

The Pathway of Biosynthesis of Nicotinamide-Adenine Dinucleotide in Rat Mammary Gland

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1. The pathway of NAD synthesis in mammary gland was examined by measuring the activities of some of the key enzymes in each of the tryptophan, nicotinic acid and nicotinamide pathways. 2. In the tryptophan pathway, 3-hydroxyanthranilate oxidase and quinolinate transphosphoribosylase activities were investigated. Neither of these enzymes was found in mammary gland. 3. In the nicotinic acid pathway, nicotinate mononucleotide pyrophosphorylase, NAD synthetase, nicotinamide deamidase and NMN deamidase were investigated. Both NAD synthetase and nicotinate mononucleotide pyrophosphorylase were present but were very inactive. Nicotinamide deamidase, if present, had a very low activity and NMN deamidase was absent. 4. In the nicotinamide pathway both enzymes, NMN pyrophosphorylase and NMN adenylyltransferase, were present and showed very high activity. The activity of the pyrophosphorylase in mammary gland is by far the highest yet found in any tissue. 5. The apparent K_m values for the substrates of these enzymes in mammary gland were determined. 6. On the basis of these investigations it is proposed that the main, and probably only, pathway of synthesis of NAD in mammary tissue is from nicotinamide via NMN.

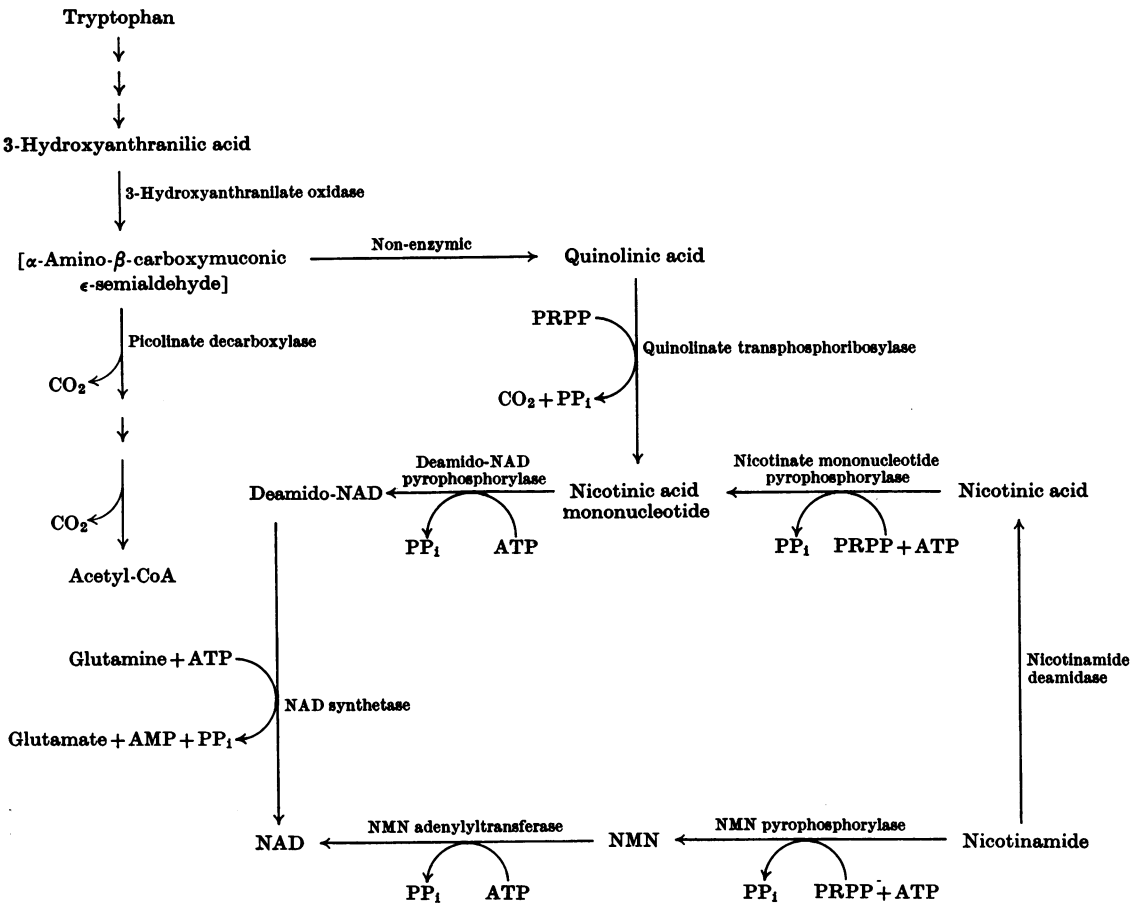
It is now widely accepted that the main pathway for the biosynthesis of NAD in erythrocytes, liver and certain micro-organisms is that described by Preiss & Handler (1957*a*, 1958*a,b*), i.e. via nicotinic acid mononucleotide and deamido-NAD (see Scheme 1). The enzymic conversion of nicotinic acid into nicotinic acid mononucleotide by nicotinate mononucleotide pyrophosphorylase has been demonstrated in liver and other tissues (Ikeda *et al.* 1965), and the formation of nicotinic acid mononucleotide by the tryptophan pathway (via 3-hydroxyanthranilic acid and quinolinic acid) has also been observed in liver (Nishizuka & Hayaishi, 1963*a,b*; Nakamura, Ikeda, Tsuji, Nishizuka & Hayaishi, 1963; Gholson, Ueda, Ogaswara & Henderson, 1964).

