismissal of a possible systemic function of this cycle is not justified on the basis of their observations. The existence of a systemic pathway involving the participation of gastrointestinal microorganisms is supported by the early work of Ijichi *et al.* (73) and strengthened by more recent reports (75,76) which appear to demonstrate that NAm in the gut (stomach) does not survive digestion. It is possible that a mechanism favoring NAm transport to the gastrointestinal tract exists (73). If such a system were functioning, the hepatic capacity to incorporate NAm into NAD via NMN under normal physiological conditions might well be of no *in vivo* significance.

Nicotinamide Deamidase

The functioning of the pyridine nucleotide cycle is dependent on the presence of an active NAm deamidase system. This enzyme is localized chiefly in the microsomes of mammalian liver (105,109). Its activity is difficult to detect, even in liver, the apparent K_m for NAm being reported as 40 mM to 900 mM, depending on the purity of the preparation. One of the primary objections to the existence of a physiologically functional pyridine nucleotide cycle is that most of the supporting data were collected under challenge conditions (500 mg NAm/Kg), a dose sufficient to cause a marked elevation in hepatic NAD and circulating NAm. Under such conditions it is possible that even an enzyme with a high K_m for substrate could bring about significant deamidation of NAm to NA. Indeed deamidation does occur *in vivo* and at

doses well below challenge levels. Whether it is totally attributable to the hepatic enzyme is not certain. Dalton et al. (110) best demonstrated the extent of this deamidation by the measurement of changing serum NA content following varying doses of NAm. They reported an elevation of serum NA with as little as 10 mg/Kg doses.

Petrack et al. (99) presented in vitro evidence that rat liver contained sufficient deamidase activity to account for the increased synthesis of hepatic NAD observed under challenge conditions. They also observed a stimulation of activity by addition of bovine serum albumin (BSA) to their assays. This finding was interpreted as being the result of the removal of some endogenous inhibitor of the enzyme. They hypothesized that the deamidation of NAm was the first and rate limiting step in its conversion to hepatic NAD. It appears that this is indeed the route of hepatic NAD synthesis followed under challenge conditions (73). The commonly used dosage of 5 mg/Kg, while insufficient to markedly increase hepatic NAD content, is still sufficient to allow tissue dependent deamidation to occur in both normal and germ free animals (22, 35). It is noteworthy that the quantities of NA and NAgly which were found in the urine of germ free animals treated with NAm were equal to, if not greater than, those observed in normal animals. Even this dose is excessive in comparison to normal physiological exposure of the animal to NAm, however.

Hormonal influences may be involved in the activity of NAm deamidase. Greengard et al. (111) observed a three-fold increase in rat liver deamidase following hypophysectomy. This increased activity appeared to be due to a decrease in content of an endogenous inhibitor. Petrack et al. (112) purified the enzyme to the point where the BSA

stimulatory effect noted in crude homogenates was lost and found only a slight decrease in K_m. The K_m observed did not approach that observed in avian liver (pigeon) where BSA stimulation is not observed and where NA and NAm metabolism may be grossly different from mammalian species. Dietrich et al. (113) showed that the effects of hypophysectomy could be reversed by replacement therapy with somatotropic hormone but that the ability of hexesterol to depress NAm deamidase noted previously in normal animals (114) was lost in hypophysectomized animals. Other estrogens, ACTH, and adrenocorticoids had no influence on the enzyme activity observed in hypophysectomized animals.

Kirchner et al. (105) showed that rabbit liver homogenates also contained inhibited deamidase which was stimulated by the addition of BSA. Purification yielded an enzyme with a K_m of 40 mM which was not influenced by BSA. The degree of inhibition of isolated enzyme was shown to depend upon the isolation conditions and the nutritional status of the animal. Su et al. (115) purified the enzyme and found two fractions with activity. The most abundant one was characterized and found to be a glycoprotein (MW 222,000) containing 36-38 moles of mannose and 7-8 moles of glucosamine per mole of enzyme. They demonstrated that NA was a competitive inhibitor of the enzyme (K_i approx. 10 mM) and that high levels of thyroxine were also inhibitory.

Greengard et al. (116) isolated an inhibitory fraction from liver homogenates and found it to be predominantly polyunsaturated fatty acids.

It is quite possible that the inhibition and high K_m of NAm deamidase observed in vitro in homogenates is artifactual. The mammalian enzyme has not been studied for cofactor requirements (e.g., anions,

cations, phospholipids). Rajagapolan et al. (117) studied NAm deamidase in mammalian species and could find no activity in the liver of the rat, guinea pig, sheep, hog, or cattle. They detected some activity in rabbit liver and found high levels of activity in pigeon liver, pigeon kidney, and chicken kidney. The same workers subsequently reported the inhibition of NAm deamidase by EDTA, 8-quinolinol, and 2,2' bipyridine in preparations obtained from pigeon liver, chick kidney, Lactobacillus arabinosus (plantarium), N. crassa, Aspergillus niger, and S. cerevisiae (118). They noted that the addition of Fe++ was effective in reversing the inhibition of the pigeon and chick preparations, Mg++ was effective in the A. niger system, and Mn++ in the N. crassa. Further studies (119) demonstrated NAm deamidase in the kidneys of 6 and the livers of 3 avian species but none in the liver or kidneys of the monkey, bat, or mouse. No effort has been made to incorporate metals into the assays of mammalian preparations and no studies verifying the metal requirements of this enzyme in avian or microbial species have been published. In this respect, it is worthy of note that the simple addition of metal to an assay may not be sufficient to permit the observation of an enzyme activity which is dependent upon that metal. A case in point is 3-hydroxyanthranilic acid oxygenase. In this system, incubation under rather rigorous pH conditions (pH 3.5) or heating (3-5 min. at 55°C.) in the presence of ferrous iron are required before one observes significant in vitro activity (120,121). The enzyme, once activated, then appears to have the capacity to carry out only a limited amount of catalysis before it must be again recharged with ferrous iron (122). This would indicate the possibility that, in vivo, another reaction is catalyzed by this enzyme or some specialized conformational change

takes place which allows for the reduction of the metal or its replenishment from the media.

Although the absence of high NAm deamidase activity makes such studies difficult and unpromising, little or nothing is known about the potential for allosteric or feedback inhibition phenomena involving the end products of this vitamin's metabolism. The possibility exists that a loosely bound protein or peptide is required for enzyme activity such as is the case with lactose synthetase (123). It is interesting that Erlich ascites cells (see below) are the only cells of mammalian origin demonstrated to possess a NAm deamidase having a low K_m. This cell line was derived from a spontaneous mouse mammary carcinoma, an organ where the enzyme is supposed to be ineffective or lacking (103). It is therefore interesting to speculate that this enzyme may be present as an inactive apoenzyme or that its synthesis is repressed in normal liver when more than sufficient dietary tryptophan, NA, and/or NAm are available, and that a prolonged dietary restriction would be accompanied by a release of the cryptic activity. NAm deamidase has not been thoroughly studied. Though the weight of *in vitro* evidence argues against its having any significance *in vivo*, at physiological concentrations of NAm, this evidence does not prove the case.

Studies with Erlich Ascites Cells

The first strong argument against the function of the pyridine nucleotide cycle was raised by Grunicke and coworkers. Their argument was based on the poor apparent kinetic character of mammalian NAm deamidase, the fact that all studies supporting the function of this cycle utilized high levels of NAm, and primarily on their finding (102)

that Erlich ascites cells appeared to convert NAm to NAD via NMN in preference to the utilization of the NA pathway. Greenbaum and Pinder (103) further fueled this argument by demonstrating the direct, NAm deamidase independent, incorporation of NAm into NAD in rat mammary tissue.

Holzer et al. (124) reported that Erlich ascites cells preferentially incorporated label from NAm into NAD relative to label from NA. Subsequently, Dietrich and coworkers (125) demonstrated that significant incorporation of labelled NA into dNAD and NAD occurred in this cell type. dNAD was preferentially labelled in the absence of a glutamine supplement and NAD became the primary repository of label in the presence of such a supplement. This indicated that a glutamine deficiency was probably responsible for the previously observed preference shown for NAm by these cells (124). No net NAD synthesis occurred from either NA or NAm in these experiments (125). The above findings were verified by Grunicke et al. (102). Their data demonstrate that the incorporation of NA into NAD in the presence of glutamine was essentially equal to that of NAm. They, however, apparently ignored the potential implications of this observation.

It was also shown that significant incorporation of labelled NA into NAD occurred at levels of 10^{-7} and 10^{-8} M NA whereas NAm at these concentrations produced much less extensive labeling (125). It was further observed that the optimum concentration for NA incorporation was 1×10^{-5} M. Above this level inhibition occurred, whereas with NAm, parallel increases in the specific activity of NAD and dNAD occurred as NAm concentrations were increased up to 1×10^{-3} M. It is perhaps noteworthy that although NMN was not measured or at least not

reported, these authors did observe measureable labeling in dNAD even at the level of 1.6 x 10^{-7} M NAm (125). The inhibition of NA incorporation above 1 x 10^{-5} M involved both labeling of NAD and dNAD implicating the inhibition of one or both of the first two steps in the conversion of NA to NAD. The potential for feedback inhibition of the first of these enzymes, NARTase, has been demonstrated (16).

Dietrich and Ahuja (126) reported that the mechanism of NA uptake differed from that of NAm in these cells. It had been previously reported by Holzer and Boltze (127) that the two vitamers were equally well absorbed by Erlich ascites cells. It would appear that these cells have the energy independent capacity to concentrate NA. This concentrating mechanism is temperature sensitive and does not involve the rapid incorporation of NA into nondiffusible intermediates as is the case in erythrocytes (128). NAm on the other hand is absorbed by passive diffusion (126) and the two vitamers do appear to be equally well absorbed under neutral or alkaline conditions. It is of interest however that these cells apparently can under certain conditions distinguish between NA and NAm. If such a condition existed *in vivo* in the liver it would fit well with the previously mentioned hypothesis put forward by Dietrich *et al.* (107).

Another point used by Grunicke *et al.* (102) to argue the predominance of the NMN pathway in this cell line was the finding by Dietrich *et al.* (100) that Erlich ascites cells apparently contained low levels of NARTase activity relative to NAmRTase activity. However, such an extrapolation of *in vitro* assay findings to the situation existent is intact cells in not necessarily valid, which is apparent from the above discussion.

The most convincing piece of evidence presented by Grunicke et al. (102) is the demonstration that 5 x 10^{-2} M 5-MNAm essentially eliminates the activity of NAD glycohydrolase and simultaneously reduces both the NAmRTase activity and the incorporation of label from NAm by 50%. The above compound (5-MNAm) has been shown to inhibit another enzyme (QARTase) related to NAD biosynthesis (These are unpublished data obtained by Streffer and Benes and commented on by these authors in another publication (108)). Due to the striking similarity between the reactions catalyzed by NAmRTase, QARTase, and NARTase one might expect the latter to be inhibited by 5-MNAm. This apparently is not the case. In other work (129), evidence is presented which indicates that 5-MNAm does not influence the incorporation of NA into NAD in these cells.

Marki and Greengard (130) demonstrated the presence of a low K_m (10^{-5} M) NAm deamidase in Erlich ascites cells which, in combination with the demonstrated formation of dNAD at low concentrations of labelled NAm in this cell type (125), convincingly demonstrated the potential of these cells to deamidate NAm and make use of the pyridine nucleotide cycle. The presence of such an enzyme casts some doubt upon the interpretation of the above experiments with 5-MNAm, since the latter may be an effective NAm deamidase inhibitor.

A final point used by Grunicke to argue in favor of the NMN pathway in Erlich ascites cells was the demonstration that NA incorporation was extremely sensitive to azaserine while that of NAm was relatively insensitive to its effects. Azaserine is a glutamine inhibitor and functions by blocking the NAD synthetase reaction (131) and results in a marked depletion of hepatic NAD (18). Since the data for the actual NAD content of the cells are not reported by Grunicke et al. (102), one

can only presume that the same effect was obtained in the tumor cells. In the presence of pyridine nucleotide depletion one would certainly expect the activity of the NMN pathway to be observed since the feedback inhibition of NAmRTase mediated by the coenzymes (16,104) would be relieved, and thus the experiment has no bearing on the problem being investigated.

The presence of a low K_m (4×10^{-5} to 1×10^{-4} M) NAmRTase has been demonstrated in Erlich ascites cells (101). There is little doubt that this cell line can produce NMN from NAm (102) and that under some conditions the NMN pathway may be dominant in the formation of NAD. However, these experiments, which led to the claim that the NMN pathway was dominant in the intact mammalian system, cannot be considered as definitive. The data indicating that the pyridine nucleotide cycle is of little importance in Erlich ascites cells themselves, appear to be more the result of a glutamine deficiency in the medium than an enzymatic preference for one or the other of the two pathways. Indeed it would appear that these cells possess all of the required enzymes for a functional pyridine nucleotide cycle and that one or more of these enzymes is under some sort of substrate and/or product related control.

The most complete and dependable data available indicate that, contrary to the claims of Grunicke et al. (102) and despite the presence of a low K_m (101) and active (101,102) NAmRTase system, the pyridine nucleotide cycle in Erlich ascites cells is possibly the preferred pathway in the presence of adequate glutamine. Dietrich and Fuller (133), utilizing NAm as the substrate, demonstrated that the specific activity in the NMN moiety of NAD, measured by the incorporation of ^{32}P, peaked at an NAm concentration of approximately 1×10^{-5} M and decreased

with further increases in concentration. Their previous studies (125) had shown a similar pattern for NA incorporation and that the incorporation of label from NAm continued to rise up to 1 x 10^{-3} M (the maximum concentration studied). The fact that ^{32}P incorporation shows an optimum well below this level when NAm is the substrate strongly suggests that the continued increase in NAD labeling previously observed with labelled NAm was due to an isotopic exchange, catalyzed by NAD glycohydrolase, since the incorporation via NMN would require a continued increase in the ^{32}P specific activity observed in the NMN moiety of NAD over the entire concentration range. These findings do not in themselves demonstrate that the pyridine nucleotide cycle is the preferred biosynthetic pathway. However, the fact that no net synthesis occurred in the experiments of Dietrich et al. (125) and the similarity in pattern between ^{32}P and NA incorporation is more easily explained on the basis of the function of this cycle combined with the essentially total, pyridine nucleotide mediated, inhibition of the NMN pathway which has been demonstrated by Dietrich et al. (16,104).

Thus, the only well documented system wherein the NMN pathway is clearly dominant is the bovine mammary gland (103), and in this tissue the activities of three of the enzymes of the pyridine nucleotide cycle have been shown to be very low or absent. Further, it has been demonstrated that this high mammary NAmRTase activity, the highest then documented, was present only in lactating mammary tissue and it is therefore directly or indirectly subject to multiple general hormonal influences (134).

There is evidence that the direct conversion of NAm to NAD via the NMN pathway occurs in the brain and skeletal muscle (135). However, no

evidence was presented which can be used as the demonstration of a preference for NAm or the exclusion of NA utilization in these tissues. Indeed, brain has been shown to utilize NA quite readily (136) and to respond to NAm challenge in a manner similar to liver though less markedly (137). In no case, however, could such information have any true bearing on the concept of a functional hepatic or systemic pyridine nucleotide cycle. This becomes evident if one considers the postulations of Dietrich and coworkers (16,107) with respect to NAm metabolism.

Dietrich (16,104) presented *in vitro* data indicating that NAmRTase is strongly inhibited by physiological levels of pyridine nucleotides. The potential for stringent metabolic control and low K_m of this enzyme are more satisfying conditions for an NAD synthesizing system, and add theoretical weight to the argument against at least the hepatic pyridine nucleotide cycle and in favor of the NMN pathway. Several very recent reports from three different sources (71,72,108,135) claim that the NAmRTase pathway is indeed the only one functioning under physiological conditions. However, in each case, either the experimental data presented do not definitively support the arguments made by the authors, or the validity of the interpretations presented are open to question.

Liver Perfusion Studies

It should be pointed out immediately, that one major shortcoming of the perfusion studies to be discussed is that they are closed loop systems. As a consequence there is no opportunity for competition for substrates from other organs as would occur under *in vivo* conditions.

Grunicke *et al.* (71,72) perfused rat livers with either labelled NAm or NA in the presence and absence of 1 mM NA in the perfusate. In

a 2.5 hour experiment (71) the inclusion of 1 mM NA resulted in a 95% reduction in the amount of label incorporated into NAD/gm of liver when 5×10^{-6} M NA was the labelled substrate. A similar experiment with 20×10^{-6} M NAm resulted in no change in label incorporation into NAD. Unfortunately no data are given for the NAD content of the liver after the various treatments, and no data are given concerning the composition of the perfusates. It is therefore difficult to interpret these results. Apparently no effort was made to measure NA ^{14}C in the NA pool when NAm was the source of label. The absence of labelled NA in the NA pool, in itself, cannot prove that NAm deamidase was nonfunctional under normal conditions, since the enzyme may be under strict control by NA. In the absence of data concerning the concentration of NAD in the livers it is also impossible to comment definitively on the function of the NMN pathway (see below). In a similar experiment perfusing for 10 minutes (72) it was shown that a 1 mM NA trap resulted in a 99.5% reduction in the specific activity of NAD when perfusing with NA and only a 50% reduction when perfusing with NAm-^{14}C. These data are taken to indicate that a considerable fraction of the NAm incorporation occurred via NMN. Again no data are given showing the presence or absence of NA-^{14}C in the perfusate when NAm-^{14}C was the label source, or the NAD content of the livers. A comparison between the two experiments is essentially impossible and at most the data can be taken to indicate that a portion of the NAD synthesis from NAm is possibly occurring via NMN, and this under conditions of unknown pyridine nucleotide content.

In Grunicke's experiments utilizing azaserine it is not clear whether the inhibitor was added when perfusion began, or with the labelled substrates some 30 to 40 minutes later. The effect of azaserine is

to markedly inhibit the incorporation of NA into NAD, and to cause large, rapid, and persisting reductions in hepatic NAD content (18,71,72,98, 131). Under conditions of depressed NAD concentration one would expect the feedback inhibition of NAmRTase (16,104,138) to be released and incorporation via this pathway to be increased. In a 20 minute experiment, Grunicke et al. (72) showed that azaserine resulted in a 90% reduction in the specific activity of NAD when perfusing with 5×10^{-6} M NA but essentially no change in the specific activity of NAD when perfusing with 10×10^{-6} M NAm. However, the concentrations of NAD and total incorporation of each labelled substrate are not reported. In a 60 minute experiment (71) a 70% reduction in NAD specific activity was accompanied by an 87% reduction in the incorporation of label per gram of liver and a 71% reduction in NAD content when perfusing with 5×10^{-7} M NA. On the other hand, a 225% increase in the specific activity of NAD accompanied by a 45% reduction in incorporation of label per gram of liver and an 87% reduction in NAD content was observed when perfusing with 1×10^{-5} M NAm. These data seem to indicate that NAm is being incorporated into NAD via a pathway independent of both NAm deamidation and of a NAD glycohydrolase mediated exchange reaction, i.e., via the NMN pathway. However, this is expected under conditions of reduced NAD content, and such findings cannot be taken as being indicative of the synthetic activities under normal liver conditions. Further, the azaserine mediated inhibition of NA incorporation into NAD does not reflect on hepatic capacity for NAm deamidation, the primary point in question. Moreover, these experiments indicate that under conditions of azaserine inhibition the NMN pathway of NAD synthesis is incapable of maintaining normal hepatic NAD levels in the presence of physiological concentrations of NAm.

In this latter respect, it is of interest that Hagino et al. (139), perfusing livers with high concentrations of NA (approx. 2×10^{-2} M), demonstrated a marked decrease in hepatic NAD content during a three hour perfusion period whereas they were able to measure a 2.5 fold increase when perfusing with high concentrations of NAm (approx. 4×10^{-2} M). 2×10^{-2} M NA is in excess of that concentration which would result from a 500 mg/Kg injection of NA (110). Under these conditions, the NA pathway is highly inhibited and any possible NAm deamidase activity is without consequence. Here again, with the NA pathway blocked, the NMN pathway was insufficient to maintain normal hepatic NAD concentrations. The NAm concentration perfused is about 8 times that observed following 500 mg/Kg NAm injection (99). Under these conditions, hepatic NAD was elevated in the perfused liver, paralleling in vivo observations, and this would be expected to be mainly due to a pyridine nucleotide cycle function, i.e., via deamidation, as is the case in vivo.

5-MNAm is a potent inhibitor of NAD glycohydrolase (102,129,140). Using only NAm-^{14}C, Grunicke et al. (71) perfused rat liver with 5×10^{-2} M 5-MNAm for a period of 2.5 hours. The result was reported as an apparent increase in the specific activity of the NAD to 150% of that observed in a control accompanied by a simultaneous increase in NAD content to about 118% of the control. The control liver in this experiment was perfused with 2×10^{-5} M NAm and the 5-MNAm perfused liver was perfused with only 1×10^{-5} M NAm. The data were interpreted as indicating that the incorporation of NAm was not due to an NAD(P) glycohydrolase mediated exchange reaction and the conclusion made that it must therefore have occurred via the NMN pathway. Although the evidence seems to support this conclusion, it is not a definitive experiment,

since no previous experiment has, in this author's opinion, clearly eliminated a significant contribution from the pyridine nucleotide cycle. The only apparently safe conclusion which can be drawn from the results of this experiment would be that the observed incorporation of label into NAD was not the result of an exchange reaction. However, closer analysis of the data presented seems to make the support of even this conclusion rather tenuous.

The above comparisons, concerning the effects of 5-MNAm were made on the basis of the final NAD content in two livers, one treated and one untreated. No replication of the experiment was reported. It should be pointed out that all the above perfusion experiments (71,72) were performed without replication (at least, none is implied). In the instances where it is reported, the final NAD content in the livers varied over a range of at least 35%, not allowing for time differences in the perfusions. For example, in the experiments using azaserine, after 1 hour of perfusion with 1×10^{-5} M NAm the NAD content in the control liver was reported to be 0.64 μmoles/gm liver. In the same experiment, perfusing with 5×10^{-7} M NA, the NAD content in the control liver was 0.42 μmoles/gm liver. In the 5-MNAm experiment, after 2.5 hours of perfusion the control liver contained 0.42 μmoles/gm liver while the 5-MNAm perfused liver contained 0.49 μmoles/gm liver. In this latter experiment, the data indicate that the NAD was disappearing at the rate of about 0.14 μmoles/gm liver/hr in the control liver and at the rate of 0.10 μmoles/gm liver/hr in the 5-MNAm perfused liver, if one presumes that both livers contained 0.64 μmoles/gm liver after one hour of perfusion. Thus, the reported 18% increase in NAD synthesis in the 5-MNAm experiment is most probably due to a decrease in the rate of NAD loss and not an increased synthesis.

These authors previously demonstrated that the NAD glycohydrolase exchange reaction catalyzed by the enzyme obtained from Erlich ascites cells was almost completely eliminated by 0.05×10^{-2} M 5-MNAm (102). It has also been clearly demonstrated that this compound strongly inhibits the net hydrolysis of NAD catalyzed by the enzyme from mammalian liver (108,140). Therefore, on the basis of the above calculations, one is drawn to either of two conclusions. First, that 5-MNAm does not extensively inhibit NAD glycohydrolase activity in perfused rat liver. If this were the case, the exchange reaction could have accounted for an indeterminate amount of the incorporation of NAm-^{14}C into NAD observed in this experiment. Therefore, the above findings would be relatively meaningless and the conclusions drawn unfounded. Second, one might conclude that 5-MNAm did indeed eliminate the NAD glycohydrolase exchange reaction to the extent that one would expect on the basis of previous studies. In this case, it would be predicted that little change would occur in the NAD content of the 5-MNAm perfused liver during the experimental period. Since the levels of NAD observed in a liver after 1 hour perfusion with NAm are significantly higher than those observed in the 5-MNAm perfused liver after 2.5 hours of perfusion one could conclude that the initial levels of NAD were markedly different. If this were so, any comparisons based on the quantitative aspects of the above perfusion studies based only on the final NAD contents of the livers would be of questionable value, since, quite obviously, the final specific activities of the NAD would be highly dependent upon both the initial NAD content in the livers and the changes in that content which occur during the perfusion.