Two objections to the acceptance of the Preiss-Handler pathway, the early observation by Kaplan, Goldin, Humphreys & Stolzenback (1957) that nicotinamide is a better precursor of NAD than is nicotinic acid, and the fact that nicotinamide is the predominant form of the vitamin in the blood (Chaykin, Dagani, Johnson & Samli, 1965; Chaykin, Dagani, Johnson, Samli & Battaile, 1965), were largely resolved by Greengard and his associates by their finding of an active nicotinamide

deamidase in liver (Petrack, Greengard, Craston & Kalinsky, 1963; Greengard, Quinn & Reid, 1964; Petrack, Greengard, Craston & Sheppy, 1965). What remains, however, as a cogent objection is the distribution and activity of this deamidase. Such studies of the distribution of the deamidase as are available (e.g. Kirchner, Watson & Chaykin, 1966) appear to confirm that, though the deamidase is present in liver in amounts that could account for the observed rates of NAD turnover, this is not true of the other tissues tested and, indeed, Kirchner *et al.* (1966) raised the possibility that deamidation need not be an obligatory step in the biosynthesis of NAD from nicotinamide. However, Ijichi, Ichiyama & Hayaishi (1966) have put forward an alternative explanation for this lack of deamidase in some extrahepatic tissues, suggesting that nicotinamide is transferred to the intestine (where the deamidase is active) and that, after deamidation, nicotinic acid is released to the blood stream. Presumably this nicotinic acid could act as an NAD precursor in various tissues.

Nevertheless, evidence is beginning to accumulate that the extrahepatic tissues may not use the Preiss-Handler pathway for NAD synthesis. Grunicke *et al.* (1966) have produced evidence that a large part of the NAD synthesis from nicotinamide in Ehrlich ascites-tumour cells is through NMN and that this incorporation of nicotinamide

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Scheme 1. Pathways of NAD synthesis. PRPP, 5-phosphoribosyl 1-pyrophosphate.

into NAD occurs even when the nicotinamide concentration is as low as $5\ \mu\text{M}$, and Dietrich, Fuller, Yero & Martinez (1965, 1966) have found that a wide range of tissues (including rat kidney, brain, heart and muscle as well as ascites-tumour cells, an adenocarcinoma and chick-embryo tissue) have the ability to convert nicotinamide into NMN at a reasonable rate with K_m about $2\ \mu\text{M}$ for nicotinamide. It is noteworthy that both of these groups of investigators found a low K_m for nicotinamide, since an early objection to the nicotinamide pathway had been the high K_m ($0.1\ \text{M}$) observed by Preiss & Handler (1957*b*) and Dold, Mielsch & Holzer (1963). There is also evidence (Bonasera, Mangione & Bonavita, 1963) that the pathway of NAD synthesis in brain is different from that in liver. A summary of these three pathways is given in Scheme 1.

In the present study the pathway of NAD

synthesis in rat mammary gland was investigated. The results suggest that neither the tryptophan nor the nicotinic acid pathway is active in mammary gland, but that this tissue can synthesize NAD from nicotinamide, via NMN, at a high rate.

METHODS

Animals

Adult female albino rats in their first lactation period were used. They were given a stock diet (41B; Bruce & Parkes, 1949) *ad lib*. The number of pups in each litter was restricted to eight; rats with less than six pups were rejected.

Reagents

NAD⁺, NADH, NADP⁺, NADPH, NMN, ATP, ADP, AMP, quinolinic acid, nicotinic acid, phenazine methosulphate, rattlesnake (*Crotalus adamanteus*) venom and

Dowex 1 (Cl⁻ form) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Yeast alcohol dehydrogenase (EC 1.1.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and glucose 6-phosphate were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). 5-Phosphoribosyl 1-pyrophosphate was supplied by P-L Biochemicals Inc. (Milwaukee, Wis., U.S.A.), salmine sulphate (protamine) by British Drug Houses Ltd. (Poole, Dorset) and 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)-thiophen by Ciba Ltd. (Duxford, Cambs.).

[*carboxy*-¹⁴C]Nicotinic acid, [*carbonyl*-¹⁴C]nicotinamide and [6-¹⁴C]quinolinic acid were obtained from The Radiochemical Centre (Amersham, Bucks.). The radioactive samples were diluted in water to give a concentration of 10 μ C/ml. and stored at -15°. 5-Methylnicotinamide was a gift from Eli Lilly and Co. (Indianapolis, Ind., U.S.A.).

Where possible all other chemicals were of A.R. grade.

Preparation of substrates

Deamido-NAD. This was prepared from NAD by the transglycosidation reaction with nicotinic acid catalysed by ox spleen NAD nucleosidase (EC 3.2.2.5) (purified according to the procedure of Zatman, Kaplan & Colowick, 1953). The incubation was as described by Honjo, Ikeda, Andreoli, Nishizuka & Hayaishi (1964). Deamido-NAD was isolated from the reaction mixture by chromatography on a Dowex 1 (formate form) column and eluting with formic acid by the procedure of Preiss & Handler (1958a). The product was freed of formic acid by repeated freeze-drying.

Nicotinic acid mononucleotide. This was prepared from deamido-NAD by a modification of the procedure of Kaplan & Stolzenback (1957). Pyrophosphatase was prepared from *Crotalus adamanteus* venom essentially by the method of Butler (1955), except that the column was packed with Whatman CF2 cellulose powder. The pyrophosphorolysis of the deamido-NAD was followed on a titrator (type TTT1c; Radiometer, Copenhagen, Denmark). The product was isolated by chromatography on a Dowex 1 (formate form) column (25 cm. \times 1.5 cm.) and eluting with formic acid by the procedure of Preiss & Handler (1958a). The fractions containing nicotinic acid mononucleotide were repeatedly freeze-dried to remove formic acid.

Characterization of substrates

The compounds were identified unequivocally by the determination of their R_F values in three different solvent systems on paper chromatograms, the solvents B and C of Preiss & Handler (1958a) and in an isobutyric acid-aq. NH₃-water system (P-L Biochemicals Circular OR-18, p. 11), by spectral analysis in the presence and absence of CN⁻ (Preiss & Handler, 1958a; Pallini & Ricci, 1965) and by showing that the spots did not react with CNBr after treatment with *p*-aminobenzoic acid, or fluoresce with butan-2-one unless they had been pretreated with methanolic KCN (Kodicek & Reddi, 1951; Pallini & Ricci, 1965). Finally, deamido-NAD and nicotinic acid mononucleotide were hydrolysed in both acid and alkali and shown to give rise only to nicotinic acid, as tested for by the above methods, in contrast with NAD and NMN, which both gave rise to nicotinamide under identical conditions.

Preparation of tissue extracts

Mammary gland. The abdominal glands from both sides were dissected out and, after being chopped with scissors, homogenized in the cold in an Emmanuel-Chaikoff homogenizer (Microchemical Specialities Co., Berkeley, Calif., U.S.A.) with clearances of 64 μ and then 25 μ . The homogenizing medium was 2 vol. of 0.25 M-sucrose. The homogenate was strained through two layers of cheesecloth and was either used directly or fractionated by the procedure of Slater & Planterose (1960).