Perfusion under aerobic and anaerobic conditions was also reported by Grunicke and coworkers (72). The data show that under anaerobic conditions hepatic ATP concentrations are rapidly diminished. Evidence is presented showing that NA accumulates in the anaerobic liver when perfused with NA. Livers perfused with NA accumulated NAm more rapidly than under aerobic conditions indicating that NAD glycohydrolase continued to function under anaerobic conditions. When livers were perfused with NAm under anaerobic conditions they accumulated NAm; however, apparently no effort was made to measure NA in these latter experiments. Had the authors made such a measurement and found no labelled NA one would expect strong emphasis to be placed on such evidence. This would be the only experimental situation in which, barring the use of high concentrations of NA as a trap, any NA would be expected to accumulate. Indeed it is the only experiment performed by these workers which is capable of looking directly at NAm deamidase activity in the intact perfused liver in absence of any complications involving the other enzymes in the system and/or the use of incompletely characterized inhibitors. The absence of NA accumulation would be strong evidence for the lack of NAm deamidase activity in perfused livers. It would not be totally definitive, however, since it is possible that the deamidase could be directly or indirectly oxygen dependent.

Data concerning the appearance of label in the various intermediates in the synthesis of NAD from NAm have been used to argue that only the NMN pathway is of physiological significance (71,72). These arguments are based primarily upon the labeling of NMN by NAm, while NA intermediates are not observed to be as heavily labelled and the fact that NA gives rise to NMN labeling particularly in the later stages of the

experiment when most of the NA has been converted to NAD. The possibility that labelled NMN might arise as a result of an exchange reaction is ignored. The same enzyme is apparently responsible for the conversion of NAMN to dNAD and of NMN to NAD (95,102,129). The reaction has been demonstrated to be reversible in the presence of PP_i *in vitro* and it is quite feasible that labelled NMN can arise from labelled NAD via the following reaction sequence:

$$NAMN + ATP \longrightarrow dNAD + PP_i$$

$$PP_i + NAD \longrightarrow NMN + ATP$$

without the direct synthesis of NMN from NAm. NMN could also arise from cleavage of NAD to NMN and AMP by nucleotide pyrophosphatase. Either of these possibilities could explain not only the formation of NMN from NA but also the time dependent nature of that formation.

The weight of evidence presented by Grunicke *et al.* (71,72) indicates that liver is indeed capable of the synthesis of NAD *via* NMN at physiological concentrations of NAm. However, there is no definitive evidence presented to show that the pyridine nucleotide cycle is an insignificant pathway in hepatic utilization of NAm. Further, evidence is presented that throws doubt on the ability of the hepatic NMN pathway to maintain hepatic NAD levels in perfused livers at normal physiological concentrations of NAm. Finally, once traces of NA are formed in, or introduced into, an actively synthetic system, even trace concentrations of this vitamer will be rapidly incorporated into NAD. Labeling in NA, NAMN, and dNAD would be difficult to detect in such a system. In both short and long term perfusions with labelled NAm, Grunicke *et al.* (71,72) did indeed observe some labeling in these intermediates. Such a finding indicates that at least some deamidation of NAm was occurring under their conditions.

In Vivo Studies

Streffer and Benes (108) studied the incorporation of NA and NAm into hepatic NAD and its intermediates in mice. Based on the appearance of labelled NMN relative to NA and dNAD they argue that NA is rapidly utilized and converted to NAD and that NAm resulting from NAD breakdown is reutilized for biosynthesis of NAD via NMN. The basis of this argument, that the labelled NMN can only arise from NAm, is not necessarily compelling, as discussed above.

These authors previously observed that NAD formation from NA was diminished in animals receiving multiple doses of NA (141). Whether such a reduction is due to dilution of the administered label as a result of the presence of non-physiological levels of NA or is a real loss in synthetic capacity is not clear. The results presented (108) show that the total incorporation of labelled NA into liver was reduced by about 30% following such treatment; however, the total incorporation still represents a 2-fold increase over the incorporation of label seen in mice receiving an equal amount of labelled NAm. If these data are recalculated and the amount of the label in the individual components isolated is expressed relative to the total quantity of label isolated one finds that NA pretreatment had little or no influence on the relative distribution of the label among the various metabolites studied. Similar treatment of the NAm data indicates a reduction in the relative quantity of label recovered as NA and dNAD.

These authors suggest that the failure of Ijichi et al. (73) to observe NMN accumulation was due to the fact that they injected directly into the portal vein. They claim that this route of administration resulted in non-physiologically high levels of NAm in the liver leading

to an inhibition of NMN formation. Such an attempt to explain away the contradictory data of these other workers is unfounded. If the NMN pathway is inhibited, it is the end products (nucleotides) not NAm which inhibit its function. Considering the rate of uptake of NAm from the medium in the work of Hagino *et al.* (139), Grunicke *et al.* (72), and Ijichi *et al.* (73), one finds that the rate of removal of NAm from the circulation is relatively slow. Rough calculations indicate that the *in vivo* NAm concentrations attained *via* intraportal injection are unlikely to approach those used by Streffer and Benes *in vitro* (108) and therefore direct inhibition by NAm is improbable. Further, if the effective dose and time involved were enough to cause an increase in nucleotide levels sufficient to prevent the formation of NMN and NAD *via* that pathway, then the hepatic function of that pathway at physiological levels of NAD and NAm is of doubtful significance, since any changes in nucleotide concentration occurring in the 1 to 2 minute period in question are immeasurably small.

Direct incorporation of NAm into NAD *via* NMN is apparently indicated by the findings of the above authors (108). The data again, however, do not definitively eliminate a significant contribution from a pyridine nucleotide cycle and a functional NAm deamidase. Indeed the persistent observation of NA and the intermediates involved in its conversion to NAD suggest that deamidation of NAm, even at physiological levels of NAm, is occurring. Further, the much higher rate of incorporation of NA into liver relative to NAm supports the suggestion that some mechanism is operational, even at physiological levels of the vitamer, which favors the flow of NAm away from the liver to some other site where it may be utilized (107) or deamidated (e.g., gut).

Collins and Chaykin (135) have recently studied the *in vivo* metabolism of NAm and NA in mice. These authors aver that their studies are done under truly physiological conditions. However, based upon their procedures, this implies that the intraperitoneal or intragastrointestinal administration of the estimated total daily vitamin intake (3-4 mg/Kg) as a single dose, into fully *ad libitum* fed animals consuming a complete commercial diet, constitutes a normal physiological condition. That this is a highly disputable presumption has already been previously discussed at some length. Support for this argument is to be found in a comparison of the hepatic labeling patterns observed by Lee (142) following the intraportal administration of microgram quantities of NA and NAm to normal rats with those observed by Collins and Chaykin in the mouse. It should be noted that the doses utilized by Lee (9 or 25 µgms/animal) were being administered to adult rats weighing about 10 times as much as the mice utilized by Collins and Chaykin whose animals received 110 µgm doses of the vitamers.

When an intraportal dose of 25 µgm of NA was administered, Lee found a maximum of 22% of the injected dose of ^{14}C in NAD 5 minutes after the injection and this slowly decreased to a level of 15% of the dose at one hour. NAm, on the other hand, reached a level of 9% of the injected dose 10 minutes after treatment and slowly rose to a level of 12% of the dose at the end of one hour. When the dose was reduced to 9 µgms, a maximum of 22% of the dose was found in NAD after 10 minutes and the level slowly decreased to 19.8% of the dose after one hour. Simultaneously, NAm was found to contain about 2% of the dose from 10 through 40 minutes and 3.4% of the dose at the end of one hour. In contrast, Collins and Chaykin, utilizing NA and the intraperitoneal

route of administration found a maximum of 0.8% of the dose in NAD after 5 minutes and this level dropped rapidly to about 0.1% by 15 minutes and was relatively constant thereafter. NAm rose rapidly to a level of about 4.5% of the dose at 15 minutes and remained relatively constant thereafter.

Using NAm-^{14}C (25 μgm/animal), Lee observed an initial burst of NAD labeling similar to that previously observed by Ijichi et al. (73). A relatively constant amount (about 4-6%) of the dose was isolated as NAD throughout the entire experimental period, including the initial measurement made only 20 seconds following the administration of NAm. Following an initial equilibration period, NAm represented approximately 3% of the dose throughout the duration of the experiment. Collins and Chaykin (135), by comparison, reported that, after equilibration, a roughly constant level of NAm was present in the liver. The level of labeling in NAD rose through 30 minutes following an initial lag period and was subsequently maintained throughout the remainder of the hour and represented somewhat less of the dose than did NAm at all times.

Lee (142) found 28-35% of the label from NA-^{14}C to be retained in the liver after 1 hour whereas Collins and Chaykin (135) found less than 5% was present after 1 hour. Using NAm-^{14}C, Lee (142) observed 15-18% of the injected dose remaining in the liver after one hour while well under 1% of the dose was found to be present after one hour in the experiments of Collins and Chaykin (135).

The above described differences in labeling patterns and label retention accompanying marked reductions in the administered dose of each vitamer seem to quite clearly indicate that the work of Collins and Chaykin (135) is, contrary to their claim, far from being representative of the physiological events surrounding NA and NAm metabolism.

It is agreed that the differences in route of administration (and species) present in the above comparisons might influence the level of label retention observed in a given organ, but this should have little influence on the label distribution observed if no marked physiological imbalance has been imposed by the treatment. In this regard, the data reported by Streffer and Benes (108) is of interest. These workers used mice as did Collins and Chaykin but administered only about 10% of the dose that was used by the latter workers (2.7 μmoles/Kg). The vitamers were administered by the intraperitoneal route. It was found that the proportion of the dose retained in the liver as NAD was about three times that found in NAm, 10 minutes after the administration of NA-^{14}C. This quite clearly is a reversal of the findings of Collins and Chaykin (135) and in agreement with the findings of Lee (142).

Based on the results of their experiments, Collins and Chaykin concluded that in all tissues studied, with the exception of liver, NAm was a better precursor of NAD than was NA. They further concluded that the metabolism of the mouse is designed for the utilization of NAm as a (systemic) precursor of NAD and that NA is converted to NAm in the liver for this purpose. Studies performed in vivo and involving the incorporation of NAm-^{14}C, must be interpreted with great caution because of the potential for an NAD glycohydrolase catalyzed exchange of the label into the NAD pools. Because of this exchange reaction it is impossible to state with certainty that any observed incorporation of NAm into NAD was necessarily due to the action of a pathway with the capacity of bringing about the net synthesis of NAD. In this regard, Deguchi et al. (136) have reached a conclusion which is contrary to that of Collins and Chaykin (135). They found that both NA-^{14}C and NAm-^{14}C were readily

incorporated into brain. They were able to detect all of the intermediates of the Preiss-Handler pathway and to show their appearance in the proper time sequence. However, they were unable to detect any NMN even though NAm was readily incorporated into brain NAD. They therefore concluded that the incorporation occurred as the result of an exchange reaction and that NA was the only precursor utilized for net synthesis in brain. It is granted that the failure to observe an intermediate of a synthetic pathway does not necessarily preclude the function of that pathway. On the other hand, neither can the ^{14}C incorporation data obtained by Collins and Chaykin, indicating that NAm is the better precursor of brain NAD than is NA, be taken as being necessarily representative of net NAD synthesis.

The conclusions reached by Collins and Chaykin (135) support a portion of the hypothesis put forward by Dietrich et al. (104) in 1968 and expanded and restated by him in 1971 (16). The primary difference between these two hypotheses is that the latter workers do not preclude the function of the systemic pyridine nucleotide cycle or the extrahepatic significance of NA as a precursor for NAD biosynthesis. Collins and Chaykin, on the other hand, (135) claim that the pyridine nucleotide cycle (hepatic or systemic) is of no consequence in the intact mouse. The argument is based on their failure to observe either NA or nicotinuric acid in the 60 minute urines of animals dosed with NAm. The validity of an argument based on excretion data, in particular short term excretion data, has already been previously discussed and the same argument applies here. The other portion of their argument is based on the observation that the deamidation of NAm is not prerequisite to the absorption of the vitamer from the digestive tract. However, the direct

absorption of NAm from the digestive tract after the administration of non-physiological doses of this vitamer does not reflect upon the potential function of a systemic pyridine nucleotide cycle. As will be discussed below in detail, no claims have ever been made that the deamidation of NAm is prerequisite to its absorption from the gastrointestinal system. Indeed, there is previous evidence in the literature which demonstrated the direct absorption of NAm from the intestine (79). The proponents of a systemic pyridine nucleotide cycle have merely shown that the per os administration of physiological quantities of labelled NAm (i.e., the gastric introduction of μgm quantities, about 5 μgms) leads to the gastrointestinal deamidation of the vitamer, that this deamidation is relatively rapid and extensive, and that the short term labeling observed in liver NAD is identical for both vitamers when administered per os but much slower in the case of NAm when they are administered intraperitoneally.

Possible Involvement of the Gastrointestinal Tract in the Metabolism of NAm to NAD

Ijichi et al. (73) reported that a large portion of a loading dose of NAm (400 mg/Kg) was rapidly excreted in the urine, but that 20% was located in the gastrointestinal tract at one hour while only 3% was found in the liver and 2% in other tissues. The greatest recovery of label in the gut was in the stomach. When the route of administration was changed from IP to IV (intraportal), and the dose reduced to 200 mg/Kg, 50% of the dose accumulated in the gastrointestinal tract at one hour. About 90% of the gastrointestinal label was found in the lumen and the amount of labelled NAm decreased while that of NA increased with

time. Little label was found in the feces, indicating essentially total resorption of the vitamin from the tract. The fact that a decreased dose of NAm resulted in increased recovery of label in the gut suggests some mechanism which is concentrating NAm *in vivo* in the gastrointestinal system. Further support for this is given by Deguchi *et al.* (136). These workers stated that large amounts of the label from an intraperitoneal dose of NAm-^{14}C (approx. 2.8 mg/Kg) were found in the gastrointestinal tract of mice.

Tanagawa *et al.* (75) demonstrated the presence of microorganisms with a low K_m nicotinamide deamidase (1 x 10^{-6} M) in the *pars preventricularis* of normal rat stomach. Upon administration of 45 nmoles of labelled NAm *per os* they found that all the gastrointestinal label was in the form of NA at 2 and 4 hours following administration. They also reported that NAm and NA gave rise to liver NAD at the same rate when administered *per os* but that NAm was a much slower precursor than NA when the two were administered intraperitoneally.

Shimoyama *et al.* (76) isolated several organisms from the preventricular region of the stomach and showed all to have deamidase activity (at least two of these, *E. coli* and *Streptococcus faecalis*, are also common to the intestinal microflora). After administration of 45 nmoles of NAm-^{14}C, these workers also noted that as the label in the stomach decreased, the intestinal and liver label content increased in parallel. From this observation they conclude that NAm is deamidated in the stomach and that the resultant NA enters the intestine for absorption. Unfortunately the time course of these experiments is too long (2 hours) and therefore such a conclusion concerning absorption of the vitamer(s) is not necessarily valid. One hour following the *per os* administration

of labelled NAm the only labelled compound isolated in the stomach or intestines was NA. These workers conclude that physiological quantities of NAm do not survive passage through the gut. This is in contrast to Chaykin et al. (32,135). However, the latter workers utilized much higher doses of NAm and/or based their conclusion on the finding that the metabolite excretion patterns were similar when NAm was administered either per os or IP and that these patterns were different from those observed with NA. As previously mentioned, such excretion data do not necessarily reflect hepatic metabolism of the vitamers in the NAD biosynthetic pathways.

Grunicke et al. (72) observed only a very small loss of isotope in the bile from perfused livers. These authors assume that the isotope appearing in the gastrointestinal tract reported by Ijichi et al. (73) must enter through the bile. Since they observed very little influx of label into the bile they concluded that the findings of Ijichi et al. are artifacts of the high NAm levels used and that the involvement of the gastrointestinal tract is of no significance in the metabolism of NAm or NA. Although this too may be the correct conclusion, it cannot be made on the basis of their evidence. They overlook the fact that in the studies of Ijichi et al. (73), the concentration of label in the gut increased with decreasing dose and that the primary concentration of the label occurred in the stomach above the point of bile entry into the gastrointestinal tract.

In spite of the fact that the time periods involved in the studies of Tanagawa et al. (75) and Shimoyama et al. (76) were too long for definitive statements to be made concerning the site of vitamer absorption, their evidence for the gastrointestinal deamidation of NAm at physiological concentrations is still very convincing.

As shown by the data of Petrack et al. (33), and as previously mentioned, differences in metabolite distribution disappear rapidly with time in animals receiving 5 mg/Kg doses of NAm and NA. It appears probable that if physiological doses of the vitamer were administered, the differences in excretion patterns observed for the two vitamers at higher levels would essentially if not totally disappear. Such a conclusion is supported by the perfusion studies of Hagino et al. (139), who could detect no differences in the urinary metabolites found in the perfusates from livers perfused with physiological concentrations of NAm and NA.

The Control of NAD Biosynthesis

The consistency of tissue NAD levels has often been mentioned as one of the most striking phenomena of living cells. The efforts to elucidate the control mechanism(s) involved in maintaining these consistent levels have been manifold. Many factors have been implicated in the control of NAD biosynthesis as a result of these studies. These can be classified into two general catagories, substrate and/or product related effects and hormonal effects.

The ATP Theory

It has been suggested by several groups that NAD synthesis was controlled by the intracellular concentration of ATP. This hypothesis was based on the observation that lowered pyridine nucleotide levels were accompanied by lowered ATP levels in hyperthroid rats (143,144). Glock and McLean (145) have attempted to relate thyroxine and the depression of NAD levels in hyperthyroidism to ATP synthesis. The theory

received experimental support from *in vitro* enzymatic evidence indicating the conversion of NAMN to NA in the absence of ATP (146,147) which was believed at the time to represent a reversal of the NARTase reaction (146). Finally, ethionine, which is known to cause a marked reduction in ATP (148), was reported to result in a transient decrease in hepatic NAD content (149). Much of this apparently supportive evidence has now been retracted or proven inconsistent. The enzymatic evidence was retracted (150) upon the demonstration that enzymes other than NARTase were responsible for the original observations. The experiments with ethionine have been repeated. Though a rapid decrease in ATP levels to 50% of normal was demonstrated, essentially no change in either endogenous NAD levels or in NAm induced NAD levels was observed (151).

Since every enzyme in the Preiss-Handler and NMN pathways is dependent upon ATP as a substrate or an allosteric effector, a drastic depletion in intracellular ATP will eventually limit these pathways. It would seem, however, that any mechanism which does not elicit an effect during a decrease of ATP content to half its normal levels is of no physiological significance.

Other Substrate and Product Influences

As has been previously mentioned, small quantities of NA are rapidly incorporated into hepatic NAD, whereas similar quantities of NAm enter the NAD pool more slowly and to a lesser extent. This observation has led to the acceptance of the idea that NA is the preferred precursor for NAD synthesis. Chaykin (7) has questioned this concept. The basis of his quite convincing argument is that the biosynthesis of NAD from NAm is possibly under the tight restraint of some physiological control

mechanism whereas that from NA is not. That is, the availability of a proper substrate is not necessarily the only requirement for the initiation of NAD synthesis. It is interesting that this 1967 argument was conveniently forgotten when claiming NAm was superior to NA as a precursor in all tissues excepting liver (Collins and Chaykin, 1972 (135)).

The study of the efficacy of the various precursors for the promotion of NAD biosynthesis yielded an apparent paradox. Early work by Feigelson et al. (70,152) demonstrated the ability of NA, NAm, and tryptophan to increase hepatic NAD content well above normal when supplied in high dietary amounts or by single injection. Kaplan et al. (8,9) demonstrated that hepatic NAD was markedly elevated by NAm challenge. Subsequently several groups have investigated the efficacy of these and related compounds as precursors of NAD biosynthesis (33,73,97,136,153).

At doses of up to 50 mg/Kg, NA is a better precursor of NAD than is NAm. Beyond this dose the response to NA begins to decrease while that to NAm continues to increase. At approximately 250 mg/Kg NAm becomes equivalent to an optimum dose of NA, but the response to NAm continues to increase markedly with increasing dose until a maximum is attained at a dose of between 500 and 1000 mg/Kg. Further, it has been demonstrated that equimolar doses of tryptophan are essentially equal to NA up to 50 mg/Kg, at which point tryptophan becomes the superior of the two as a precursor for NAD synthesis (152).

Langan et al. (97) demonstrated that a 250 mg/Kg dose of NA resulted in only one-third of the increase mediated by an NAm challenge. Combining the treatments was less effective than NAm alone, indicating that NA was inhibiting NAD synthesis from NAm. The fact that NAm is a better precursor of NAD than NA at higher levels of administration has

been ascribed to two factors. It has been argued that the longer half-life of NAm is a significant factor (33) and it no doubt is under single dose conditions. However, the primary factor is slow release of NA from NAm. Such an explanation was put forward by Petrack et al. (99), who argued that the high K_m NAm deamidase was the first and rate limiting step in NAD biosynthesis from NAm. Regardless of the validity of this statement, such a mechanism does permit the slow release of NA into the Preiss-Handler pathway, preventing the accumulation of inhibitory levels of NA and/or related nucleotides. That such inhibition is the primary factor contributing to the results observed is supported by the finding that 6 mg/Kg of NA administered every 15 minutes for 4 hours (90 mg/Kg total) yielded the same levels of hepatic NAD as NAm challenge (104). This is further supported by the demonstration that multiple doses of NAm (500 mg/Kg at zero time followed by 100 mg/Kg every 2 hours) resulted in a decrease in the maximum level of NAD obtained (34).

Dietrich (16) presented in vitro data demonstrating the specific feedback inhibition of NARTase by dNAD. In this work, 5×10^{-4} M dNAD was utilized (an unlikely in vivo concentration in the absence of compartmentalization). An 87% inhibition of NARTase activity was observed when NA was present at physiological concentrations (1×10^{-5} M). This inhibition was reduced to 54% by increasing the NA concentration to 1×10^{-4} M. It is possible that an accumulation of dNAD might contribute to the reduction of NA utilization at high concentrations of this substrate. However, the fact that the inhibition is substrate reversible implies that other factors are also involved.

It is apparent that some control of NAD biosynthesis from NA exists. The mechanism(s) only come into play under pharmacological circumstances.

They appear to be represented mainly by substrate and/or product mediated inhibition. The degree to which this inhibition occurs is dependent upon the dosing conditions and the extent to which they promote the rapid accumulation of NA, NAMN, and/or dNAD.

The contribution of variable half-life, under challenge conditions, is due primarily to marked differences in the rate of excretion of the two vitamers. NA is very rapidly excreted, having a half-life of about one hour. NAm is excreted much more slowly, having a half-life of approximately 5 hours (33). One major factor contributing to this difference is the effective tubular resorption of NAm (154), which does not appear to occur in the case of NA. Superimposed upon this is the stimulation of renal filtration resulting from an increase in renal blood flow (155,156). Whether NA causes this circulatory effect directly, through the stimulation of glucocorticoid and/or thyroxine output or both, is not clear. The net result, in any event, is a more effective removal of NA from the system and a consequent reduction in the duration of its availability to the synthetic pathways.