Liver. This was homogenized at 0° with 2 vol. of 0.25 M-sucrose in a Potter & Elvehjem (1936) homogenizer. The homogenate was either used directly or fractionated by the procedure of Schneider & Hogeboom (1950).

Enzymic procedures

Tryptophan pathway. (a) 3-Hydroxyanthranilate oxidase (3-hydroxyanthranilate-oxygen oxidoreductase, EC 1.13.1.6). This enzyme activity was measured by the method of Decker, Kang, Leach & Henderson (1961). Although the activities obtained when liver was used were comparable with those in the literature (Ikeda *et al.* 1965), no activity could be obtained under similar conditions from mammary-gland homogenates or from any of the fractions obtained from them. Variations in the concentrations of FeSO₄ or (NH₄)₂SO₄ had no effect on mammary-gland activity, nor did variations in the pH of the reaction mixture. The factors studied by Decker *et al.* (1961) were also varied, but these also failed to produce any detectable activity in preparations of mammary-gland tissue.

(b) Quinolinate transphosphoribosylase. This enzyme activity was measured essentially by the method of Nakamura *et al.* (1963), except that [6-¹⁴C]quinolinic acid was used in place of [U-¹⁴C]quinolinic acid, and the products were separated by paper chromatography in isobutyric acid-aq. NH₃ (sp.gr. 0.88)-water (660:17:330, by vol.), pH 3.8. The observations of Nakamura *et al.* (1963) and of Ikeda *et al.* (1965) that this enzyme is active in liver and kidney were confirmed, but we failed to find any activity in mammary gland, a finding consistent with the observation of Ikeda *et al.* (1965) that liver and kidney were the only tissues active among the ten they investigated.

Nicotinic acid pathway. (a) Nicotinamide deamidase. This enzyme activity was assayed by measuring the rate of conversion of [6-¹⁴C]nicotinamide into [6-¹⁴C]nicotinic acid by the method of Petrack *et al.* (1963). The products of the reaction were separated by paper chromatography with butan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (340:3:57, by vol.) and the chromatogram was then cut into 2 in. strips and scanned in a Packard Radiochromatogram scanner (model 7200). The identified spots were cut out, placed in vials containing 18 ml. of 4% (w/v) 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiopen in toluene and the radioactivities were determined in a Nuclear-Chicago scintillation counter (type 724). Petrack *et al.* (1963) have described the activation of the deamidase by the inclusion of bovine serum albumin in the incubation mixture. We have confirmed that this activation occurs with liver preparations, but we have failed to observe it with mammary-gland extracts despite the use of a variety of preparations of serum

albumin, including one generously supplied by Dr P. Greengard.

(b) Nicotinate mononucleotide pyrophosphorylase (nicotinate mononucleotide-pyrophosphate phosphoribosyltransferase, EC 2.4.2.11). In liver this enzyme activity was measured by the procedure of Ikeda *et al.* (1965), but the conditions described by them gave only a small activity when applied to mammary gland. For this latter tissue it was found that, as for liver, 100 μM -nicotinic acid and 5 mM-ATP were optimum, but the enzyme only attained maximum velocity when the concentration of 5-phosphoribosyl 1-pyrophosphate was increased to 0.5 mM and that of MgCl_2 to 2.5 mM. The optimum pH values were found to be slightly different (pH 7.2 for mammary gland and pH 7.4 for liver). The final assay systems used were: for liver, as described by Ikeda *et al.* (1965); for mammary gland, [6- ^{14}C]nicotinic acid, 0.1 mM (1 μC); 5-phosphoribosyl 1-pyrophosphate, 0.322 mM; ATP, 5 mM; MgCl_2 , 2.5 mM; potassium phosphate buffer, pH 7.2, 40 mM; tissue extract, 0.1 ml.; the final volume was 1 ml. The tissue extract was the supernatant fraction [prepared from a 1:3 (w/v) liver or mammary-gland homogenate] after being stirred with charcoal (1.5 g./10 ml.). The extract was added to 0.9 ml. of incubation mixture and incubated for 1 hr. at 37°. The reaction was stopped by adding 0.1 ml. of 2N-acetic acid and heating for 2 min. in a boiling-water bath. After the precipitate had been removed, the products of the reaction were isolated by paper chromatography on Whatman 3MM paper with the isobutyric acid-aq. NH_3 (sp.gr. 0.88)-water solvent system, and the radioactive spots were identified and counted as above. Under the conditions of this assay the rate of formation of nicotinic acid mononucleotide was linear with time up to 2 hr. and with extract from up to 40 mg. wet wt. of tissue. In general the supernatant fraction used for this assay was prepared from a 0.25 M-sucrose homogenate as such preparations were slightly, but uniformly, more active than supernatants prepared from homogenates in 0.15 M-KCl-0.16 M- KHCO_3 . In this assay mixture the 5-phosphoribosyl 1-pyrophosphate was used at less than the optimum concentration, because of the high cost of this material, but the concentration used gives not less than 90% of the maximal activity.