The possibility that the mammalian system may have a capacity to adapt to the continued presence of hyperphysiological levels of NA or tryptophan has been eliminated by the work of Feigelson et al. (70). These authors incorporated high concentrations of NA and/or tryptophan into the diet of rats for a period of two weeks. At the end of this time, the hepatic pyridine nucleotide levels were found to be elevated above normal. The findings indicate that tryptophan is capable of overriding the proposed mechanism for the control of its input into the Preiss-Handler pathway (see below). Such a finding should not be permitted to detract from the significance of this mechanism, in as much

as high levels of tryptophan are not naturally occurring and the purpose of such a mechanism is presumably to spare tryptophan under conditions of limited supply.

The concern voiced by Chaykin (7) over the lack of an effective physiological control on NA input into NAD becomes important only if prevention of NAD synthesis beyond a given level is to be desired. To the best of this author's knowledge, it has not been demonstrated that the marked elevation of NAD which occurs in response to the various pharmacological conditions discussed results in any metabolic disruption or physiological damage to the animal.

The level of precursors utilized in all of the above studies are unattainable under natural conditions and, as a consequence, no selective pressure has been placed on the organism requiring these dietary precursors to develop any such control mechanism. The basic problem confronting such species is one of maintaining the minimum levels of cofactors required for normal metabolic function. This is the point where maximal selective pressure would be exerted. Thus, mechanisms which minimize the loss of diffuseable intermediates, provide for their proper interstitial distribution and restrict the metabolic turnover of the nucleotides would be most likely to develop.

In this sense, the frugal use of tryptophan would be advantageous to the organism. The potential for feedback control on the input of tryptophan into the "uncontrolled" Preiss-Handler pathway has been demonstrated (12-14). Of particular interest is the work of Cho-Chung and Pitot (12,13). These workers have demonstrated a strong inhibitory effect of pyridine nucleotides, NADH and NADPH in particular, on tryptophan oxygenase. Further work demonstrated that the tryptophan

mediated elevation of this enzyme (157,158) was eliminated by NAm challenge. It appears that the pyridine nucleotides bring about an allosteric modification of tryptophan oxygenase. This interferes with the substrate protection afforded by tryptophan (157,158) and simultaneously depresses the activity of the enzyme. The entry of tryptophan into this pathway is thus inhibited. This mechanism appears to be sensitive enough to contribute at physiological levels. The classical nutritional evidence concerning the tryptophan sparing effect of NA and NAm also supports the function of such a mechanism.

Excellent supportive evidence for the constraint on NAm incorporation into NAD hypothesized by Chaykin (7) has been presented by Dietrich et al. (16,104). In vitro evidence demonstrated that a marked inhibition of NAmRTase was effected by normal physiological concentrations of a mixture of pyridine nucleotides (104). Utilizing 5×10^{-4} M NAD, a 65% decrease in NAmRTase was observed with NAm at a concentration of 1×10^{-4} M and an 88% inhibition was observed when NAm was present at 1×10^{-5} M. NMN at 5×10^{-4} M was found to be an equally effective inhibitor (16). However, its concentration is not likely to reach such a level in vivo in the absence of compartmentalization. The feasibility of the in vivo operation of this mechanism is easily based on NAD alone since the levels of NAD utilized in the experiments are lower than those normally observed in vivo. The mechanism of this inhibition has been recently studied (138). All oxidized pyridine nucleotides containing a "β" nicotinamideribose linkage were found to be potent competitive inhibitors of NAmRTase when NAm was the variable substrate.

The imposition of such a control mechanism on the incorporation of NAm into NAD need not merely serve the purpose of maintaining the

intrastitial levels of NAD as suggested by Chaykin (7). By limitation of the levels of NAD which can be synthesized by the liver from NAm the organism can assure that all tissues receive a supply of precursor for NAD synthesis. The liver being the only organ capable of generation of any quantity of NAm therefore is forced to permit its exit to the general circulation for use by the other tissues. If there were no restraint placed upon hepatic reuse of NAm one would expect its very effective retention by the liver, particularly in times of dietary restriction. The net result would be that the remaining tissues would be rapidly starved for precursors. Since the net synthesis of NAD from NMN can be initiated by NAm deamidase it would be necessary that constraint be placed on this enzyme also. Regardless of the manner in which it is accomplished, this indeed appears to be the case.

Hormonal Influences

Many apparent hormonal influences on NAD biosynthesis have been observed. The data obtained from such studies are, however, often contradictory and difficult to interpret.

Greengard et al. (10,11,159) demonstrated that NAm challenge in hypophysectomized rats resulted in a marked increase in both the extent and duration of the NAD elevation observed in normal animals. Utilizing combinations of endocrinectomy, these workers demonstrated that the loss of the thyroid and adrenal functions associated with hypophysectomy made the primary contribution to this phenomenon. Combined adrenalectomy and thyroidectomy accounted for 80% of the response observed in the hypophysectomized animal, each gland being shown to contribute about equally. Either thyroxine, ACTH, or glucocorticoid

therapy resulted in a complete or partial, dose dependent, reversal of these effects. All of these studies were based on the response of hepatic NAD levels to a challenge dose of NAm.

Bosch and Harper (143) observed a similar phenomenon in rats afflicted with induced hyperthyroidism. In this study, the hyperthyroid rats responded to NAm challenge with a marked reduction in the extent and duration of the NAD elevation as compared to normal animals. Similarly, induced hypothyroidism has been shown to elevate and prolong this response (160).

Hyperphysiological doses of estrogens have been demonstrated to have a marked effect on NAD metabolism in normal and adrenalectomized animals (161,162). The estrogens decrease the response to NAm challenge. This estrogenic influence is not seen in the hypophysectomized animal. The influence of these hormones cannot be mediated through the pituitary adrenal axis since they continue to be effective in adrenalectomized animals (161). Since the above authors did not measure the effect of estrogens on the NAD response in their thyroidectomized animals, the possibility exists that the response is mediated through the pituitary via the thyroid.

In addition to apparent hormonal influences on NAm deamidase previously presented, Dietrich et al. (163) have shown that hypophysectomy and adrenalectomy result in about a 40% decrease in NAmRTase activity. In the same study, hypophysectomy was shown to depress NAD kinase by about 30% while adrenalectomy was without effect.

It would seem that the above referenced changes in enzyme activity are of little or no consequence to a consideration of the observed responses to NAm challenge. (Indeed they appear to be of no real

consequence under any circumstances.) Instead, these hormonal effects seem to be predominantly due to a change in the duration of substrate supply following NAm challenge, as is discussed below.

It has been demonstrated that the administration of NA and NAm to normal animals increases enzyme activities (24) which have previously been shown to be induced by glucocorticoids. These vitamer effects are eliminated by hypophysectomy or adrenalectomy, indicating that their influence on enzyme activity is mediated via the pituitary adrenal axis. Johnson and Kanics (164) have proven this to be the case by demonstrating a 2- to 3-fold increase in plasma corticosterone following vitamer challenge. This response was abolished by prior hypophysectomy. It is possible that the vitamers also stimulate thyroid function; however, no reports could be found which would either support or refute this conclusion. It has also been demonstrated that endocrinectomy has a marked influence on renal function. All changes that have been observed are in the direction of reduced excretory function. The renal changes observed in hypophysectomized animals have been shown to be dependent upon both glucocorticoid and thyroxine replacement for their complete reversal (154,165-173).

In this light, one of the most significant observations made by Greengard et al. (34) is that the half-life of NAm following NAm challenge was increased from about 4 to about 20 hours by hypophysectomy. It would appear that this marked increase in precursor half-life in hypophysectomized animals is responsible for the increase in the extent and duration of the hepatic NAD elevation observed following NAm challenge. This increase in NAm half-life seems to result primarily from changes in kidney function. That is, hypophysectomy, in itself,

leads to a decrease in renal function and, at the same time prevents any ancillary stimulation thereof which might be effected by a pharmacological dose of the vitamer in the intact animal. If these conclusions are valid, one would predict that an increase in the extent and duration of the NAm induced elevation of hepatic NAD should occur in any endocrinectomized animal where thyroid or adrenal function is decreased or eliminated. Indeed, the above predicted changes in hepatic NAD have been observed in all studies involving such endocrinectomy or hypoendocrine function. Conversely, in the instance of hyperendocrine function, one would expect the NAD response to be decreased in both extent and duration and this too was found to be the case in induced hyperthyroidism. Data will be presented herein which support the above conclusions. An exception to the above predicted results in terms of the changes in NAD content but not in precursor half-life will be pointed out in connection with the discussion of glucocorticoid influences on NAD biosynthesis.

Insulin has been implicated as being important in the control of tryptophan incorporation into NAD. Alloxan diabetic rats were observed to have a slight (15%) reduction in hepatic pyridine nucleotide content (174). Mehler et al. (175) observed a ten-fold increase in picolinic acid decarboxylase activity in alloxan diabetic rats. It was subsequently proposed that this enzyme syphoned away the tryptophan intermediates normally available for NAD biosynthesis (176), an hypothesis which subsequently received extensive experimental support (106). Clearly this is a direct hormonal influence on the synthesis of NAD from tryptophan. However, the implication that this is a mechanism which has the purpose of controlling NAD biosynthesis is not obvious. It

may well be that the stimulation of tryptophan catabolism via the glutarate pathway is a reflection of an overall physiological response aimed at the generation of glucose and maintenance of anaplurotic intermediates. In this case, insulin would not be functional in the purposeful control of NAD biosynthesis under normal physiological circumstances, and the small decrease in hepatic NAD content observed in the diabetic rat would be purely coincidental.

The influence of glucocorticoids on NAD metabolism presents a further paradox in the study of this area. As noted above, glucocorticoid therapy results in at least a partial reversal of the response of hypophysectomized animals to NAm challenge (11). Greengard et al. (177) also demonstrated that chronic glucocorticoid therapy prevented the symptoms of NA deficiency in the dog and the reproductive disruption therefrom in pregnant rats. This effect was subsequently demonstrated to be due to a glucocorticoid mediated increase in the availability of tryptophan, presumably due to a stimulation of protein catabolism (178). This conclusion was based on the observation that glucocorticoid therapy resulted in an increase in plasma tryptophan and a maintenance of normal liver NAD levels in animals fed a tryptophan-niacin deficient diet. Further, in animals with depleted NAD levels, a single dose of glucocorticoid resulted in an increase in hepatic NAD which was preceded by an increase in plasma tryptophan. This response was definitely not due to a glucocorticoid mediated increase in tryptophan oxygenase since this enzyme activity was found to be doubled in pregnant rats regardless of their nutritional status (179).

After 30 days of dietary deficiency, the hepatic NAD content of rats had fallen to about 60% of normal. Half of this loss occurred

during the first 3 to 4 days following exposure to the deficient diet. The remaining decrease took place during the next 2 to 3 weeks with a sudden drop recorded at the 12th to 14th day. Hepatic NAD levels remained essentially constant through the last 10 days of these trials. The NADP/NADPH content of the liver did not change during the entire period. Daily injections of glucocorticoid were capable, in a dose dependent manner, of maintaining hepatic NAD levels at normal or above normal levels during the entire period of deficiency.

The most impressive finding made in the above experiments is the total failure of the reproductive function of the animals on the deficient diet. Pregnant rats were placed on the deficient diet on the day of conception. On the 13th day of pregnancy, the uteri of these animals bore essentially no viable fetuses and no young were ever delivered. The sensitivity of such a vital species survival function to a relatively small decrease in an essential cofactor is indeed amazing.

It is tempting on the basis of the dual effects of glucocorticoids to propose, as Greengard and coworkers did, that the pituitary adrenal axis plays a significant role in NAD homeostasis. However, hypophysectomized and adrenalectomized rats maintain normal hepatic NAD levels when supplied with an adequate diet in the absence of a functional pituitary adrenal axis. This fact, combined with the above demonstrated inability of the rat to successfully maintain so vital a function as reproduction, in the presence of such a potentially effective survival mechanism, makes the concept that glucocorticoids play an important role in NAD homeostasis rather tenuous. The ability of endocrinectomized animals to maintain normal NAD levels also seems to eliminate the above mentioned enzyme changes as being of any significance.

The Influence of Drugs

Burton et al. (180) demonstrated, in normal animals, that reserpine and chlorpromazine increased the duration of the NAD elevation which occurs in response to NAm challenge. These drugs have been shown to have a marked influence on pituitary-adrenal function (181). The above findings were confirmed by Greengard et al. (182). Subsequently, Fratta et al. (183) demonstrated that chlorpromazine and impramine had the ability to prevent fetal death in the pregnant rat being fed a deficient diet.

These tranquilizing drugs have been shown to stimulate adrenal glucocorticoid output (184). It is interesting that these drugs are capable of preventing reproductive failure via stimulation of glucocorticoid production on the one hand while, when administered at the same dose level, they appear to counteract a glucocorticoid mediated response on the other. In this apparent contradiction seems to lie the answer to the paradoxical effects of the glucocorticoids.

In a more recent study (185), utilizing normal rats, the effects of ACTH, chlorpromazine, and physical stress, induced by limb ligation, on the changes in hepatic NAD following NAm challenge were investigated. All ancillary treatments resulted in a marked elevation of hepatic NAD as compared to those observed in rats receiving only the NAm challenge. After 24 hours, these levels had begun to return to normal. The ACTH, physically stressed, and chlorpromazine treated animals were found to have NAD levels which were, respectively, 1.6, 2.1, and 3 times those found in NAm challenged animals. The results of all treatments were the same, differing only in degree. It is clear that the drug effects

are due to a stimulation in glucocorticoid output which in turn induces an increase in the hepatic NAD levels.

The explanation of the "Greengard glucocorticoid paradox" seems to lie in differences in the duration and potency of the glucocorticoid levels resulting from any given treatment. There is no doubt that these hormones reverse the differences in NAm excretion observed between normal and hypophysectomized rats when using radiotracer methodology and that this effect is a result of glucocorticoid stimulation of renal function in the endocrinectomized animal. On the other hand, it is obvious that glucocorticoids stimulate NAD synthesis by increasing the availability of tryptophan for that purpose (178). It is probable that the influence of chlorpromazine is effected by this same mechanism and that it is more potent and long lasting in this regard than a single administration of ACTH (185). The influence of these drugs upon NAD levels following NAm challenge is therefore explicable on the basis of a marked increase in tryptophan availability for NAD synthesis, which maintains the elevated levels long after the NAm supplied by the challenge has been eliminated. Support for this explanation is to be found in the original work of Greengard et al. (11). In their studies with glucocorticoid replacement therapy, the most potent glucocorticoids were the least effective in reversing the effects of NAm challenge in hypophysectomized rats. Such a result can be explained on the basis of the above argument, i.e., the more potent glucocorticoids stimulate renal function reversing the NAm excretion phenomena while at the same time increasing the availability of tryptophan for NAD biosynthesis thus counteracting the extent of the depression observed in NAD levels by making an alternate substrate available for its synthesis.

NAD Glycohydrolase

Little attention has been given to the possible involvement of NAD glycohydrolase in the control of intercellular NAD levels. Jacobson and Kaplan (186) have demonstrated the wide distribution of this enzyme in the tissues of several mammalian species.

Clark and Pinder (151) have estimated that the rate of hepatic nucleotide hydrolysis observed *in vivo* can be accounted for by about 1.0% of the NAD glycohydrolase activity measured *in vitro*. A similar observation has been made in Ehrlich ascites cells (187). The hepatic enzyme activity has been shown to increase during fasting and the hepatic NAD content has been shown to drop simultaneously (188,189).

At least one carcinostatic drug, Trenimon (2,3,5-Tris(1-aziridynyl)-p-benzoquinone), has been shown to release NAD glycohydrolase activity of intact Ehrlich ascites cells (129) and also to markedly reduce the cellular NAD content (187,190). The action of this drug is complex. It releases NAD glycohydrolase activity, in a dose dependent manner, at concentrations above 4×10^{-6} M and simultaneously has an ability to inhibit both the NAMN and NMN adenylyltransferase activities of these cells above a concentration of 2×10^{-5} M. The drug has no effect on NAD glycohydrolase activity when measured in cell free extracts.

The now generally favored NMN pathway for the synthesis of NAD from NAm has been demonstrated to be unable to maintain hepatic NAD levels under a drug stress imposed by azaserine. In experiments utilizing this drug as an inhibitor of NA incorporation into NAD, marked decreases in hepatic NAD were found to occur in both perfused liver (71) and *in vivo* (98). In liver perfused with physiological levels of NAm, under conditions shown to permit the maintenance of hepatic NAD content, NAD

dropped rapidly to less than 30% of the initial concentration when azaserine was added to the perfusate. Similarly, *in vivo* studies demonstrated that azaserine administration to fully fed animals resulted in a rapid decrease in hepatic NAD to 25% of the normal levels. A rapid depletion in the elevated NAD levels produced by NAm challenge was also demonstrated. NAm challenge prior to or simultaneous with azaserine administration was shown to enable the liver to maintain normal NAD levels. NAm challenge following depletion permitted these levels to return to normal while those in unchallenged azaserine treated subjects remained markedly depressed.

It seems obvious that the net synthesis of NAD observed following NAm administration to azaserine treated rats must be taking place via the NMN pathway. That this synthesis occurs only until normal or slightly higher levels are reached supports this hypothesis and the concept that NAD biosynthesis via this pathway is under tight end product constraint. However, azaserine treatment does deplete hepatic NAD in the absence of an NAm challenge. Although no effect of azaserine on NAD glycohydrolase was demonstratable *in vitro* (191), it seems probable that a parallel can be drawn with the similar effects of Trenimon on NAD glycohydrolase activity *in vivo*. Since the hepatic capacity for the destruction of pyridine nucleotides far exceeds its ability to synthesize them with substrates available at normal physiological levels, one is drawn to the conclusion that some very tight constraints are placed upon the activity of NAD glycohydrolase.

Bock *et al.* (192) have attempted to study this phenomenon in Ehrlich ascites cells. It was found that the intracellular NAD content was constant (300-400 nmoles/ml of cells) during incubation. The estimated

intracellular rate of NAD degradation was 60 nmoles/hr/ml of cells. In contrast to this, the cells were found to have a capacity to degrade exogenous NAD at a rate of 3200 nmoles/hr/ml of cells. This figure was equivalent to the degradative capacity of a DNase treated sonicate of an equivalent volume of such cells. These findings led to the conclusion that the microsomal NAD glycohydrolase is localized on the outside of the plasma membrane or on the inside of the endoplasmic tubules, the latter having been theorized to have a direct connection with the extracellular space (193). How such a compartmentalization is so radically disrupted by a carcinostatic agent is not immediately apparent.

The potential importance of NAD glycohydrolase in the control of NAD content is further emphasized by the data of Brown (194). It was observed that during the development of severe niacin deficiency the NAD content of blood and liver dropped to about 50% of normal while that of brain remained unchanged. These results might be taken as being indicative of a tissue priority for precursors or of a difference in the rate of turnover in NAD which would implicate NAD glycohydrolase. Deguchi et al. (136) demonstrated that both NAm and NA were taken up only slowly by the brain and invoked the argument that this was due to the relative impermeability of the blood brain barrier. One could reverse such an argument and claim that once inside the brain, the exit of these precursors would also be slow and thus explain the observations of Brown. However, Gerber and Deroo (195) have presented data which indicate that both the brain and skeletal muscle of the mouse and rat have an extremely slow pyridine nucleotide turnover rate.

These authors injected rats and mice with traces of NA-^{14}C and followed the time course of changing tissue specific activities as was

previously done by Shuster et al. (196) utilizing NAm challenge. In addition to the tissues studied by the latter, the brain, skeletal muscle, and testis were studied. The differences in the results were dramatic. Shuster et al. (196) had observed tissue isotope half-lives measured in terms of 2 to 3 hours. Gerber and Deroo, on the other hand (195), found the labeling of liver, kidney, spleen, and intestine rapidly reached maximum values followed by a decrease in tissue specific activity with a half-life of 2 to 3 days. In contrast, the maximum specific activity of skeletal muscle and heart required 1 to 2 days to reach a maximum. This was followed by a decrease with a half-life of between 10 and 30 days. Though the blood brain barrier might be used to explain the results observed in brain, it cannot explain the similar observation in skeletal muscle.

That there are interstitial variations in the extent of NAD turnover is apparent. NAD glycohydrolase would seem to be the key to these differences. Whether the role of this enzyme in NAD regulation is under hormonal control, or whether it is simply compartmentalized in a manner suggested by Bock et al. (192) is at the moment a question without an answer.

The study of NAD biosynthesis and its control is, quite clearly, a difficult and complex one. A great deal of very careful experimentation is required before this subject can be fully understood. A comparative study of representative mammalian and avian species might yield some insight on this subject. The careful study of the enzymology of the various tissues and the kinetics of the distribution of these vitamers throughout the intact system is needed. The utilization and catabolism of these compounds under very carefully controlled physiological

conditions and perhaps conditions of mild or even relatively severe deficiency might also prove fruitful. In any case the continued investigation of this subject under the generally used conditions of dosing is probably fruitless and can yield no further useful information than has already been made available.

The concept of both the hepatic and systemic pyridine nucleotide cycles has been seriously questioned. As a result of conceptual errors and/or inadequate experiments, many of the claims made in the literature concerning the nonfunction of either of these cycles are either not applicable to physiological circumstances themselves or are not supported by definitive evidence.

The evidence in support of the existence of a systemic pyridine nucleotide cycle has been attained from a sounder set of experiments, in the physiological sense, than any of that reported by its detractors. It seems clear that at least a systemic system is operational under normal physiological conditions. What is not clear is whether this cycle plays any vital physiological role. It is possible that this system, though a biological reality, is without a purposeful function. The information that is lacking, then, is definitive evidence that NA per se is an essential metabolic entity.

The weight of currently available evidence suggests that the function of NAm deamidase is limited in mammalian liver under conditions of normal substrate supply. Thus, under such conditions, the proposed hepatic pyridine nucleotide cycle must be assumed to turn only slowly. As to whether the in vitro evidence supporting such a conclusion is correct or whether the situation is more complex than it would appear remains to be seen. This conclusion is not intended to imply that the NMN pathway

is of greater import to hepatic NAD biosynthesis than is the pyridine nucleotide cycle.

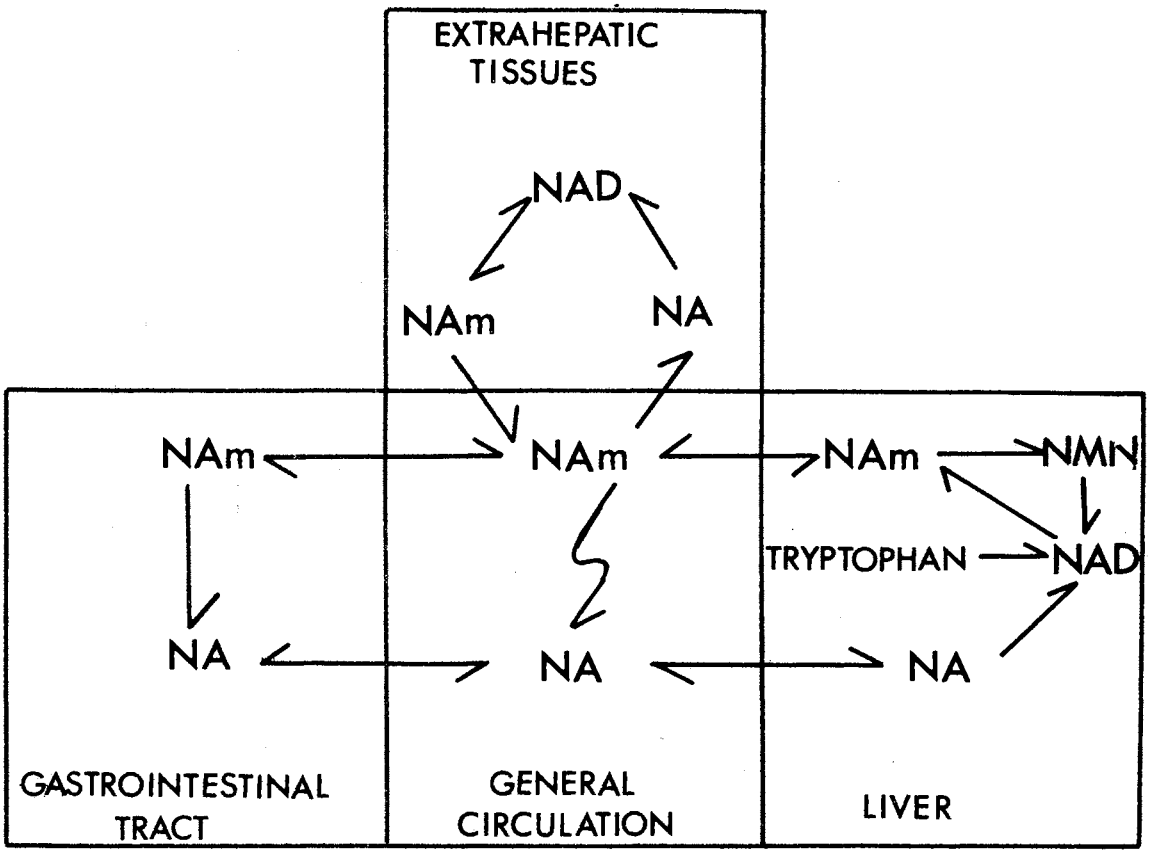
There is little doubt that NAm is the vitamer form of central importance when considering the systemic synthesis of pyridine nucleotides. This conclusion is reached predominantly on the basis of its preponderance as the circulating form of the vitamer. Having reached this conclusion, it is easy to disregard the pyridine nucleotide cycle concept and accept the NMN pathway as the primary salvage pathway being utilized in mammalian systems. There is a great deal of evidence which indicates the wide distribution of the NMN pathway in mammalian tissues and the kinetic character of the system, as studied in vitro, suggests its function in the control of NAm utilization. However, this body of evidence does not preclude a real and absolute requirement for NA and thus, the concept of an hepatic and/or systemic pyridine nucleotide cycle cannot be justifiably ignored.

It would seem that the liver is predominantly a user of tryptophan and of any NA that enters the system and at the same time is the supplier of NAm to all of those tissues requiring it for NAD synthesis. With the data available to date, it is the author's opinion that the best overall conceptual consideration of NAD biosynthesis has been put forward by Dietrich (16) and an adaptation of this concept is presented in Figure 2. It should be noted that this scheme, contrary to the claims of Grunicke and Chaykin, provides for at least a systemic pyridine nucleotide cycle and also for the use of either NA or NAm by the extrahepatic tissues.

Only two currently defined control mechanisms appear to have any effect on basal NAD biosynthesis. These involve the feedback inhibition

of tryptophan oxygenase in the liver, which limits the entry of tryptophan into the Preiss-Handler and glutarate pathways, and the feedback inhibition of NAmRTase, which limits the incorporation of NAm into NAD via the NMN pathway. Both of these inhibitions are mediated by the pyridine nucleotides themselves. Though there may be other factors involved in the direct control of NAD biosynthesis, none of those proposed in the literature to date, other than the two above indicated, are supported by any compelling evidence which indicates that they are of any significance in the normal animal being fed an adequate or less than adequate diet in terms of the NAD precursors supplied.

Figure 2. The Systemic Pyridine Nucleotide Cycle.
Adapted from Dietrich (16).


EXTRAHEPATIC
TISSUES
NAD
NAm
NA
NAm
NAm
NAm
NAm
NMN
TRYPTOPHAN
NAD
NA
NA
NA
GASTROINTESTINAL
TRACT
GENERAL
CIRCULATION
LIVER

CHAPTER III

MATERIALS AND METHODS

Materials

Nicotinamide-7-^{14}C (59-60 mCi/mmole), nicotinic acid-7-^{14}C (59.1 mCi/mmole), L-methyl-^{14}C-methionine (60 mCi/mmole), S-adenosyl-L-methionine (58 mCi/mmole), and 5-hydroxytryptamine ^{3}H-(G) creatinine sulphate (8.5 Ci/mmole) were obtained from Amersham/Searle. Nicotinamide-7-^{14}C (5.0 mCi/mmole), N^1-methyl-^{14}C-nicotinamide chloride (3.86 mCi/mmole), and L-methionine (11 mCi/mmole) were obtained from New England Nuclear. Urea-^{14}C (48.2 mCi/mmole) was obtained from International Chemical and Nuclear Corporation. NAD was a gift from Dr. D. Brusca of Enzomedic Laboratories Inc. S-adenosyl-L-methionine was obtained from Sigma (Grade I, chloride salt) or from Calbiochem (B-grade, iodide salt). Nicotinamide, triamcinolone, and hydroxycorticosterone hemisuccinate were obtained from Sigma. Adenosine triphosphate and N^1-methylnicotinamide iodide were obtained from Calbiochem. N-acetylserotonin was purchased from the Regis Chemical Co. and BioSolv-BBS-3 from Beckman Instruments Inc. PPO (2,5 diphenyloxazole) and POPOP (1,4-bis-2-(5-phenyloxazole) benzene) were obtained from the Packard Instrument Co. Quenched Liquid Scintillation Standards were obtained from the Nuclear Chicago Corp.

CM-82 (Carboxymethylcellulose), P-81 (phosphocellulose), and DE-81 (diethylaminoethylcellulose) ion exchange papers were obtained from the

Reeve Angel Co. Cellex-P (phosphocellulose powder) was purchased from Biorad Laboratories. Amberlite CG50(H) (now Rexyn 102 H) was obtained from the Fisher Chemical Co. All other chemicals were reagent grade.

Bovine pineal glands were purchased from Pel-Freeze Biologicals Inc.

Liquid Scintillation Counting

All liquid scintillation counting was done utilizing a toluene ethanol cocktail unless otherwise specified. This cocktail contained 400 ml of absolute ethanol, 600 ml of toluene, 4 gm of PPO, and 0.2 gm POPOP per liter. All counting data were converted to disintegrations per minute (dpm) utilizing the channels ratio correction method. A set of acetone quenched sealed toluene-^{14}C standards was used as the master reference throughout.

Animals

All experiments were performed with male rats. Normal animals were obtained from the Holtzman Co. These animals were held in these laboratories for 3-7 days prior to use at which time they weighed between 90 and 110 gm. Hypophysectomized animals were obtained from Hormone Assay Laboratories. These animals were committed to surgery at a weight of 90-100 gms. They were maintained in these laboratories for at least one week and were not utilized until at least 10 days after surgery at which time their weights ranged between 80 and 100 gms.

All animals were supplied with a standard pelleted commercial diet and water ad libitum. The hypophysectomized animals received water containing 5% glucose.

All compounds were either dissolved or suspended in sterile 0.9% saline and were administered intraperitoneally. The specific details of the treatments will be presented with the experimental results.

In Vivo Time Course Studies

As a result of preliminary investigations it became desirable to reinvestigate the time dependent changes in NMTase activity and hepatic NAD which had been previously observed while simultaneously monitoring any changes which might occur in hepatic SAM and methionine adenosyltransferase (MATase) activity and the effects of glucocorticoid therapy thereon.

Normal animals and hypophysectomized animals were divided into groups of three animals each. Animals were sacrificed at 0, 4, 8, 12, 24, 36, and 48 hours after receiving a nicotinamide challenge. A second set of hypophysectomized animals received a single injection of hydrocortisone hemisuccinate simultaneously with NAm challenge and were sacrificed at 8, 12, 24, 36, and 48 hours following treatment. A third set of hypophysectomized animals, run subsequent to the main portion of the experiment, was subjected to chronic glucocorticoid (triamcinolone) therapy for a period of 5 days prior to the administration of a NAm challenge. This group of animals is represented by only the 24, 36, and 48 hour time points. The animals allocated to the earlier time periods died during a period of extremely high temperatures resulting from an air conditioning failure and analysis of the data indicated that little would be gained by repeating the experiment.

At the appropriate time each animal was sacrificed by decapitation and the carcass allowed to bleed out while held under cold running water

(approximately 30 seconds). The liver was then rapidly excised, rinsed with cold water, blotted, and weighed. A 0.5-0.7 gm sample of the liver was removed and a 25% (w/v) homogenate was prepared, using a Potter-Elvehjem homogenizer, for use in the assay of MATase activity. Details of this preparation and assay procedure are given below. Approximately 1.0 gm of the remaining liver was taken and a 20% (w/v) homogenate was prepared in ice-cold 0.4 M $HClO_4$. This preparation was centrifuged at 0-4°C. for 10-15 minutes at full speed in a clinical centrifuge. The resulting supernate was assayed for both NAD and SAM as described below.

The liver remaining after the above two samples were removed was immediately frozen in liquid N_2 and stored in a deep freeze for use in subsequent determination of its NMTase activity. Two people were involved in the above procedure which permitted the completion of all of the above sampling and homogenizing steps within a period of 3-5 minutes following sacrifice of the animals.

All reagents utilized in the above experiments were prepared in a single batch, subdivided and stored in a deep freeze until required.

All animals were treated in groups of 6/day, such that they would be sacrificed at 30 minute intervals between the hours of 9:00 and 11:30 AM. The MATase assay was routinely initiated 45 minutes after each animal was sacrificed. The SAM and NAD analyses were completed by 2:00 PM on the afternoon of the same day.

The Determination of Hepatic SAM

The measurement of hepatic SAM was based on the method reported by Baldessarini and Kopin (197). This method is based on a double label isotope dilution analysis measured by the incorporation of ^3H from

N-acetylserotonin (N-acetyl-5-hydroxytryptamine) and ^{14}C from the methyl group of SAM-methyl-^{14}C into melatonin (N-acetyl-5-methoxyserotonin). This incorporation was catalyzed via the enzyme hydroxyindole-O-methyl transferase (E.C.2.1.1.4) (HIOMTase). The quantity of SAM in the sample was determined by obtaining the ratio of $^{3}H/^{14}C$ in the isolated melatonin and comparing it to a standard curve.

The Synthesis of N-Acetylserotonin. N-acetylserotonin-^{3}H-(G) was prepared from 5-hydroxytryptamine-^{3}H (G) creatinine sulphate using the method of Kopin et al. (198). The procedure involves formation of N,O-diacetylserotonin with acetic anhydride in triethylamine, followed by mild alkaline hydrolysis in sodium carbonate to remove the O-acetyl-group. Unhydrolyzed N,O-diacetylserotonin is extracted with chloroform, the solution acidified with 6N HCl and the N-acetylserotonin extracted with ethyl acetate. The ethyl acetate extract of the above reaction mixture was found to yield only one significant peak of radioactivity following thin layer chromatography on activated silica gel in a chloroform:methanol:glacial acetic acid (93:7:1) solvent system (199). This peak co-chromatographed with authentic carrier N-acetylserotonin. The ethyl acetate was removed under a stream of dry nitrogen. The residue was dissolved in water to the isotopic concentration desired and utilized without further purification since no measureable chloroform extractable radioactivity was present. The solution was kept frozen until needed and protected from light at all times.

The Synthesis and Quantitation of Double Labelled Melatonin. To 1.0 ml of the above described $HClO_4$ liver extract, 0.2 ml of SAM-methyl-^{14}C (0.3 µCi, 50 µCi/mmole) was added. A fixed, predetermined volume of

a 3 M potassium carbonate: 0.5 M triethylamine mixture was then added with vigorous stirring in order to neutralize the extract and precipitate the perchlorate (final pH of 6.5-7.0). Two 0.5 ml aliquots of the resulting supernate were transferred to HIOMTase assay tubes containing 1.0 ml of phosphate buffer (0.067 M, pH 6.7) in 0.05 M sodium bicarbonate and 0.1 ml of N-acetylserotonin-^{3}H (G) (0.4 μCi, 14 mCi/nmole). The assay was initiated by the addition of 0.2 ml of bovine pineal gland extract. The HIOMTase preparation was obtained by centrifugation of a 25% homogenate at 35,000 x g for 1 hour at 2°C. (N.B. If a crude enzyme preparation is used, it must be dialyzed prior to use.) The reaction was terminated after 60 minutes of incubation at 38°C. by addition of 2.0 ml of 1N sodium hydroxide. A series of standard assay tubes containing 0, 1, 2, 3, 4, or 6 μgm of added SAM were run with each set of livers assayed.

The alkaline assay mixtures were extracted twice with 5 ml aliquots of chloroform. The combined extracts were washed with 2 ml of 1N sodium hydroxide, transferred to scintillation counting vials, and evaporated to dryness. Toluene-ethanol cocktail (10 ml) was added and the samples counted.

The Determination of Hepatic NAD

The remainder of the perchlorate extract was neutralized by the addition of a predetermined proportion of 3 M potassium carbonate: 0.5 M triethanolamine and the potassium chlorate was allowed to precipitate. The NAD content of the neutralized extract was determined in duplicate by the alcohol dehydrogenase method (200).

Assay for L-Methionine Adenosyltransferase (MATase) Using P-81

Because of the limited amounts of liver available and the number of analyses required per liver, it was necessary to utilize a sensitive assay requiring only small quantities of tissue. Several workers have developed assays for the estimation of this enzyme based on spectrophotometric or radioisotopic methods; however, they all are either cumbersome, time consuming, insensitive and/or have been developed and used at substrate concentrations (L-methionine) well below K_m levels (201-205).

Biochemical assays for several kinases and phosphoribosyltransferases using DEAE cellulose ion exchange papers have been developed during recent years (206-210). These assays, in general, are rapid and sensitive. This type of analysis is feasible if any limitations imposed by the ion exchange capacity and selectivity of the ion exchange paper can be overcome. Newsholme *et al.* (207) suggested that carboxymethylcellulose and phosphocellulose papers make possible the extension of such assays to enzymes with cationic products. Apparently no advantage has been taken of this possibility. It was the purpose of the experiments described below to develop a method making use of a cation exchange paper in the analysis of a radioisotope incorporation assay for MATase, while at the same time optimizing the assay conditions for use in comparing the levels of enzyme activity present in the livers of rats treated as described above.

The Enzyme Assay. The assay was performed with crude homogenates of rat liver. The liver was homogenized in 1 x 10^{-2} M Tris-HCl buffer

(pH 7.6), 0.2 M KCl, 0.15 M $MgCl_2$, 0.02 M ATP (neutralized to pH 7.0-7.4 with KOH). The specific activities of L-methionine-^{14}C used ranged from 20-200 μCi/mmole. The assay was incubated at 37°C. for 30 minutes using enzyme representing 20% of the final assay volume. Final assay volumes were 0.25 ml. Incubations were terminated by addition of 10% perchloric acid (20% of the assay volume). The tubes were neutralized to approximately pH 6-7 by addition of a fixed volume of a 3 M potassium carbonate: 0.5 M triethanolamine mixture with rapid stirring to avoid localized highly alkaline conditions. The samples were kept frozen or at 0-4°C. until assayed. Protein concentration was determined by the Biuret assay (211) using bovine serum albumin as a standard.

Quantitation of S-Adenosylmethionine-^{14}C (SAM-^{14}C). The sample to be assayed (normally 0.02 ml from a clinically centrifuged assay tube) was applied with an Eppendorf micropipet (Brinkman Instruments Inc.) to the center of a square (3 cm x 3 cm) of P-81 ion exchange paper, supported by its edge on a stainless steel test tube rack. The syringe tip was rinsed twice with deionized water and the square was just saturated by the dropwise application of deionized water to the original site of application. This square was placed on top of a second square supported in the same manner and both layers were saturated by the further dropwise application of water to the center of the original square. The two layers of ion exchange paper were placed in a Buchner funnel (size 0), over two layers of Whatman No. 1 filter paper (4.25 cm discs), and mounted on a 500 ml Buchner flask. The squares were rinsed with six volumes of deionized water (approximately 300 ml). Six samples were run simultaneously. This procedure permitted 24-30 samples to be run per hour. The water rinsing was performed under vacuum by connecting the six flasks in parallel to a water aspirator.

After rinsing, the squares were removed and allowed to air dry. The two stacked squares were cut in half and the resulting 1.5 x 3 cm pairs of strips were placed on edge in a counting vial so that they formed an almost continuous double layer around the walls of the vial. Scintillation cocktail (10 ml of BioSolv-BBS-3 cocktail) was added and the vials were shaken for 15 minutes on a reciprocal shaker (240 excursions per minute) to solubilize the radioactivity (212). The samples were counted in a Model 3003 Packard Liquid Scintillation Spectrometer.

The BBS-3 scintillation cocktail contained 170 ml of BioSolv BBS-3, 60 ml of deionized water, 770 ml of toluene, 0.2 gm of POPOP and 4 gm of PPO per liter. A noneluting toluene-ethanol cocktail previously described was also used in this work.

The counting data were converted to disintegrations per minute using the channels ratio correction method. The influence of the solid support on this determination was studied and will be discussed below.

Several methods of assay termination were attempted during the development of this procedure and are discussed. Duplicate internal standard tubes were prepared in the normal manner for each set of assays and immediately heat inactivated (2 minutes at 90°C.) upon addition of the enzyme. These tubes were centrifuged in a clinical centrifuge at 2°C., a quantity of SAM-^{14}C was added to the resulting supernatant solutions, and these were kept in an ice bath. Subsamples of these tubes were put through the same assay termination and analytical procedures as the enzyme assays in question. The relative values of these control tubes were used to correct for any product losses occurring during the termination procedures.

The Binding of SAM and L-Methionine to P-81. An assay tube was run using the conditions of Mudd and Cantoni (202), inactivated by two minutes of heating, and centrifuged. A small quantity of SAM-^{14}C or L-methionine-^{14}C was added to a portion of the chilled supernatant solution (approx. 1 x 10^5 dpm/ml in each case) and the binding capacity for each compound on the paper assay system previously described was tested. Ten samples were run in each test. The percent retention was calculated by comparing the disintegration rate of samples that had been washed to that of samples which were not washed. Counting was done in 10 ml of the toluene-ethanol cocktail.

SAM was retained on P-81 at a level of 95.5 $\pm$ 2.9% (standard deviation) when assayed at a level of 0.02 ml and 96.0 $\pm$ 2.5% when assayed at a level of 0.04 ml. Up to 5% of the retained label was found in the second layer of paper at the lower level and 10% at the higher level of sampling. The use of a double layer system yielded more consistent results than did a single layer system.

Under the conditions of high ionic strength used in this assay system, carboxymethylcellulose (CM-82) proved unsuitable. Less than 50% of the product was retained at a sampling level of 0.02 ml and less than 30% at a level of 0.04 ml.

Approximately 0.5% of the original L-methionine-^{14}C was retained on P-81. This value led to high background counting rates in early trials. This problem was largely eliminated by prepurification of the methionine. The purification was accomplished by passing the labelled substrate through a Cellex-P(Na^+) column eluted with water. Presoaking the squares of P-81 with unlabelled 200 mM L-methionine or the rinsing of the samples into the P-81 with this solution and doubling the volume of water used

in washing the assay papers yielded no further background reduction. Prepurification reduced substrate retention to about 0.1%. This accompanied by an increase in substrate specific activity resulted in background values being reduced to about 5% or less of the total incorporation of label observed in a normal assay. The background activity observed was time independent.

Quantitation of S-Adenosyl-L-Methionine-^{14}C. An assay tube was prepared and heat inactivated and SAM-^{14}C (diluted to approximately the same specific activity as the methionine substrate) was added to a portion of the supernatant solution to yield a disintegration rate well in excess of that of typical assays. A dilution series was prepared using the remainder of the unlabelled supernate as the diluent. This preparation was done in plastic tubes since it was found that SAM bound to glass resulting in erroneous results. The original sample and the dilution were assayed in triplicate at each of three volumes (0.01, 0.02, and 0.05 ml). The samples were counted in the non-eluting toluene-ethanol cocktail. The results are shown in Figure 3. The observed disintegration rate was found to be linear and independent of the quantity of sample bound. This result is in agreement with the findings of Nunez and Jacquemin (213) and it permits the use of a single correction factor for all assays when accounting for the self-absorption effect in a non-eluting counting system.

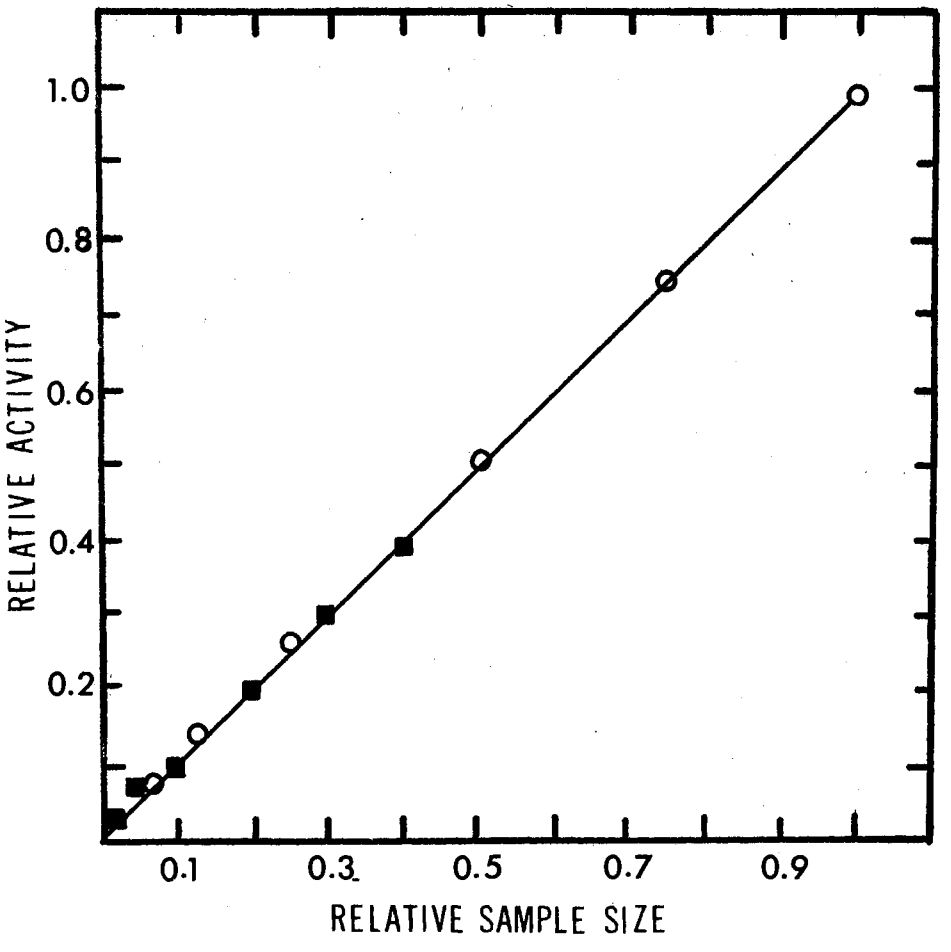
As mentioned above, it was found that SAM bound to glass. This was first observed when an effort was made to determine the proportion of counts unaccounted for by channels ratio correction due to self-absorption. It would be expected that the disintegration rate calculated from the sample on the solid support would be lower than for the same sample

Figure 3. The Proportionality of Disintegration Rate to the Quantity o
SAM Bound on P-81.

○— indicates the dilution series sampled at 0.05 ml.

□— indicates the dilution series sampled at 0.02 ml.

The data for 0.01 ml sampling are not shown.
Each point represents the average of three determinations.
Methods are described in the text.