(c) Deamido-NAD pyrophosphorylase (nicotinate mononucleotide adenyltransferase, EC 2.7.7.18). The activity of this enzyme, reported by Preiss & Handler (1958b) to be identical with NMN adenyltransferase (EC 2.7.7.1), was measured by the same procedure as that adopted for this latter enzyme (see below) with NMN as substrate. Though some doubt has arisen about the identity of these two enzymes, the report of Dahmen, Webb & Preiss (1967) appears to confirm the original finding that they are, in fact, the same.

(d) NAD synthetase [deamido-NAD-L-glutamine amidoglycylase (AMP), EC 6.3.5.1]. NAD synthetase activity was measured by essentially the method of Preiss & Handler (1958b) except that the NAD formed was determined by the method of Greenbaum, Clark & McLean (1965a). The activity of mammary gland was very low and many variations in reaction conditions including substitution of NH_4Cl for glutamine as amino group donor (Preiss & Handler, 1958b; Imsande, 1961), failed to increase enzyme activity. It does not seem likely that the failure to detect this enzyme was due to the presence in mammary-gland

extracts of an inhibitor, since the addition of such extracts to liver preparations had no effect on the activity of the latter.

(e) NMN deamidase. Attempts were made to assay this enzyme by measuring the extinction of the cyanide complex of NMN at 325 m μ by the method of Sarma, Rajalakshmi & Sarma (1961).

Nicotinamide pathway. (a) NMN pyrophosphorylase (NMN-pyrophosphate phosphoribosyltransferase, EC 2.4.2.12). The method of Dietrich *et al.* (1966) was used for the determination of the activity of this enzyme in liver. The conditions described by these authors were, however, not optimum for mammary gland and were therefore modified. The final assay system was as follows: tris-HCl buffer, pH 7.6, 50 mM; 5-phosphoribosyl 1-pyrophosphate, 0.322 mM; nicotinamide, 0.15 mM; ATP, 5 mM; MgCl_2 , 1.5 mM; enzyme, 0.2-0.5 ml.; the total volume was 1.0 ml. The enzyme was obtained from the supernatant fraction of 1:10 (w/v) mammary-gland or liver homogenate in 0.25 M-sucrose; 0.7 ml. of a 1% (w/v) protamine sulphate solution was added/10 ml. of supernatant and the precipitate discarded. The assay mixture was incubated for 30 min. at 37° and the reaction stopped by heating in a boiling-water bath for 2 min. After cooling and centrifugation, 100 μl . of the supernatant was used for chromatographic separation on Whatman 3MM paper in isobutyric acid-aq. NH_3 (sp.gr. 0.88)-water, and the spots were identified and their radioactivities determined as described above. Under these conditions the enzyme activity was proportional to the amount of enzyme added, at least up to 50 mg. of tissue equivalent, and was constant for at least 40 min. Sucrose homogenates were superior to those obtained in the tris-HCl medium described by Dietrich *et al.* (1966). The pH-activity curve for this enzyme showed a very broad optimum between pH 7.5 and 8.3. The enzyme was stable overnight at -20°, but lost about 30% of its activity on storage at -20° for 7 days. As with all the tissues tested by Dietrich *et al.* (1966), the mammary-gland enzyme was restricted to the cytoplasmic fraction. Dietrich *et al.* (1966) showed that the supernatant fractions obtained from liver homogenates were activated by pretreatment with protamine sulphate. We have confirmed that a large increase in activity results from the treatment of liver supernatants in this way, but the effect on mammary gland is much smaller, though significant.