1.0
0.8
0.6
0.4
0.2
RELATIVE ACTIVITY
0.1
0.3
0.5
0.7
0.9
RELATIVE SAMPLE SIZE

in free solution. It was found, however, that SAM-^{14}C samples counted on P-81 and in a free solution yielded essentially the same disintegration rate, if not somewhat higher for the P-81 bound material. It was further shown that less than 2% of the counts were eluted from the paper by the toluene-ethanol cocktail, indicating a glass binding phenomenon and not the release of labelled product from the support into solution was responsible. Further, the addition of unlabelled SAM resulted in an increase in counting rate of samples in "free" solution. Counting the liquid samples in polyethylene vials also resulted in an increased counting rate relative to the same samples counted in glass vials. SAM is very sensitive to heat and heating the samples in a boiling water bath for 30 minutes prior to addition of the cocktail resulted in an increased counting rate. It was finally determined that the disintegration rate calculated for samples bound on the P-81 support was 78% $\pm$ 2% of the total observable in free solution when the binding phenomena had been reversed. This value, being independent of sample size, was routinely used in accounting for self absorptive losses when counting in the toluene-ethanol cocktail.

Elimination of Counting Losses Due to Self-absorption. All of the results reported herein have been quantitated with the method described above. Recently, however, a procedure was developed which permits the in-vial elution of the sample from the ion exchange paper, thus permitting a direct calculation of the results from the observed counting rates and affording about a 20% increase in assay sensitivity (212).

The BBS-3 cocktail described above results in essentially a total release of the SAM from the P-81 into solution. Thus, the disintegration rate obtained from a previously bound sample duplicates that of an

equivalent sample in free solution and the channels ratio method yields the true disintegration rate without further correction. In practice it was found that the disintegration rates obtained for samples bound on P-81 and released by shaking in BBS-3 cocktail range between 96-99% of those for similar samples in free solution in the same cocktail.

Both of the above methods of quantitation yield equally satisfactory results when working with ^{14}C. Although a simple non-eluting toluene cocktail is the most economical, the choice as to which is the most desirable is left to the preference of the user. On the other hand, self-absorption losses associated with counting ^{3}H on a solid support are very large. When counting ^{14}C on a solid support the disintegration rate is underestimated by about 20-30% (about 22% in this instance) due to self-absorption. Under the same conditions, disintegrations rates obtained when counting ^{3}H are underestimated by 75-95% (212). Therefore, the added cost of using an eluting cocktail such as the one described in the present study is easily justified on the basis of the increase in sensitivity and precision of the estimation.

Conditions for an Optimal Assay. The above experiments indicated that the P-81 analytical system was suitable for a sensitive comparative assay and a series of experiments were run in order to determine the conditions required for an optimal assay. The assay composition given above yielded results equally as good as those obtained using the conditions of Mudd and Cantoni (202). The lowering of the L-methionine concentration from 0.02 M to 0.01 M and the $MgCl_2$ concentration from 0.3 M to 0.15 M resulted in no reduction in the observed activity. This permitted a reduction in ionic strength and also doubled the sensitivity of the assay without increased use of labelled substrate. The addition

of unlabelled SAM or sulfhydryl reagents to the assay system using crude homogenates resulted in no stimulation of enzyme activity in contrast to the findings of Mudd et al. (203). However, much of the work done by these authors was performed with partially purified enzyme and the L-methionine content of their assay was below K_m levels. Either of these conditions might explain their findings.

Assay Termination. Due to the high ionic strength of the assay and the limited exchange capacity of the paper, the initial experiments were terminated by two minutes of heating at 90°C. in order to avoid further increases in ion content. It was found, however, that heating resulted in a large and rapid loss of product as measured by loss of binding on P-81. The time course of this loss is shown in Figure 4. As would be expected, it is a pseudo first order decay with a rate constant $k = -0.235$ min^{-1}. For this reason acid termination was attempted in the manner described above. A comparison of the results for acid and heat termination is shown in a typical time course plot (Figure 5). Both methods yielded excellent results in terms of linearity and reproducibility. The acid termination, however, is easier and results in little or no loss of product, thus eliminating the need for a large, potentially variable, correction factor in the calculations. Any increase in ionic strength that occurred caused none of the problems expected. The acid termination procedure therefore became the method of choice. Both methods yielded preparations which were stable to storage for at least 10 days. Care must be taken, however, to keep the pH below 7.6 when neutralizing the acid terminated assay, otherwise some degree of alkaline hydrolysis of SAM can occur.

Figure 4. The Time Course of Thermal Degradation of SAM.

SAM-^{14}C was added to the supernatant solution from a heat terminated label free standard assay. Triplicate aliquots of this supernatant were then placed in a water bath at 90°C. for the times specified and the recovery of SAM determined by the P-81 assay described. Each point represents the average of 6 assays run in duplicate on the triplicate aliquots.

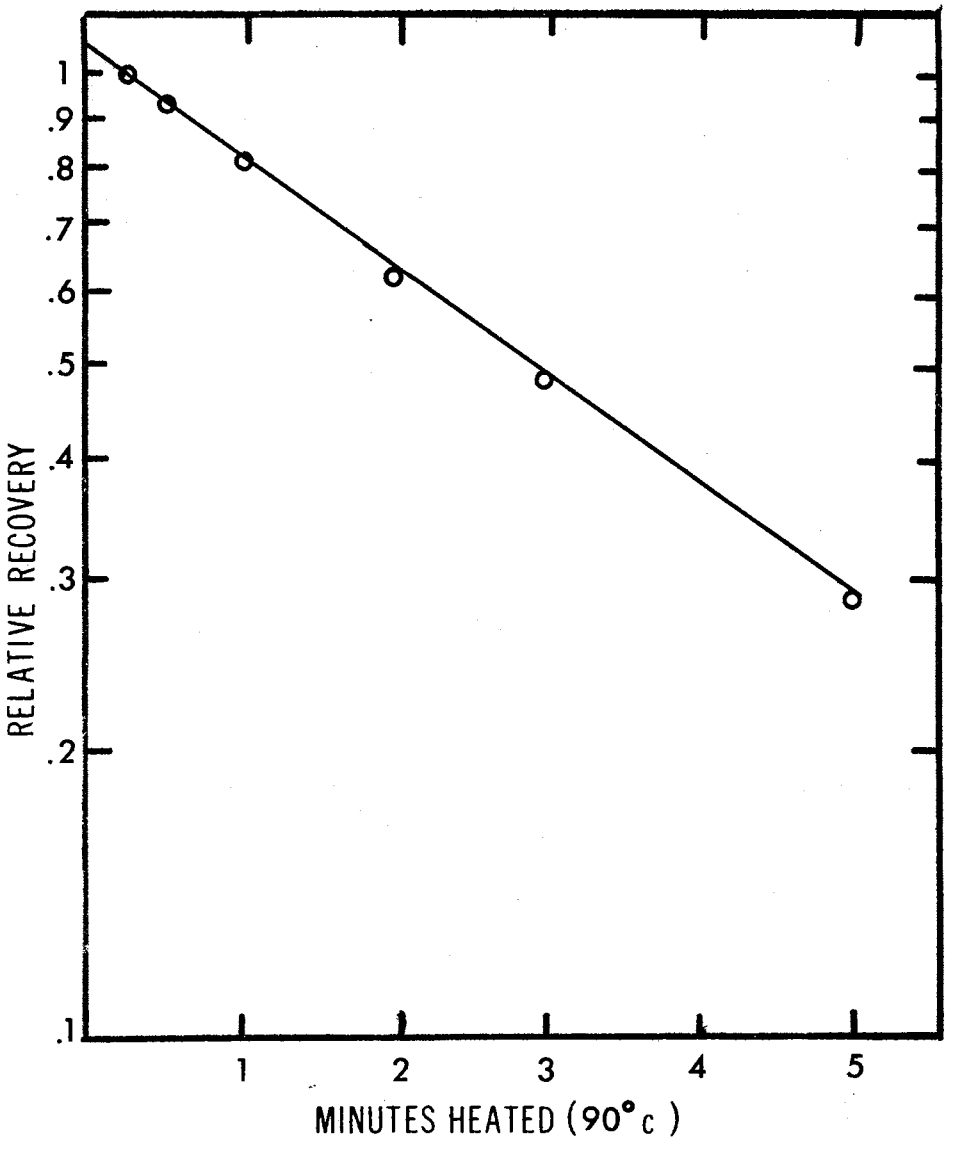
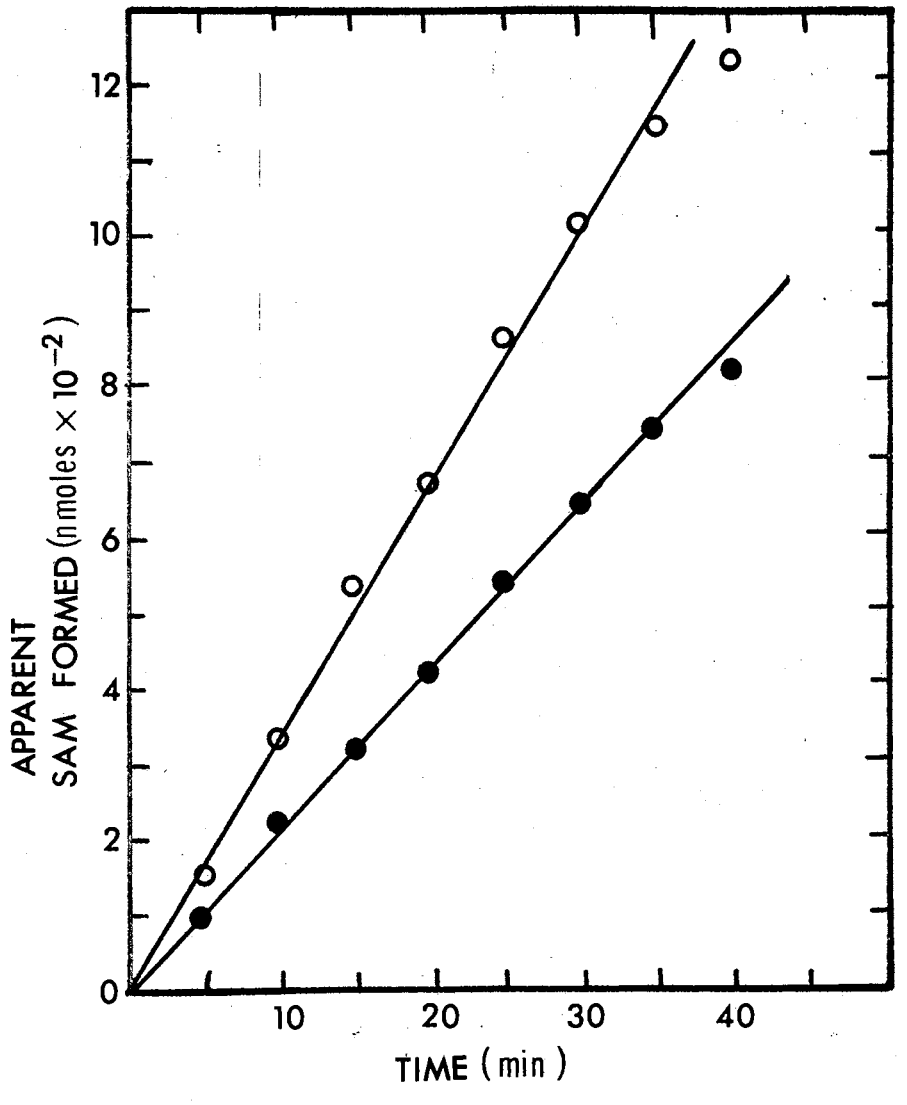

RELATIVE RECOVERY
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
1
2
3
4
5
MINUTES HEATED (90° c)

Figure 5. Comparison of Heat vs. Acid Termination of the MATase Assay

○ — indicates acid terminated assays.

● — indicates heat terminated assays.

The assay contained 16 mg of protein/ml.
Acid terminated standards were 98.1% and heat terminated standards were 61.6% of the internal standard SAM control. The specific activity of the enzyme was calculated to be 129.3 and 130.7 nmoles/mg protein/hr by the acid and heat terminated assays respectively. Assay preparations and conditions were as reported under methods.


APPARENT
SAM FORMED (nmoles × 10⁻²)
0
2
4
6
8
10
12
10
20
30
40
TIME (min)

An effort was made to terminate the assays by chelating the magnesium with EDTA. This method proved unsuitable, since EDTA at a concentration of 0.075 M (sufficient to supply a maximum of only one potential binding site per magnesium ion) resulted in almost a complete loss of SAM binding on P-81.

Assay Linearity. The relationship between enzyme activity and assay incubation time and protein concentration are shown in Figures 6 and 7, respectively. The assay is linear for about 25-30 minutes and over the entire range of protein concentrations studied. The upper curve in each figure represents the data obtained with the BBS-3 cocktail and the lower curve in each case represents the same series of assays counted in the non-eluting toluene-ethanol cocktail. The latter data have not been corrected for the self absorption losses. The activity measured on the toluene-ethanol derived line is approximately 79% of that from the BBS-3 derived line.

The Enzyme Preparation. This enzyme is relatively unstable in the supernatant solutions obtained from crude homogenates. A relatively rapid loss in activity (8-10%/hr.) was noted when the supernatant solutions were held at 0^{o}C. in an ice:water bath. When 1 ml aliquots of freshly prepared supernates were placed in a deep freeze, this loss occurred to essentially the same extent during the first three to four hours. The substitution of 5×10^{-3} M dithiothreitol or dithioerythritol for mercaptoethanol in the homogenizing media did not prevent or reduce the rate of this activity loss.

In order to minimize errors introduced by this loss of enzyme activity when making comparisons between animals, all preparative procedures

Figure 6. SAM Formation as a Function of Incubation Time.

○— indicates samples counted in the BBS-3 counting syste (each point is the average of 6 assays run in triplicate on duplicate incubations).

●— indicates samples counted in the toluene:ethanol system (each point is the average of duplicate assays on a single incubation).

A fresh preparation of enzyme was used. All assays were terminated with acid. The assays contained 7.9 mg protei ml. Assay conditions and preparations were as reported i the text.

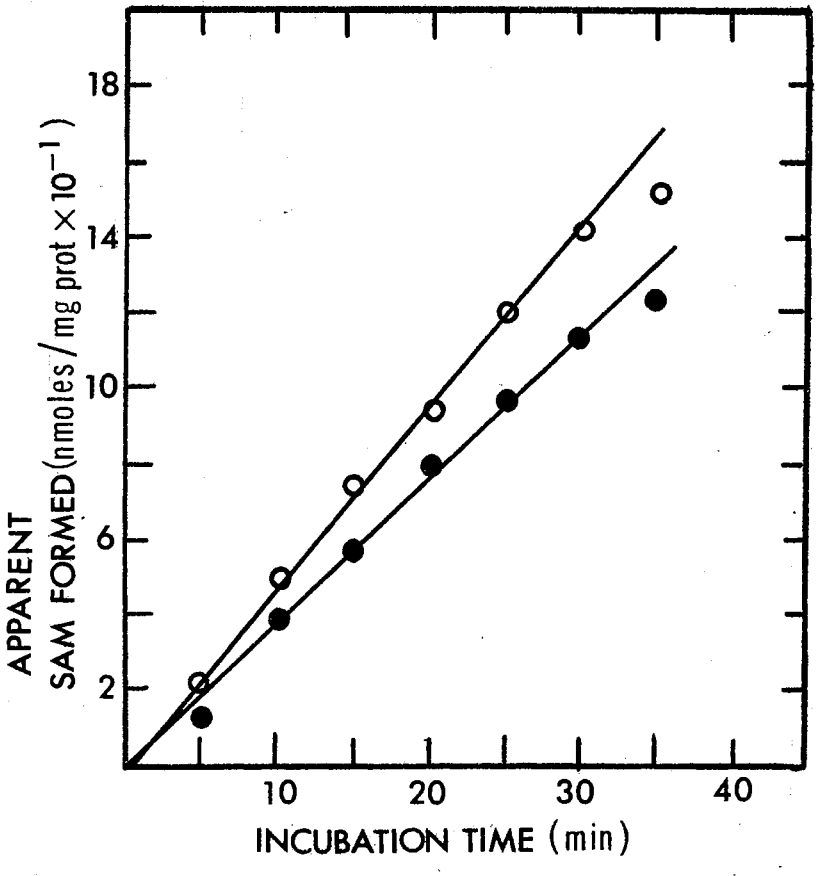
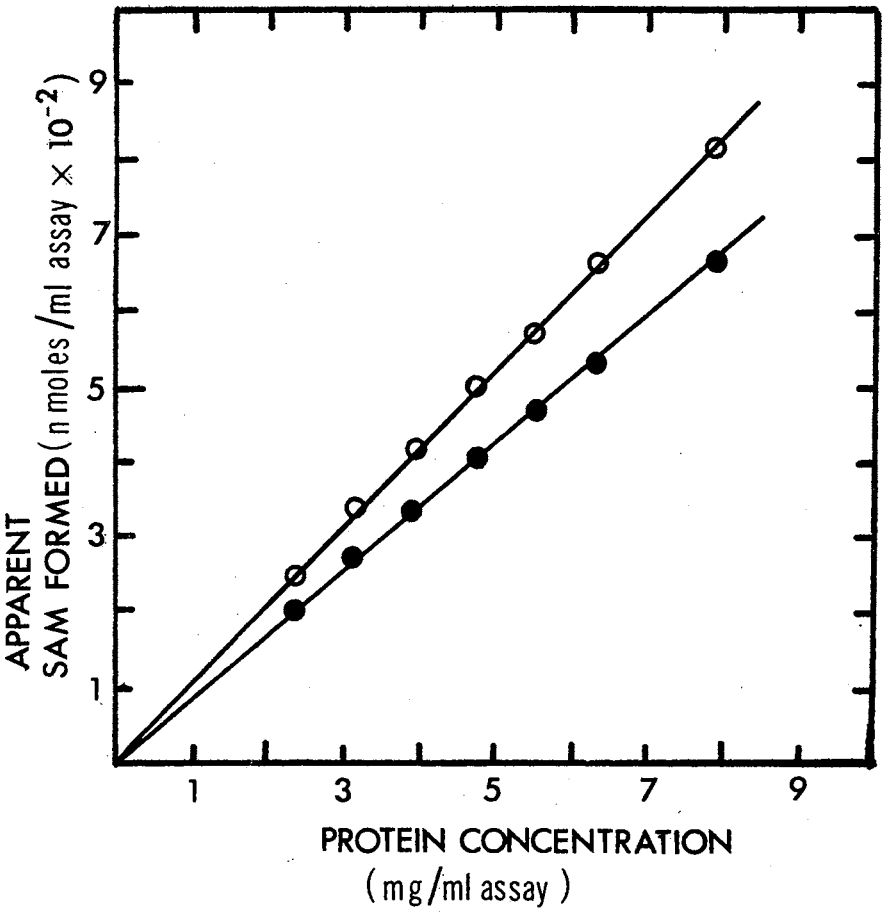

APPARENT
SAM FORMED (nmoles / mg prot × 10⁻¹)
18
14
10
6
2
10
20
30
40
INCUBATION TIME (min)

Figure 7. SAM Formation as a Function of Protein Concentration.

○- indicates samples counted in the BBS-3 counting system (each point is the average of 6 assays run in triplicate on duplicate incubations).

●- indicates samples counted in the toluene:ethanol system (each point is the average of 4 assays run in duplicate on duplicate incubations).

A fresh preparation of enzyme was used. All assays were incubated for 20 minutes and terminated with acid. Assay conditions and preparations were as reported in the text.


APPARENT
SAM FORMED (n moles /ml assay × 10^{-2})
9
7
5
3
1
1
3
5
7
9
PROTEIN CONCENTRATION
(mg/ml assay)

were done as quickly as possible and at fixed times relative to the time of sacrificing each animal as was described previously.

Assays for Nicotinamide-N^1-Methyltransferase (NMTase)

Two assay systems and two analytical procedures were used in measuring the NMTase activity in the above experiments. The assays were initiated by the addition of enzyme, incubated at 38°C. for 60 minutes, and terminated by 2 minutes of heating in a boiling water bath regardless of the assay conditions or analytical systems used.

Sodium Acetate Buffered Assay. This assay, with the exception of the termination procedure, was run under conditions identical to those used previously by Flesner (23) and essentially as reported by Cantoni (214). The final reaction mixture contained 1×10^{-2} M NAm (0.2 mCi/mmole NAm-7-^{14}C if an isotope incorporation assay was being used) and 2×10^{-3} M SAM. Sodium acetate buffer (0.1 M, pH 5.0) was added at a level of 20% of the assay volume and the NMTase preparation was added at a level of 40% of the assay volume. The enzyme preparation used was the supernate obtained from a 20% (w/v) liver homogenate. This homogenate was prepared in ice cold sodium acetate buffer (0.1 M, pH 5.0) using a Potter-Elvehjem homogenizer, and centrifuged at 25,000 x g for 30 minutes at 2°C.

Tris-HCl Buffered Assay. This assay was identical to that described above in terms of NAm and SAM content. Tris-HCl buffer (0.2 M, pH 7.6) was substituted for sodium acetate buffer. The enzyme was prepared in exactly the same manner except that Tris-HCl (0.05 M, pH

8.1) was used as the homogenizing medium (215). Both sets of assay conditions reported above have been previously demonstrated to be linear with respect to time and protein concentration ranges used (23,215). This was checked and confirmed.

Fluorescence Analysis of NMTase Activity. The spectrofluorometric determination of NMNAm was based on a procedure reported by Huff and Perlzweig (216). A 0.1 or 0.2 ml aliquot of the assay supernate was made to 1.0 ml with glass distilled water and 0.5 ml of 2-butanone were added. To this mixture 0.2 ml of 6N sodium hydroxide were added with immediate vigorous mixing and the reaction mixture was incubated at room temperature for 5 minutes. Then, 0.3 ml of 6N hydrochloric acid were added and the mixture placed in a boiling water bath for 3 minutes. The reaction tubes were cooled to room temperature, 1.0 ml of 20% potassium phosphate (monobasic) were added and the mixture diluted to 11 ml with glass distilled water. SAM free incubations were run and processed in the same manner and utilized to correct for the fluorescence contribution made by any compounds other than NMNAm which were present in the reaction mixture, in particular NAD. All assays were analyzed in duplicate and every tenth reaction tube was an NMNAm standard.

The relative fluorescence was determined in an Aminco Bowman Spectrofluorometer at 25°C. A 365 nm excitation wave length was used and the fluorescence output measured at 460 nm.

Radioisotopic Incorporation Analysis of NMTase Activity. The radioisotope incorporation analysis for NMTase activity was performed in exactly the same manner as that for MATase activity above. The binding and counting characteristics of NMNAm-^{14}C on P-81 ion exchange paper

were found to be identical to those reported for SAM-^{14}C. However this assay was not as precise as that for MATase due to the presence of a relatively high and variable background counting rate. This background was time independent. Unlike the MATase assay, prepurification of the NAm-^{14}C on Cellex-P columns had little effect on the background counting rate observed. Presoaking the P-81 with 0.3 M NAm did not significantly improve the background counting rate observed. Although this assay was sufficiently accurate for use with the saturation assays performed in the time course studies, the variability in, and the extent of, the background counting rate resulted in this methodology being unsuitable for enzyme kinetic analysis in the vicinity of K_m concentrations of either substrate. Recently, some promising results were obtained when paper strip chromatography on P-81 with buffer elution was used in an effort to reduce the background. This system, however, has not yet been perfected.

Excretion Studies

These studies were aimed at determining the basis for the differences observed between normal and hypophysectomized rats subjected to NAm challenge.

Urine Collection

The animals were housed in individual plastic cages (4" x 4" base) with wire mesh floors, supported in the mouth of a large polyethylene funnel (6.5 in. ID) which was loosely plugged with glass wool. The 24 hour urine samples were collected in large test tubes which were held in plastic beakers packed with powdered dry ice. The animals were

fasted during the period of urine collection but had access to water ad libitum. At the end of the collection period, the funnels were thoroughly rinsed and the sample plus washings kept frozen until analyzed.

Determination of the Total Isotope Excretion

The urine samples were thawed and filtered through Whatman No. 1 filter discs into a 125 ml Buchner flask under a slight vacuum supplied by a water aspirator. The volume of the sample was determined and triplicate 0.05 or 0.1 ml aliquots were placed in 10 ml of scintillation cocktail for the determination of the total radioactivity excreted.

Determination of the Isotope Excreted as NAm and NMNAm

The determination of specific catabolites was restricted to NAm and NMNAm since they were the two major components found in the urine and showed the largest and therefore most easily measurable differences. A 0.1 ml aliquot of each urine sample was spotted in a band across the entire width of each of 4 Whatman No. 1 paper strips (4 x 50 cm, 8 cm from one end). The strips were developed by descending chromatography. Two were developed with n-butanol saturated with 3% ammonium hydroxide and two with n-butanol saturated with water to which glacial acetic acid was added just prior to use (60:1). The strips were developed for a period of 14-18 hours which permitted a frontal migration of 30-35 cm.

The two solvent systems were utilized because they afforded a more certain separation of the compounds of interest. The alkaline solvent system moved NAm well in front of the next fastest migrating labelled component (R_f 0.7 vs 0.5) but did not separate NMNAm from NAgly. The

acidic solvent, on the other hand, permitted the separation of NMNAm from all other catabolites; however, a clean separation of NAm was not so certain.

The radioactive peaks were located with a Nuclear-Chicago Actigraph III 4 π paper strip scanner. The peaks corresponding to NMNAm on the acid developed and NAm on the alkali developed strips were cut out, placed in scintillation vials containing 10 ml of cocktail, and counted.

CHAPTER IV

RESULTS AND DISCUSSION

The studies reported here were initiated as a result of the paradoxical outcome of previous research in these laboratories.

It was observed by Lee et al. (22) that marked differences existed in the metabolite excretion patterns of normal and hypophysectomized rats following the administration of a NAm challenge. In particular, the excretion of NMNAm by hypophysectomized rats was markedly lower than in normal rats and the time course of its excretion was radically different (Figure 8).

These observations led to the hypothesis that NMTase might play a significant role in the control of NAm metabolism. It was thought that the synthesis of NMTase might be mediated by glucocorticoids, in a manner similar to that previously observed with tryptophan oxygenase (24). One would thus predict that glucocorticoids would stimulate an increase in NMTase activity and that a similar response would be observed in the normal but not in the hypophysectomized animal following NAm challenge. A series of experiments was performed to test this possibility and a partial summary of results obtained is presented in Table 1. The basal levels of NMTase activity were found to be considerably higher in the livers of hypophysectomized rats than in those of normal rats. Of the treatments attempted, only NAm challenge was found to have any effect on the NMTase activity. The effect observed, an apparent

Figure 8. The Time Course of NMNAm Excretion in Normal and Hypophysectomized Rats Following NAm Challenge.

Solid bars represent NMNAm excretion by hypophysectomized rats and the open bars that by normal rats. The methodology used was essentially identical to that described in Chapter III and has been previously reported in detail (142). Adapted from Lee et al. (22).

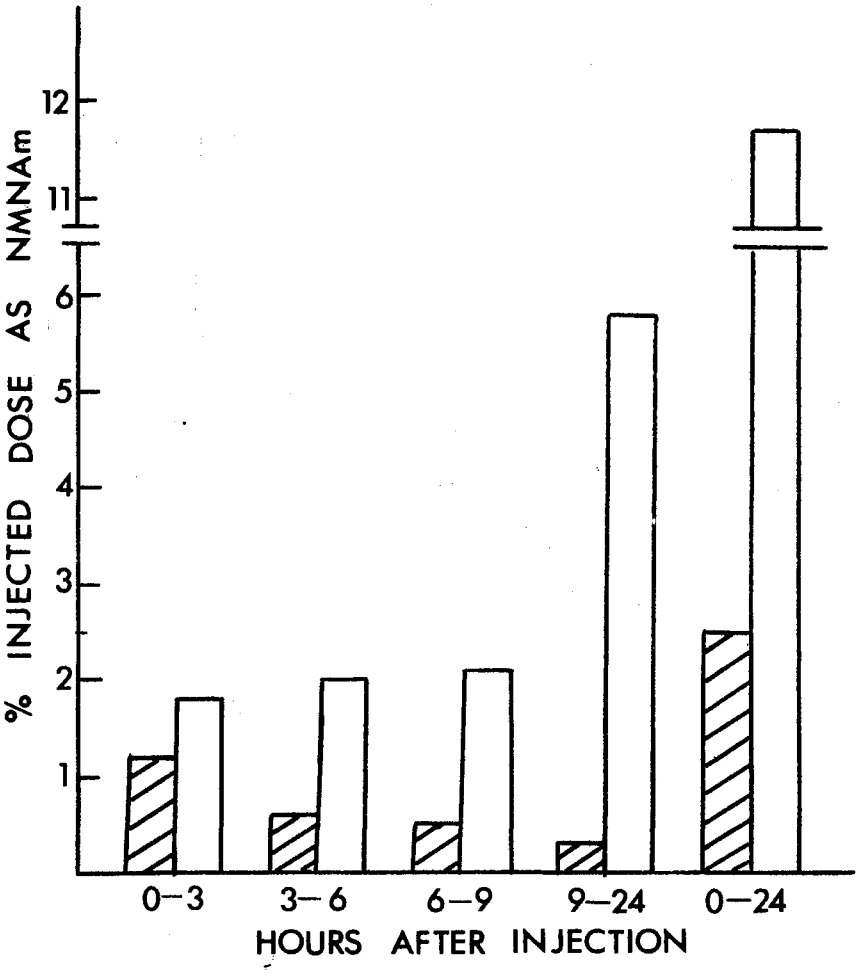

% INJECTED DOSE AS NMNAm
12
11
6
5
4
3
2
1
0–3
3–6
6–9
9–24
0–24
HOURS AFTER INJECTION

TABLE I

THE TIME COURSE OF CHANGES IN NMTASE ACTIVITY FOLLOWING VARIOUS TREATMENTS

Animals/ Treatment	NMTase Activity nmoles/mg protein/hr				
	Hours After Treatment				
	0	4	8	12	24
Normal/ 500 mg NAm/Kg (6)	24.2	47.6	59.0	57.6	43.7
Normal/ 50 mg NA/Kg (3)	27.1	24.2	24.3	26.4	24.3
Normal/ 5 mg HC/Kg (3)	23.5	29.6	22.6	27.7	23.6
Hypophysectomized/ 500 mg NAm/Kg (3)	37.2	68.0	61.8	70.9	78.5

The enzyme was prepared and assayed using the sodium acetate buffer system described under Methods. The assays were terminated by the addition of two volumes of 10% trichloroacetic acid and analyzed fluorometrically as previously described with the exception that instead of running SAM free control assays, the assays were corrected for the fluorescence contribution from NAD by determining the latter compound separately on the basis of its fluorescence in alkali (217). Adapted from Flesner (23).

two-fold increase in enzyme activity, was the same in both hypophysectomized and normal animals.

The results of these experiments are inconsistent with the hypothesis mentioned above. The potential for hormonal influences is essentially eliminated by the observation of an increase in enzyme activity in the hypophysectomized rat. It was concluded, therefore, that the elevation of enzyme activity was directly related to the increase in hepatic NAD content which results from NAm challenge. This is the only obvious factor common to both the normal and hypophysectomized rat under such conditions. Thus, the working hypothesis was changed to assume that NAD is an allosteric modifier of NMTase, and preliminary experiments were performed to test the effect of NAD on the *in vitro* activity of the enzyme (23). These experiments indicated that NAD had no influence on the enzyme activity observed. However, the levels of NAD added to the assays were insufficient to approximate those normally observed *in vivo*, and were much less than those observed following NAm challenge.

Influence of NAD on the *In Vitro* Activity of NMTase

The studies reported here were initiated with an attempt to determine the *in vitro* effects of appropriately high concentrations of NAD upon NMTase.

Upon addition of high concentrations of NAD to the assays, the fluorescence methodology previously employed for the analysis of enzyme activity became unuseable. The added NAD became the primary contributor of fluorescence in the reaction mixture, making an accurate estimate of NMNAm impossible. Rather than attempting to modify the fluorescence methodology, it was decided that the use of a radioisotope incorporation

assay would be simpler and would simultaneously afford an independent confirmation of the previous findings.

The original analyses were conducted by placing 0.1 ml aliquots of heat terminated assay supernatant solutions on Amberlite CG50(H) columns (0.6 x 6.0 cm, 1.5 ml bed volume) which were washed with 12-13 column volumes of deionized water to remove the NAm-^{14}C. The NMNAm-^{14}C was then eluted in 8.0 mls of 0.5 M HCl and the radioactivity present determined. This system was tedious, relatively insensitive, and was subsequently abandoned in favor of the previously described methodology. However, using this assay methodology it was determined that the addition of exogenous NAD at levels of up to 0.02 M had no significant influence on the in vitro activity of NMTase. Representative data drawn from experiments demonstrating this point are presented in Table II.

Not only was the addition of NAD found to be without effect, but dialysis of the enzyme supernate against homogenizing buffer to remove endogenous NAD was also found to be without influence on the activity observed.

The above experiments did nothing to help explain the in vivo observations. It was therefore presumed, perhaps too quickly, that the observed phenomena were due to in vivo effects.

MATase is the enzyme responsible for the synthesis of SAM, which is, in turn, the methyl donor for the NMTase catalyzed reaction. The activity of this enzyme is depressed in adrenalectomized animals and is inducible by glucocorticoid therapy (218). It was thought possible that the supply of SAM might be depressed in hypophysectomized rats and thus become limiting under NAm challenge. The time course of NMNAm elimination (Figure 8) shows a decreasing rate of excretion with time

TABLE II

THE INFLUENCE OF NAD ON NMTASE ACTIVITY IN VITRO

Source of Liver	NAD ADDED (final concentration) (mmolar)	NMTase Activity[a] nmoles/mg prot/hr. undialyzed homogenate	dialyzed homogenate[e]
Normal Rat[b]	0	13.4	
	0.5	13.0	
	1.0	13.0	
	2.0	15.0	
Hypophysectomized Rat[c]	0	24.1	
	0.5	23.7	
	1.0	23.7	
	2.0	23.3	
Normal Rat[d]	0	11.5	12.5
	1	10.5	12.5
	5	12.6	11.9
Normal Rat[d]	0	10.0	12.0
	1	11.9	12.9
	5	12.1	13.0

a) Assay procedures are described in the text
b) Injected with 1.0 ml of saline and sacrificed immediately
c) Sacrificed 12 hours after NAm challenge
d) Sacrificed 8 hours after injection of 1.0 ml saline
e) Homogenates were dialyzed against 100 volumes of homogenizing buffer at 4°C. for eight hours
b),c) Assayed in sodium acetate buffer system, d) Assayed in Tris-HCl buffer system

in the hypophysectomized rat as compared to the relatively constant output by the normal animal. Such a pattern fits with the possibility of a developing methyl donor deficiency and such an occurrence would permit an explanation of the apparently contradictory findings of Lee (22) and Flesner (23).

Preliminary Excretion Studies

If the above hypothesis were correct, it would dictate that glucocorticoid therapy would reverse the previously observed differences in the excretion patterns of normal and hypophysectomized rats. Greengard et al. (11) had already demonstrated the reversal of the increased extent and duration of the NAm induced elevation of hepatic NAD in hypophysectomized rats by both glucocorticoid and thyroxine therapy. Therefore, a partial repetition of the excretion studies with the addition of glucocorticoid treatment to the experiments was initiated.

In these experiments, all animals (5/treatment group) received an NAm-^{14}C challenge (500 mg/Kg, approximately 5 μCi-^{14}C). Those rats receiving glucocorticoid therapy simultaneously received a single dose of hydrocortisone hemisuccinate (175 mg HCHS/Kg). This was equivalent to the dose previously utilized by Greengard in reversing the hepatic NAD response. For the purposes of this study it was thought sufficient to measure only total excretion and the excretion of NAm and NMNAm. The results of this experiment (with standard deviations) are presented in Table III.

The administration of glucocorticoids completely reversed the excretion differences between normal and hypophysectomized animals. However, while manipulating these data it became apparent that the

TABLE III

THE EXCRETION OF NAM-^{14}C, NMNAM-^{14}C, AND TOTAL RADIOACTIVITY BY NORMAL AND HYPOPHYSECTOMIZED RATS FOLLOWING AN NAM-^{14}C CHALLENGE WITH AND WITHOUT HYDROCORTISONE THERAPY[a]

Animal/ Treatment	% of Injected ^{14}C Excreted			% of Total ^{14}C Excreted	
	Total	as NAm	as NMNAm	as NAm	as NMNAm
Normal/ 500 mg NAm/Kg	72.7 ±6.5	43.3 ±4.3	6.5 ±0.5	59.5 ±4.4	9.0 ±1.2
Normal/ 500 mg NAm/Kg 175 mg HCHS/Kg	77.5 ±5.7	50.0 ±3.4	5.4 ±0.9	64.9 ±3.9	7.0 ±0.9
Hypophysectomized/ 500 mg NAm/Kg	46.9 ±8.5	27.8 ±4.8	2.7 ±0.8	57.8 ±7.6	5.7 ±0.8
Hypophysectomized/ 500 mg NAm/Kg 175 mg HCHS/Kg	83.0 ±1.6	47.2 ±3.8	6.1 ±1.0	56.7 ±4.7	7.3 ±1.1

[a]All results are given with standard deviations. Experimental methods are described in the text.

relative excretion of NAm was essentially the same in all animals. The differences in NMNAm excretion when expressed on a relative basis were reduced by half. It should be pointed out that the excretion of NMNAm observed in the normal animals was only about half of the amount previously recorded (Figure 8). In a subsequent experiment, to be reported below, hypophysectomized rats receiving glucocorticoid therapy excreted quantities of NMNAm at the levels previously observed by Lee (22) in normal rats.

The expression of the data on a relative basis seemed to indicate the possibility that much of the difference in the observed excretion patterns, in the case of NAm at least, was attributable to nothing more than a decrease in the relative rate of excretion in the hypophysectomized rat. Such an argument was rather cursorily suggested by Greengard et al. (34) in an effort to explain their observation of a marked increase in the half life of NAm in hypophysectomized rats.

Despite the paucity of data concerning the effects of glucocorticoids and endocrinectomy on renal function, a review of the literature supports such an argument. White et al. demonstrated a depression of renal function in the hypophysectomized dog (166). While sodium and potassium excretion remained normal, there was an increase in plasma urea and nonprotein nitrogen. This is indicative of a reduced renal clearance of these compounds. Sodium and potassium excretion is controlled by metallocorticoids produced by the zona glomerulosa of the adrenal cortex. This region of the adrenal gland does not atrophy following hypophysectomy. On the other hand, glucocorticoids are produced by the zona fasciculata of the adrenal cortex. Extensive atrophy of this region occurs following hypophysectomy and is reversible by ACTH therapy

(165). Ingabar et al. (167), studying the renal influences of adrenal insufficiency in man, demonstrated that ACTH and cortisone therapy increased both glomerular filtration rate and renal plasma flow, both of which were depressed under these conditions. Similar evidence in support of adrenal involvement in urine production is to be found in reports by Gaudinio and Levitt (169) and Boss et al. (170).

Earle et al. (172) demonstrated that ACTH and glucocorticoid replacement therapy in the hypophysectomized dog could only account for about one half to two thirds of the observed depression in renal function. This implies a contribution from other hormonal sources. Braun-Mendex (171) demonstrated that thyroid extract had the effect of reversing the reduction in kidney weight observed in hypophysectomized rats and showed that thyroidectomy alone was sufficient to account for this weight loss. Davis et al. (173) showed that the decrease in renal blood flow accompanying hypophysectomy was not due to changes in adrenocortical function. This too may be a thyroxine mediated effect. GSH has also been demonstrated to have an influence in renal function in hypophysectomized animals (168). A brief review of this area (168) indicates that in all cases of adrenalectomy, hypophysectomy, thyroidectomy, or hypoendocrine activity of these glands, a general reduction in renal function occurred. Conversely, in cases of hyperendocrine activity there was a stimulation of renal activity. There is a direct parallel between the observed changes in the extent and duration of the NAm induced elevation of hepatic NAD and the influence of these endocrine factors on renal function, as has been previously discussed.

Beyer et al. (154) have shown that NMNAm is excreted by both glomular filtration and tubular secretion mechanisms. It has subsequently

been demonstrated that the tubular secretion of NMNAm is inhibited by other basic compounds and by general alkalosis (219-221). The ability of basic compounds to inhibit NMNAm secretion decreases with decreasing basicity. Thus, it is feasible that the differences in NMNAm excretion are also due only to changes in kidney function. In light of this possibility, experiments aimed at determining the influence of glucocorticoid therapy on the NMNAm excretory capacity of the hypophysectomized rat were performed.

In the first of these experiments, five hypophysectomized rats were challenged with NAm. Simultaneously, trace doses of urea-^{14}C (approximately 2 μCi, 2 μmoles) and NMNAm-^{14}C (approximately 1.5 μCi, 1.7 μmoles) were administered. A second group of five animals received identical treatment and, in addition, 175 mg HCHS/Kg were administered. Urine samples were collected for 24 hours. Aliquots of each sample were chromatographed in the butanol-ammonia solvent system previously described and the peaks corresponding to urea and NMNAm cut out and counted. In addition, samples of each urine were incubated with urease for 30 minutes at 38°C. after the method of Conway (222). The incubations were then acidified with 6 N HCl, placed in a vacuum desicator for 30 minutes to allow for the removal of $^{14}CO_2$ and the residual ^{14}C determined. The results of this experiment are presented (with standard deviations) in Table IV.

Though not the ideal test compound, urea-^{14}C was used on the presumption that the blood urea of the hypophysectomized rats would be elevated and that the dilution of urea-^{14}C therein would reflect any differences in glomerular filtration (urea clearance) caused by glucocorticoid therapy. The excretion of urea-^{14}C was considerably lower in

TABLE IV

THE EFFECT OF HYDROCORTISONE REPLACEMENT THERAPY ON THE ABILITY OF NAM CHALLENGED, HYPOPHYSECTOMIZED RATS TO EXCRETE TRACER DOSES OF UREA-^{14}C AND NMNAM-^{14}C[a]

	% Injected Dose Recovered in the Urine[b]		
	Urea-^{14}C (chromatographic)	NMNAm-^{14}C (chromatographic)	(after urease)
Hypophysectomized	72±5.2	83.7±4.3	79.2±5.1
Hypophysectomized (175 mg HCHS/Kg)	98±5.9	88.8±5.5	83.5±7.7

a) All experimental details are described in the text.

b) The % of the injected dose recovered in each compound is based on the radioactivity originally injected as that compound.

the hypophysectomized rat. This indicates that urea clearance is depressed in hypophysectomized rats and that this function can be stimulated by hydrocortisone therapy. There was also a slight increase in the excretion of NMNAm following glucocorticoid administration, however, this change was small and does not appear to be sufficient to account for the differences observed in the excretion of NMNAm arising from a NAm-^{14}C challenge.

The above experiment was criticized. It was argued that a trace dose of NMNAm-^{14}C administered with an NAm challenge might not be sufficient to demonstrate a difference in the renal capacity for its elimination. In retrospect, the methods of quanitation were also open to some question. In response to this criticism, the experiment was modified and rerun in two parts. In the first part of the experiment, the animals were treated as above with the exception that only a tracer dose of urea-^{14}C was administered. One week later, the same two groups of animals were again treated and at this time they received NMNAm-^{14}C (60 mg/Kg, 1.5 μCi). This dose is equivalent to the amount excreted by a normal rat in the first 24 hours following an NAm challenge (Figure 8). The excretion of the label was quantitated simply by counting aliquots of the urine samples. The results, with standard deviations, are presented in Table V. Again no apparent difference in the capacity to excrete NMNAm is indicated. The differences in urea excretion, although somewhat smaller, remain.

The above described excretion experiments indicated that the primary differences (NAm and total excretion) between the excretion patterns of normal and hypophysectomized rats were probably due to a depression of renal function in the hypophysectomized animals. Further, this

TABLE V

THE EFFECT OF HYDROCORTISONE REPLACEMENT THERAPY ON THE ABILITY OF HYPOPHYSECTOMIZED RATS TO EXCRETE UREA AND N-METHYLNICOTINAMIDE[a]

	% of Injected Dose Recovered in Urine	
	Compound Injected	
	Urea-^{14}C	NMNAm-^{14}C
Hypophysectomized (5)	84±4.5	90±8.4
Hypophysectomized + Hydrocortisone (5)	92±5.3	91±9.9

a) Complete experimental details are given in the text.

function appeared to be sufficiently stimulated by a single dose of glucocorticoid to permit the general reversal of these patterns. At the same time, no evidence was obtained supporting the conclusion that the differences in NMNAm excretion were due merely to changes in renal activity. It was concluded, therefore, that the basis for this difference might lie elsewhere.

In Vivo Time Course Studies

A preliminary experiment had failed to uniformly reproduce the NMTase response previously observed by Flesner in NAm challenged animals (Table I). It was thus decided to reinvestigate the in vivo time dependent changes in NMTase. The experiment was designed for the simultaneous measurement of NMTase, NAD, MATase, and SAM. Hypophysectomized animals received either no treatment, single doses of hydrocortisone, or chronic triamcinolone therapy. Thus, while checking the influence of NAm challenge on NMTase, this experiment afforded a simultaneous measurement of the influence of glucocorticoid therapy on the NAm induced changes in hepatic NAD in hypophysectomized rats. This phenomenon had previously been studied by Greengard et al. (11). Also, the experiment permitted a test of the hypothesis that the decreased NMNAm excretion in hypophysectomized rats might be due to the development of a methyl donor deficiency. In the event that such a change was noted, the influence of glucocorticoid replacement therapy would be known. The general procedures and methodology used in this study have been previously described.

The Responses of Hepatic NAD

The time course of the changes occurring in hepatic NAD levels are presented graphically in Figure 9, and the numerical data, with standard

Figure 9. Changes in Hepatic NAD Levels of Normal Rats and of Hypophysectomized Rats (With and Without Glucocorticoid Therapy) Following NAm Challenge.