(b) NMN adenyltransferase (ATP-NMN adenyltransferase, EC 2.7.7.1). The method of Kornberg (1950) was used for the determination of this enzyme activity in liver and was modified slightly for use with mammary gland. For this latter tissue the final conditions were: glycylglycine-KOH buffer, pH 7.6, 50 mM; NMN, 3 mM; ATP, 25 mM; MgCl_2 , 4 mM; nicotinamide, 0.2 M; homogenate (in 0.25 M-sucrose), 0.2 ml.; the total volume was 0.28 ml. Incubation was for 15 min. at 37°, after which 0.5 ml. of 0.15 N-HCl was added and the mixture was heated at 100° for 2 min. and then cooled in ice. After the addition of 0.2 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, the mixture was neutralized and then centrifuged in the cold. The NAD formed was determined by the method of Greenbaum *et al.* (1965a). The concentration of NMN used in this assay does not give maximum activity, for which 4 mM is required. However, the lower concentration used (for reasons of cost) gives 90% of the maximum rate.

Results with this lower concentration of NMN have therefore been corrected to maximum activity. The assay system is linear with time up to 40 min. and is proportional to enzyme concentration up to 70 mg. of tissue. Under the conditions described here less than 1% of the NAD⁺ formed was reduced to NADH during the incubation and no NADP⁺ or NADPH could be detected. It was also established that the amount of nicotinamide present was sufficient to prevent the loss of any of the NAD formed.

The adenylyltransferase preparations used in these assays were whole homogenates in 0.25 M-sucrose. Hogeboom & Schneider (1952) have shown that, in liver, NMN adenylyltransferase is located in the nuclei, and Branster & Morton (1956) have found that this is also true of mammary gland. We have confirmed this latter observation and shown that the activity of the whole homogenate is the same as that of the purified nuclear fraction obtained from it.

RESULTS AND DISCUSSION

Tryptophan pathway

The pathway from tryptophan to nicotinic acid has been closely studied in a number of Laboratories, notably those of Hayaishi and Gholson. Though most of the steps in this pathway have now been elucidated, only two of them were studied in the present work. The first of these, 3-hydroxyanthranilate oxidase, was measured because it is normally by far the most active enzyme of the pathway and its presence in mammary gland should have been the most easy to demonstrate. The second enzyme, quinolinate transphosphoribosylase, is the key enzyme for entry from the tryptophan pathway to the nicotinic acid pathway.

3-Hydroxyanthranilate oxidase. The activity of this enzyme was measured in liver and values in good agreement with those in the literature (Ikeda *et al.* 1965; Shimoyama, Kori, Usuki, Lan & Gholson, 1965) were found. No activity, however, could be detected in mammary gland. Decker *et al.* (1961) have studied the factors effective in the conversion of this enzyme into, or its maintenance in, its most active form. In particular, they drew attention to the role of acid and heat, ammonium sulphate and Fe²⁺. The effects of each of these factors, either alone or in combination, were investigated and none of the variations tried produced measurable activity. Though it is possible that, in this investigation, ideal conditions for the assay of this enzyme were never achieved, it seems unlikely that conditions giving a reasonable fraction of the potential activity were not obtained, and it is probable that the failure to find any activity of the oxidase in mammary gland probably reflects a genuine absence of the enzyme.

It seems valid to conclude either that 3-hydroxyanthranilate oxidase has negligible activity in mammary gland or, less likely, that it occurs but the product, β -amino- β -carboxymuconic ϵ -semi-

aldehyde, never enters the pathway to NAD owing to rapid decarboxylation by a very active picolinate carboxylase.