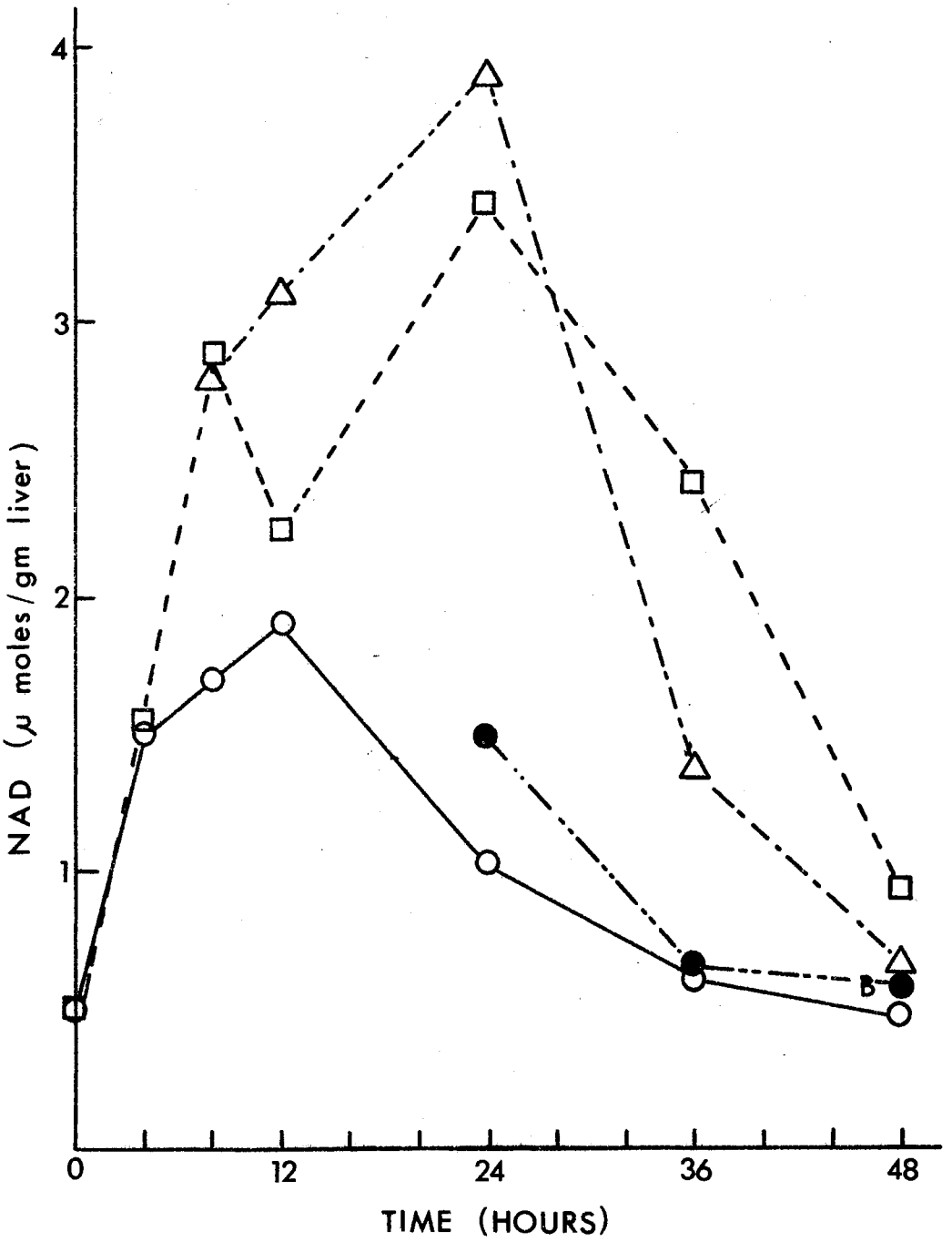
○ Normal Rats

□ Hypophysectomized Rats

△ Hypophysectomized Rats (175 mg HCHS/Kg)

● Hypophysectomized Rats (chronic triamcinolone 5 days at 60 mg/Kg/day)

Experimental details are described in the text.


4
3
2
1
0
12
24
36
48
NAD (μ moles/gm liver)
TIME (HOURS)
B

deviations are presented in Table VI. The general pattern of the changes in the normal and hypophysectomized animals are in reasonable agreement with those previously reported by Greengard et al. (10). Contrary to his findings is the fact that in normal animals, hepatic NAD did not return to basal levels within 24 hours. The influence of glucocorticoid therapy in hypophysectomized animals was to shorten the duration of the elevation of hepatic NAD. Triamcinolone was more effective than hydrocortisone in this respect. This too is in contrast to what would be predicted on the basis of the data of Greengard et al. (11). It is possible that an explanation of this contradiction might be found in the differences in methodology used in the two studies. Greengard et al. administered both hormones at the level of 125 mg/Kg in a single subcutaneous dose. By comparison, an equivalent dose of hydrocortisone and chronic triamcinolone (60 mg/Kg for 5 days) were used in the present study. Both were administered intraperitoneally. The change in the route of administration, in particular, may be the critical difference. This might lead to the much more rapid inactivation of these hormones, thus lessening the extent and duration of their relative effectiveness. If this is the case, the reversal of the effectiveness in the two hormones in depressing hepatic NAD levels is of no consequence. That is, the previous hypothesis put forward in explanation of the paradoxical effects of glucocorticoids on hepatic NAD levels would still be valid, since one can argue that as administered in these experiments, neither drug was capable of invoking a sustained increase in circulating tryptophan.

The Responses of Hepatic MATase

Prior to the initiation of this study, a report was uncovered which indicated that the activity of MATase was not depressed below normal in

TABLE VI

CHANGES IN HEPATIC NAD LEVELS OF NORMAL RATS AND OF HYPOPHYSECTOMIZED RATS (WITH AND WITHOUT GLUCOCORTICOID THERAPY) FOLLOWING NAM CHALLENGE

	HEPATIC NAD (μmoles/gm liver)			
Animals	Hypophysectomized			Normal
Ancillary Treatment[a]	none	HCHS	T	none
TIME (HOURS)				
0	0.49 ±0.09			0.49 ±.06
4	1.55 ±0.14			1.49 ±0.25
8	2.88 ±0.88	2.75 ±0.19		1.69 ±0.61
12	2.24 ±0.38	3.13 ±0.19		1.90 ±0.49
24	3.42 ±1.25	3.89 ±1.02	1.48 ±0.30	1.02 ±0.39
36	2.42 ±0.27	1.36 ±0.66	0.65 ±0.08	0.61 ±0.09
48	0.93 ±0.18	0.66 ±0.04	0.70 ±0.04	0.47 ±0.05

[a]HCHS; hydrocortisone hemisuccinate (175 mg/Kg); T, chronic triamcinolone (60 mg/Kg/day for 5 days).
Experimental details are described in the text.

hypophysectomized rats as had been shown in adrenalectomized rats (223). If this were the case, the basis of the hypothesis relating reduced NMNAm excretion to a reduction in methyl donor availability would be eliminated. In the absence of a decrease in MATase activity, there would be no reason to suspect a methyl donor deficiency. It had been previously demonstrated that large doses of NAm did not deplete the SAM pools in normal rat liver (224). Therefore, it is absolutely mandatory to the above hypothesis that MATase activity be depressed to a level where the capacity for SAM synthesis becomes limiting.

The observation that MATase levels were normal in the hypophysectomized rat was unpredicted and without obvious explanation. It had been demonstrated that thyroidectomy resulted in an increase in hepatic MATase above normal levels and that thyroxine therapy could reduce the activity present in thyroidectomized, hypophysectomized, adrenalectomized, and normal rat liver (223). Growth hormone was also shown to depress MATase activity in the liver of hypophysectomized rats. On the surface, this seems to indicate that hypophysectomy eliminated the depressant effects of these hormones and thus the enzyme remained at normal levels. However, glucocorticoids stimulate the synthesis of this enzyme (218,223). In the absence of a normal glucocorticoid stimulus, it did not appear reasonable that removal of the depressant effect of other hormones should necessarily permit enzyme production to proceed at normal levels. Thus, before discarding the hypothesis and eliminating this portion of the time course study, a preliminary experiment was performed. The MATase activity present in normal and hypophysectomized rat liver was determined. In contrast to Pan and Traver (223), the enzyme activity in hypophysectomized rat liver was

found to be only about 50% of that in normal rat liver. The MATase study was therefore retained and the results are reported in Figure 10 and Table VII (with standard deviations).

The data obtained using chronic triamcinolone therapy is in excellent agreement with the findings of Pan and Traver (218). These results also confirmed the preliminary observation that the basal MATase activity was depressed in the liver of hypophysectomized rats.

When expressed on a per mg protein basis, the activity of MATase in hypophysectomized rat liver is lower than in the normal rat. This activity was elevated to, or in excess of, normal by the administration of a single dose of hydrocortisone. Such a pattern of enzyme activity and influence of hydrocortisone would be predicted if the SAM hypothesis were valid. However, presuming all other factors to be equal, the most important figure is the total enzyme activity available. This is more clearly reflected by the expression of the enzyme activity on the basis of tissue weight. When expressed on this basis, the patterns of MATase activity appear to change. The influence of hydrocortisone therapy does not seem to be nearly so marked. The level of MATase in the hypophysectomized rat is essentially unchanged during the course of the experiment. The enzyme activity in normal rat liver, on the other hand, after an apparently brief elevation appears to decrease through the first 24 hours following NAm challenge.

It is interesting to speculate that the apparent changes in total MATase activity in the liver of the normal rat are a reflection of a changing balance of hormonal events induced by NAm challenge and mediated via the hypophysis. However, though [redacted] and NAm have been demonstrated to elevate the circulating levels of glucocorticoids (164), there is no

Figure 10. Changes in Hepatic MATase Activity in Normal Rats and in Hypophysectomized Rats (With and Without Glucocorticoid Therapy) Following NAm Challenge.

○ Normal rats

□ Hypophysectomized rats

△ Hypophysectomized rats (175 mg HCHS/Kg)

● Hypophysectomized rats (chronic triamcinolone, 5 day at 60 mg/Kg/day)

Experimental details are described in the text.

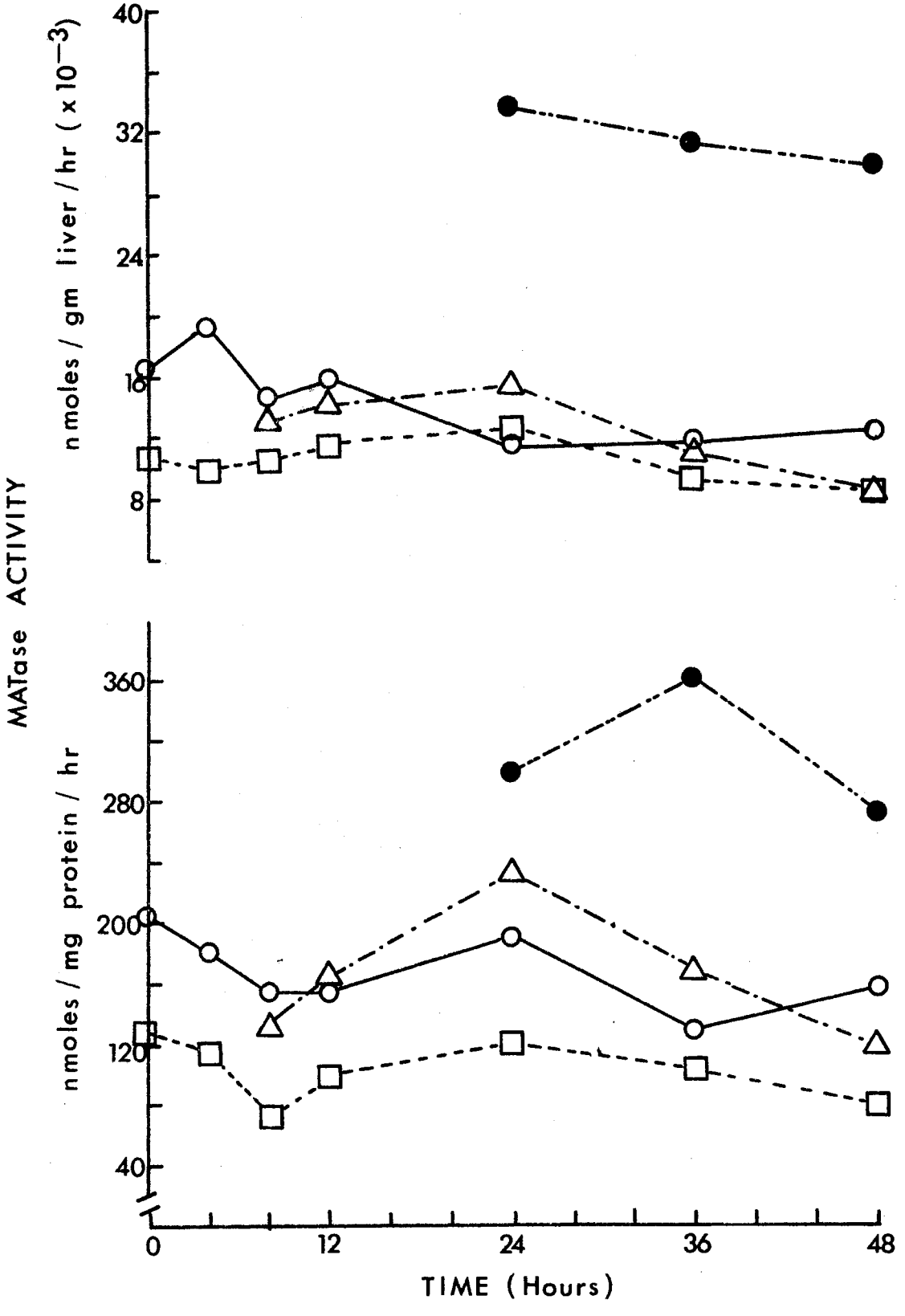

MATase ACTIVITY
n moles / gm liver / hr (x 10⁻³)
40
32
24
16
8
n moles / mg protein / hr
360
280
200
120
40
0
12
24
36
48
TIME (Hours)

TABLE VII.

CHANGES IN HEPATIC MATase ACTIVITY IN NORMAL RATS AND IN HYPOPHYSECTOMIZED RATS (WITH AND WITHOUT GLUCOCORTICOID THERAPY) FOLLOWING NAm CHALLENGE.

	MATase Activity (nmoles/hr)							
Animals	Hypophysectomized						Normal	
Ancillary Treatments[a]	none		HCHS		T		none	
Units per	mg protein	mg liver	mg protein	mg liver	mg protein	mg liver	mg protein	mg liver
Time (Hours)								
0	128 ±11	10,768 ±2136					204 ±47	16,585 ±3181
4	114 ±17	9719 ±724					181 ±36	19,446 ±5620
8	72 ±11	10,548 ±1602	131 ±14	13,135 ±1364			157 ±13	14,499 ±974
12	98 ±18	11,500 ±892	164 ±10	14,089 ±1849			156 ±20	15,845 ±2136
24	120 ±32	12,600 ±1338	231 ±61	15,251 ±2598	298 ±42	33,617 ±2030	191 ±21	11,464 ±819
36	102 ±8	9210 ±812	137 ±11	10,924 ±678	359 ±17	31,324 ±3017	129 ±33	11,510 ±2489
48	78 ±5	8448 ±1176	117 ±20	8442 ±1094	272 ±43	29,982 ±2223	157 ±60	12,467 ±2285

[a]HCHS; hydrocortisone hemisuccinate (175 mg/Kg); T, chronic triamcinolone (60 mg/Kg/day for 5 days). Experimental details are described in the text.

direct evidence, of which the author is aware, in support of the presumption that thyroxine or growth hormone levels are influenced by the administration of pharmacological doses of either vitamer.

The Response of Hepatic SAM

Baldessarini and Kopin (197), who developed the methodology used in this study, found that the hepatic SAM content of rats of approximately the age and weight of those used in this study was extremely variable. The values they obtained ranged from 25 to more than 100 nmoles of SAM/gm of liver. Although one would like to argue that a naturally occurring variability could account for the range of values observed in this study (Table VIII), such is not the case. The variation within the groups of animals is quite possibly due to such an interanimal variation. However, due to an oversight in the experimental procedures, the SAM data obtained in this study has lost most of its value in terms of a time course comparison.

The data presented in Table VIII are the average values of two determinations on each liver. The disagreement between these determinations was rarely in excess of 10%. The groups between which direct comparisons are deemed appropriate are indicated by identical subscripts. The values in these groups were obtained on the same day and from a simultaneously generated standard curve. The reasons for restricting interpretation of the data in such a manner are two-fold. First, in at least two instances, (a and i) the SAM-^{14}C was not added to the samples prior to the neutralization step. Thus, any SAM degradation that might occur during this step was unaccounted for. Second, though originally intended, the HIOMTase preparations were not dialyzed prior to use. The

TABLE VIII

HEPATIC SAM CONTENT OF NORMAL RATS AND OF HYPOPHYSECTOMIZED RATS (WITH AND WITHOUT GLUCOCORTICOID THERAPY) FOLLOWING NAM CHALLENGE

	HEPATIC SAM (nmoles/gm liver)		
Animals	Hypophysectomized		Normal
Ancillary Treatment	none	HCHS	none
Time (Hours)/Animal No.			
0/1	58.8		47.5
/2	34.8 a		57.5 g
/3	30.0		46.3
4/1	35.0		43.8
/2	35.2 a		40.0 g
/3	35.0		37.5
8/1	61.0	95.0	80.0
/2	38.8 b	95.0 b	45.0
/3	73.8	78.8	66.3
12/1	87.5	56.3	76.3
/2	56.4 c	82.5 c	77.5 h
/3	100.0	61.3	81.3
24/1	90.0	65.0	25.0
/2	74.0 d	72.5 d	30.0 i
/3	100.0	68.8	31.3
36/1	48.8	38.8	77.5
/2	46.3 e	38.8 e	62.5 h
/3	42.5	45.0	76.3
48/1	47.5	36.3	42.5
/2	38.8 f	30.0 f	36.3 i
/3	42.5	48.8	42.5

HCHS, hydrocortisone hemisuccinate (175 mg/Kg)

All other experimental details are described in the text.
Groups with common subscripts were determined on the same day (a-i).

pineal gland is an organ rich in SAM and essentially unique for N-acetylserotonin. Several different enzyme preparations, from two different batches of pineal glands, were used. In several instances the preparations were used over a span of several days. Since both of the above compounds are highly unstable, their addition to the standard assays with the HIOMTase had an unquantified effect upon both the slope and intercept values of the individual standard curves. Thus, any comparison of the data obtained on different days is rather tenuous. A repetition of these measurements was not possible due to the limited amounts of tissue available.

Restricting the comparisons to the groups indicated, no patterns emerge with respect to the relative levels of SAM observed in these groups and the MATase activity found in the same livers. In particular, the hepatic SAM content of the hydrocortisone treated hypophysectomized rats is not consistently different from that of the control animals. It was thus concluded, that no marked changes in the hepatic SAM content of either normal or hypophysectomized rats occurred as a result of NAm challenge. That is, although capable of stimulating an increased formation of MATase, hydrocortisone therapy did not result in any marked changes in hepatic SAM content, while conversely the depressed levels of MATase present in hypophysectomized rat liver are sufficient to maintain adequate SAM synthesis.

Although these conclusions are considerably weakened by the poor quality of the SAM data presented, it would appear that any attempt to explain the differences in NMNAm excretion on the basis of a methyl donor deficiency is unfounded. Evidence supporting the validity of this conclusion, at least under normal physiological conditions, is presented below.

The Responses of Hepatic NMTase

The results of the NMTase study are presented in Figure 11 and Table IX (with standard deviations). When expressed on a per mg of protein basis, the curves fluctuated widely. As will be shown below, this pattern at least in the normal rat series seems to be real. That is, it is primarily a reflection of the variability in the groups of animals and not in the analytical procedures.

The data do not replicate the findings of Flesner (Table I). If any real increase in enzyme activity occurred, it was, at the maximum, about 40% of basal compared to the 100% increase reported by Flesner when expressed on a per mg of protein basis and only slightly in excess of 20% when expressed on a per gm of liver basis. In even more marked contrast to the findings of Flesner (23), the hepatic enzyme levels present in the hypophysectomized rat do not appear to respond at all.

As indicated by the previous data, the apparent basal NMTase activity in normal rat liver is lower than that in the hypophysectomized rat and this difference is by and large maintained throughout the treatment period studied, particularly through the first 24 hours. Hydrocortisone therapy appeared to have little influence on this activity regardless of the mode of expressing the data. Triamcinolone therapy, on the other hand, appeared to increase the total NMTase activity present by about 20%. However, this increase in activity is not clearly reflected in the specific activity of the enzyme.

The wide variability in the data and the almost complete lack of agreement with the previous finding promoted a partial reanalysis of the tissues. The series of normal rat livers was rehomogenized in sodium acetate buffer and the isotope incorporation assay rerun. At the same

Figure 11. Changes in the Hepatic NMTase Content of Normal Rats and of Hypophysectomized Rats (With and Without Glucocorticoid Therapy) Following NAm Challenge.

○ Normal rats

□ Hypophysectomized rats

△ Hypophysectomized rats (175 mg HCHS/Kg)

● Hypophysectomized rats chronic Triamcinolone (60 mg/Kg day for 5 days)

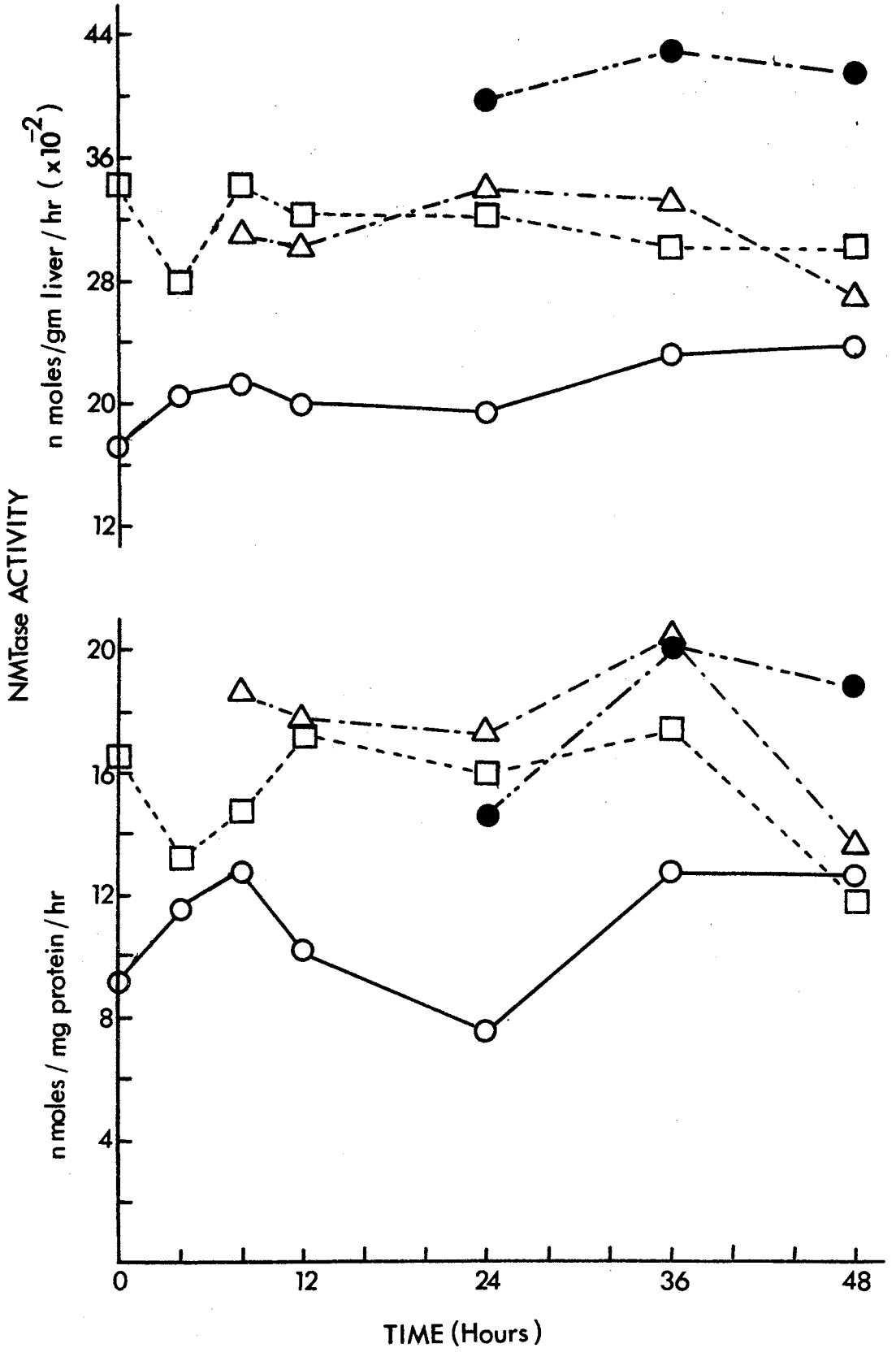

NMTase ACTIVITY
n moles/gm liver / hr (x10^{-2})
44
36
28
20
12
n moles / mg protein / hr
20
16
12
8
4
0
12
24
36
48
TIME (Hours)

TABLE IX.

CHANGES IN THE HEPATIC NMTase CONTENT OF NORMAL RATS AND OF HYPOPHYSECTOMIZED RATS (WITH AND WITHOUT GLUCOCORTICOID THERAPY) FOLLOWING NAm CHALLENGE.

	NMTase Activity (nmoles/hr)							
Animals	Hypophysectomized						Normal	
Ancillary Treatments[a]	none		HCHS		T		none	
Units per	mg protein	mg liver	mg protein	mg liver	mg protein	mg liver	mg protein	mg liver
Time (Hours)								
0	16.5 ±4.0	3420 ±550					9.1 ±1.1	1720 ±585
4	13.2 ±1.1	2780 ±220					11.5 ±1.3	2050 ±205
8	14.7 ±3.5	3420 ±240	18.3 ±2.2	3080 ±190			12.7 ±1.3	2130 ±330
12	17.2 ±1.7	3220 ±335	17.7 ±1.3	3010 ±260			10.1 ±2.0	1990 ±160
24	15.9 ±3.7	3210 ±340	17.2 ±3.4	3390 ±285	14.5 ±2.1	3960 ±295	7.5 ±2.3	1940 ±295
36	17.4 ±1.7	3010 ±285	20.3 ±2.6	3310 ±160	20.0 ±1.7	4270 ±330	12.7 ±2.5	2320 ±230
48	11.8 ±1.0	3000 ±220	13.5 ±3.6	2690 ±375	18.7 ±4.5	4130 ±415	12.6 ±5.7	2360 ±525

[a]HCHS, hydrocortisone hemisuccinate (175 mg/Kg); T, chronic triamcinolone (60 mg/Kg/day for 5 days). Experimental details are described in the text.

time, the previous Tris-HCl assays were reanalyzed fluorometrically. The results of these analyses are presented in Figure 12. The pattern of change in NMTase activity yielded by each of the analytical procedures was essentially the same. This indicates that the variation is predominantly due to real differences between the various groups of animals. No plausible explanation seems to fit this pattern. Thus, one is forced to presume that it is a result of random variation among the animals and that no real increase in NMTase activity occurred in response to NAm challenge.

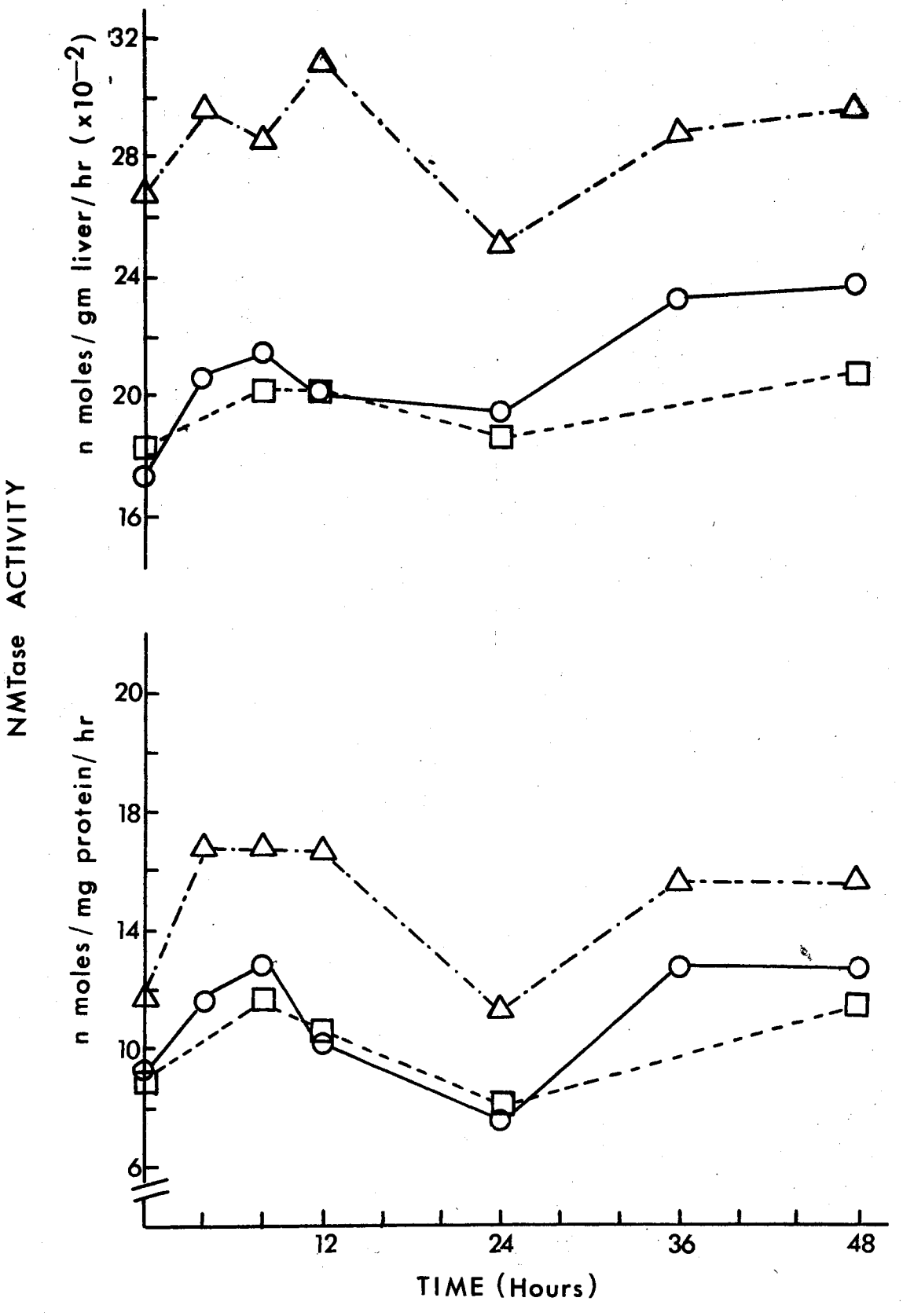
One other possibility existed which had to be checked before the original NMTase data could be found to be in complete disagreement with the present data. It was possible that the original data had been obtained from livers which had been stored for a considerable period of time. An early experiment had shown that the preincubation of either dialyzed or undialyzed enzyme preparations at 25°C. for one hour in the absence of either substrate resulted in about a 60% loss of enzyme activity. If the enzyme was preincubated in the presence of 2×10^{-2} M NAD, this loss in enzyme activity did not occur. A similar protection of NMTase activity by SAM had been previously demonstrated by Burton *et al.* (215). If the original data had been obtained from stored livers, it was possible that the differing NAD levels in the livers could have yielded differential protection against losses in enzyme activity during the storage period. Thus, after ten months of storage, samples of all the remaining livers from the series of hypophysectomized control animals were reanalyzed using the fluorescence methodology previously described. The relative fluorescence patterns in the SAM free assays paralleled the time course of NAD content originally observed in these livers, indicating

Figure 12. The Time Course of Changes in Hepatic NMTase Activity as Determined by Three Independent Analyses.

○ Tris-HCl Buffer, NAm-^{14}C Incorporation Analysis

□ Sodium Acetate Buffer, NAm-^{14}C Incorporation Analysis

△ Tris-HCl Buffer, Fluorescence Analysis


NMTase ACTIVITY
n moles / gm liver / hr (x10⁻²)
32
28
24
20
16
n moles / mg protein / hr
20
18
14
10
6
12
24
36
48
TIME (Hours)

that NAD and or an NAD related substance was still present. The NMTase activity measured in the 0, 4, 8, 24, and 36 hour treatment groups was found to be 8.8, 9.1, 9.3, 9.4, and 7.7 nmoles/mg protein/hr. These data indicate that some loss of enzyme activity occurred during the ten month storage period; however, the enzyme activities were still essentially the same in all groups tested.

Influence of Multiple NAm Challenge on Hepatic NMTase Activity

It had been previously shown by Greengard et al. (34) that multiple injection of NAm maintained the elevation of hepatic NAD in normal rat livers through 30 hours in a manner similar to that observed in hypophysectomized rat livers following a single NAm challenge. In this work, they treated normal rats with an NAm challenge and followed with the injection of 100 mg NAm/Kg every two hours.

In anticipation of the repetition of the results previously obtained by Flesner (23), a few animals were submitted to multiple NAm challenge as a preliminary test of the effects of this treatment on hepatic NMTase activity and NAD levels. It was expected that the elevated enzyme levels would fall as the NAD levels returned to normal and thus by maintaining or even increasing the extent of NAD elevation, a corresponding increase in NMTase activity would occur.

The animals received an NAm challenge every 12 hours and were sacrificed 12 hours after the final challenge dose. Normal rats were challenged two, three, or four times (one per group) and a hypophysectomized rat was challenged 4 times.

Drawing conclusions from such a limited experiment is admittedly an unsound practice. However, since the author sets no precedent in this area by so doing, the data are presented in Table X. For comparative purposes, the NMTase activity observed in the control rats (zero time) and the hepatic NAD content of the NAm challenged rats (12 hours after a single challenge dose) as obtained from the time course experiment are included. These preliminary findings indicate that elevation of hepatic NAD for extended periods of time is without marked influence on hepatic NMTase. The data also indicate that multiple challenge results in a decrease in the hepatic NAD levels observed relative to that which arises from a single NAm challenge. If this is indeed the case, it indicates that such treatment yields inhibition of the Preiss-Handler pathway probably through the generation of excessive levels of NA.

The maintenance of high levels of NAm would also be predicted to promote a maintained elevation of circulating glucocorticoids. Thus, if any influence of glucocorticoids on NMTase synthesis occurred, it would be expected to be apparent here, and no obvious effect was observed.

Influence of Chronic Triamcinolone Therapy on the Excretion of NAm and NMNAm in NAm Challenged Hypophysectomized Rats

The extent of the differences in NMNAm excretion by hypophysectomized and normal rats following NAm challenge has been found to be widely variable. Greengard *et al.* (34) observed levels of 9.2% and 6.0%; Lee (Figure 8) 11.7% and 2.5%, and the current study (Table III) 6.5% and 2.5% in normal and hypophysectomized rats, respectively. The high level

Table X

HEPATIC NMTASE ACTIVITY AND NAD CONTENT FOLLOWING MULTIPLE NAM CHALLENGE

Times Challenged	Animal: Normal NMTase[a] nmoles/hr / mg protein	Normal NAD µmoles / gm liver	Hypophysectomized NMTase[a] nmoles/hr / mg protein	Hypophysectomized NAD µmoles / gm liver
0[b]	9.1 ±2.0	0.49 ±0.06	16.5 ±4.0	0.49 ±0.09
1[b]	10.1 ±2.0	1.90 ±0.49	17.2 ±1.7	2.24 ±0.39
2	14.3	1.78		
3	10.2	1.47		
4	10.8	1.22	14.3	1.78

a) Enzyme activity determined using Tris-HCl buffer, NAm-^{14}C assay.

b) 0 and 1 challenge data is taken from zero control and 12 hour values in time course data.

observed by Greengard in hypophysectomized rats compared to the low level observed here in normal rats was bothersome. To be absolutely certain that glucocorticoid therapy was effecting a change in the excretion patterns of hypophysectomized rats, another experiment was performed. The study was restricted to hypophysectomized animals.

The more effective chronic triamcinolone therapy was substituted for the previously used hydrocortisone therapy. The results of this experiment are reported in Table XI (with standard deviations).

If one considers the triamcinolone treated rats as being normal, the results are almost an exact match of those reported by Lee on both an absolute and relative basis. No normal rats were used as controls in this experiment. Thus, the conclusion that the results obtained from the triamcinolone treated animals are a reflection of what would have been recorded in a normal control group can only be inferred. The basis for such an inference is the fact that glucocorticoid therapy in the previous experiment (Table III) resulted in a complete reversal of the excretion patterns and that similar treatment of the normal animals resulted in no radical changes as compared to the normal control. In any event, the results in themselves were sufficient to convince one that the previously observed differences were real and that the extent of the differences was probably influenced by environmental factors.

The first attempt to perform the above experiment was frustrated by the inadvertent elimination of the challenge dose of NAm. By the time the oversight was realized, the animals had already been exposed to tracer levels of NAm-^{14}C for varying periods. Rather than administer the challenge under such circumstances, the experiment was allowed to run its course and analyzed in the usual manner. Each animal had

TABLE XI

THE EFFECT OF CHRONIC TRIAMCINOLONE THERAPY ON THE EXCRETION OF NAM-^{14}C AND NMNAM-^{14}C BY HYPOPHYSECTOMIZED RATS FOLLOWING A NAM-^{14}C CHALLENGE[a]

Animal/ Treatment	% of Injected ^{14}C Excreted			% of Total ^{14}C Excreted	
	Total	as NAm	as NMNAm	as NAm	as NMNAm
Hypophysectomized 500 mg NAm-^{14}C/Kg	44.3 ±8.6	18.5 ±3.7	3.6 ±1.3	42.5 ±8.3	8.0 ±1.7
Hypophysectomized 500 mg NAm-^{14}C/Kg Triamcinolone 60 mg/Kg/day for 5 days	75.1 ±8.1	41.8 ±4.5	12.7 ±1.3	56.6 ±4.6	16.9 ±1.6

a) Experimental details are given in the text.

received a dose of approximately 110 µgm NAm/Kg (5.4 µCi). All other treatment was identical to that reported in the above experiment.

The results of this experiment are presented in Table XII (with standard deviations). Though initially surprising, they are almost totally predictable on the basis of previous literature data, as has already been discussed (Excretion, Chapter II).

The excretion of NMNAm in the hypophysectomized control rats was within 20% of that observed in the triamcinolone treated rats. NAm represented only a small portion of the excreted dose and the difference between the two treatment groups was again small (about 23%).

It should be recalled that the NMNAm and NAm values were routinely obtained from chromatograms developed in two different solvent systems. Because of the nature of the above results, the chromatographic peaks corresponding to NMNAm and NAm in the alternate chromatograms were quantitated and essentially the same results were obtained.

A similar analysis of the chromatograms from the NAm challenge experiment yielded, as had been previously observed, markedly different answers in the NMNAm region of the two chromatographic systems. The only known metabolites which chromatograph with or near NMNAm in the alkaline butanol solvent system are NA and NAgly. Therefore, the difference in the dose recovered in the two chromatography systems is a rough measure of the presence of NA and its derivatives. Applying such a calculation to the data yielded a value of $15.2 \pm 6.7\%$ and $5.9 \pm 3.6\%$ of the injected dose excreted as NA or its derivatives in the control and triamcinolone treated groups respectively. The production of NA and related metabolites from NAm-^{14}C indicated the function of NAm deamidase. In this light, the above values can be taken as being

TABLE XII

THE EFFECT OF CHRONIC TRIAMCINOLONE THERAPY ON THE EXCRETION OF NAM-^{14}C AND NMNAM-^{14} BY HYPOPHYSECTOMIZED RATS FOLLOWING A TRACER DOSE OF NAM-^{14}C[a]

Animal/ Treatment	% of Injected ^{14}C Excreted			% of Total ^{14}C Excreted	
	Total	as NAm	as NMNAm	as NAm	as NMNAm
Hypophysectomized 0.11 mg NAm-^{14}C/Kg	33.4 ±2.5	0.5 ±0.2	18.4 ±2.8	1.4 ±0.4	54.7 ±5.1
Hypophysectomized 0.11 mg NAm-^{14}C/Kg Triamcinolone (60 mg/Kg/day for 5 days)	34.4 ±1.9	0.61 ±0.1	22.6 ±0.7	1.8 ±0.1	65.8 ±4.0

a) Experimental details are given in the text.

indicative of the absolute minimum NAm deamidase activity present in these animals. The relationship between the above two averages is, perhaps fortuitously, in agreement with the approximate three-fold increase in NAm deamidase activity previously reported to occur in hypophysectomized rats (111,161).

In addition to NMNAm and NAm, the urine chromatograms obtained from the tracer dose excretion study have other minor components. In the case of the hypophysectomized controls, two small peaks were observed on the strips. One of these, which chromatographed in the region that would be expected for NAmNO, yielded an average of 2% of the injected dose. The other unknown, which chromatographed in the region that would be predicted for the pyridones, gave an average of 5.1% of the injected dose. In the case of the triamcinolone treated rats, only one ancillary peak was detectable. This peak was chromatographically similar to the suspected pyridone peak observed in the control group chromatograms, and yielded an average value of 2.7% of the injected dose. Summation of all components accounted for yields 26.0% and 25.9% of the dose injected for the control and treated groups respectively.

During the course of these studies, it became apparent that the methodology used for the quantitation of isotopic materials on paper chromatograms was inadequate. It was found, for example, that counting of pure NMNAm-^{14}C on Whatman No. 1 chromatography paper in the toluene-ethanol cocktail yielded only 71% of the radioactivity actually present. It was also found that NAm-^{14}C was recovered essentially quantitatively. A study of the problem using representative urine samples indicated that with the exception of NAm-^{14}C the average radioisotope recovery from urine chromatograms counted in a toluene-ethanol cocktail was

approximately 68% of that present. It was also found that in fluid urine samples, the average recovery of the isotope activity other than NAm was approximately 79%. The values for the isotope recovery on the chromatograms and in the urine samples obtained from the tracer dose study were corrected on the basis of the above findings. The result is an estimated total excretion of approximately 42% and 44.1% of the injected dose in the control and treated groups respectively. The correction of the total recovery of the label from the chromatograms yields 40.6 and 40.5% of the injected dose respectively. That is, the estimated quantities of metabolites recovered in the 3 or 4 peaks detected on the urine chromatograms accounts for approximately 90% of the total dose excreted.

Therefore, in at least the physiologically dosed animals, any differences in NMNAm excretion can be accounted for by a redistribution of the label into primarily two other metabolites. The major change is apparently in a shift from NMNAm into pyridones and NAmNO. If this is indeed the case, the NMNAm plus pyridone metabolism should be taken as representative of the total NMTase function. Presuming that the major unknown is indeed pyridone, little difference exists between the treated and control groups in the above study.

CHAPTER V

CONCLUSION

There were differences in the distribution of ^{14}C among the metabolites excreted by hypophysectomized rats and those receiving chronic glucocorticoid therapy following the administration of a near physiological dose of NAm-^{14}C. However, at least a portion of this apparent change appeared to be in the further conversion of NMNAm to the 2 and/or 4-pyridones and thus, is not a reflection of a change in NMTase function. Regardless of the identity of the minor components excreted, both the treated and untreated animals excreted predominantly NMNAm. The data suggest that NMTase is not a tightly controlled enzyme and that it is probably of no great significance in exerting any control on NAm or NAD metabolism. The observation of only small differences in the basal excretion patterns of these animals indicates that the marked differences observed in the NAm challenged animals were basically pharmacological artifacts. Since the original conception of this study was related to the possibility that NMTase might play a significant role in the control of NAm metabolism, no further effort was expended and the area was abandoned.

At the outset, it was proposed that NAD was an allosteric modifier of NMTase. If one considers this concept more closely, the original data demand that NAD be an allosteric inhibitor of enzyme activity. Simultaneously, NAD must protect the enzyme against degradation *in vivo*.

Further, since the basal NAD content of both hypophysectomized and normal rat liver is identical, one is forced to argue that the rate of enzyme synthesis and/or degradation in normal rats is different from that in hypophysectomized rats.

It was the intent of the initial experiments to determine what influence, if any, NAD might have on NMTase activity. The results of those experiments indicated NAD was without influence on the in vitro enzyme activity. However, since these studies were conducted only under substrate saturating conditions, they reflect only changes in V_{max}. Thus, it can only be stated that NAD had no influence on the V_{max} value of the hepatic NMTase reaction. Subsequently, a study was initiated to determine the influence of NAD on NMTase activity at subsaturating levels of substrate. Using the fluorometric assay, it was determined that the apparent K_m's for NAm and SAM in the sodium acetate buffer system were about 2.5×10^{-4} M and 1.4×10^{-4} M, respectively. These values are in good agreement with those reported by Salvador and Burton (215) in Tris-HCl buffer at pH 8.0. As before, the addition of significant quantities of NAD to the assays made any accurate estimate of enzyme activity impossible. An apparent solution to this problem suggests itself. If the assays were terminated with heat or perchloric acid followed by neutralization, the resultant supernates could be incubated with N. crassa NAD glycohydrolase. This should eliminate the NAD and the competitive fluorescence contributed by it. By the time this most obvious potential solution to the problem presented itself, this study had been terminated and the idea was never tested. Such a study was not likely to prove fruitful. If one considers the time course of NMNAm excretion in normal and hypophysectomized rats, it is seen that a decrease in

excretion rate is unique to the latter. Thus, the concept of NAD having any significant effects on NMTase activity at subsaturating substrate concentrations becomes rather tenuous.

It is more feasible that small changes in other enzyme activities contribute to the redistribution of the label under challenge conditions. In addition, the possibility exists that NMNAm might, as a result of reduced renal function, accumulate in the circulation of the hypophysectomized rat to a greater extent than in normals. In this light, the interpretation of the early tests of renal function becomes somewhat questionable. The experiments would have been more convincing if they had been done on a time course basis with attention being given to the levels of circulating NMNAm with time. A similar study of circulating metabolite levels in the original excretion studies might also have been quite revealing.

The administration of glucocorticoids has been demonstrated to reverse the changes in NMNAm, NAm, and the total dose excretion observed in hypophysectomized rats following NAm challenge. Drawing a parallel with the findings of Greengard et al. (11), it has been predicted that the differences observed in metabolite excretion and in hepatic NAD levels in hypophysectomized rats following NAm challenge are due principally to a decrease in renal function in these animals.

An effort was made to reproduce the previously observed elevation of NMTase activity in NAm challenged rats; no support for such a marked increase in enzyme activity could be obtained.

During the course of this study, the 2-butanone fluorescence procedure was found to be much more reproducible than the alkali procedure. Thus, when the fluorescence analysis was subsequently used, SAM free

control assays were used. In the original study, no SAM free controls were used (23). Instead, the NAD present in the assays was corrected for by the use of an alkaline fluorescence procedure which yielded little or no fluorescence with NMNAm. It is possible, that under the influence of NAm challenge, some fluorescence producing compound other than NAD which contributed to the 2-butanone fluorescence analysis but not to the alkaline fluorescence analysis was being accumulated in the liver. If this were the case, the lack of SAM free controls in the analysis would fail to correct for this fluorescence and the result would be a time dependent overestimate of the NMTase activity. The most obvious candidate for making such a contribution is, all too obviously, NMNAm itself. It is conceivable that NMNAm accumulates in the livers of the NAm challenged animals. Such an accumulation would not influence an isotope incorporation assay and would go unaccounted using the original methodology (23). Unfortunately, this thought comes at the time of this writing and time does not permit a check of this possibility. That such an obvious possibility should escape one for so long a time is indeed humbling.

There are obviously many questions left unanswered by this study. However, the pursuit of the answers to these questions seemed fruitless. In so far as they could be expected to yield no insight into the understanding of NAm or NAD metabolism, their solution would be purely academic. Indeed, the time and resources required for their clarification would most probably be best invested elsewhere.

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