Quinolinate transphosphoribosylase. In this case also the activities found in liver were found to correspond well with those in the literature (Ikeda *et al.* 1965), but no activity was found in mammary gland. It does not seem that an inhibitor exists in mammary gland, since adding mammary-gland extract to liver extract had no effect on the activity of the liver preparation. Appreciable activity of this enzyme has only been found in liver and kidney (Ikeda *et al.* 1965), and it is totally absent from hepatomas (Shimoyama *et al.* 1965).

Nicotinic acid pathway

Two of the three enzymes of this pathway were measured, namely nicotinate mononucleotide pyrophosphorylase, the first enzyme of the sequence, and NAD synthetase, the last enzyme. The remaining enzyme, deamido-NAD pyrophosphorylase, is about six times as active as NAD synthetase in liver and was not measured directly in this work. It is, however, identical with the NMN adenylyltransferase that occurs in the nicotinamide pathway (Preiss & Handler, 1958*b*; Dahmen *et al.* 1967), which was measured separately in that context, as described below. The values quoted for the activity of this enzyme in the nicotinamide pathway may not, however, be used directly in assessing the activity of the deamido-NAD pyrophosphorylase, as the enzyme usually shows a greater activity with nicotinic acid mononucleotide as substrate than it does with NMN (Preiss & Handler, 1958*b*); Dahmen, *et al.* 1967).

Assays of the two enzymes studied gave values for liver in good agreement with those previously reported (Shimoyama *et al.* 1965; Ikeda *et al.* 1965; Preiss & Handler, 1958*b*). In mammary gland the nicotinate mononucleotide pyrophosphorylase activity was 40 μ moles/g. of tissue/hr. at 37° compared with about 300 μ moles/g./hr. in liver. The mammary-gland activity is comparable with the values described by Ikeda *et al.* (1965) for a variety of tissues, including brain, muscle, lung, testis and intestine. The activity of the synthetase in mammary gland was not detected by the method employed, and cannot have exceeded 8 μ moles of NAD/g./hr. at 37°, the minimum sensitivity of the method.

A second point of entry into the nicotinic acid pathway is from nicotinamide via the activity of nicotinamide deamidase, an enzyme believed by Petrack *et al.* (1963) to be the controlling enzyme of the sequence in liver. Though the results obtained above on the other enzymes of the pathway suggest the conclusion that this route is

probably of minor significance in the synthesis of NAD in mammary gland, the deamidase could be of significance here as a high activity of this enzyme would decrease the availability of nicotinamide to the remaining system for NAD synthesis. For these reasons, attempts were made to measure the activity of nicotinamide deamidase in liver and mammary gland.

The values obtained with a liver microsomal fraction (80 and 510 $\mu\text{moles/g. wet wt./hr.}$ for inhibited and serum-albumin-treated preparations respectively) compared favourably with those reported by Petrack *et al.* (1965). The activity in mammary gland, however, was negligible, and certainly did not exceed 25 $\mu\text{moles/g./hr.}$

Another possible point of entry into this pathway would be via the deamidation of NMN. Attempts to demonstrate the deamidation of NMN in mammary gland by the method of Sarma *et al.* (1961) were unsuccessful. Degradation of NMN did occur, but the product was found to be free nicotinamide and not nicotinic acid mononucleotide as reported by Sarma *et al.* (1961). Petrack *et al.* (1965) also failed to confirm the occurrence of this type of deamidase in crude liver preparations.

Nicotinamide pathway

Only two enzymes are involved in this pathway and both were measured.

NMN-pyrophosphorylase. Activities of 140–150 $\mu\text{moles/g./hr.}$ were obtained for protamine sulphate-treated rat liver supernatant, in good agreement with Dietrich *et al.* (1966). The enzyme activity measured in mammary gland under the conditions described above was approx. 1 $\mu\text{mole/g./hr.}$ The apparent K_m for nicotinamide was calculated by the method of Lineweaver & Burk (1934) to be 35 μM . The enzyme in mammary-gland extracts appears to exist largely in an uninhibited form since the addition of protamine sulphate increased the activity by only about 10%, whereas the activity in liver extracts increased by 81% (see Dietrich *et al.* 1966).

The value of 1 $\mu\text{mole/g./hr.}$ quoted above is, of course, a minimal value since the mammary-gland tissue contained about 50% of retained milk. A corrected activity would therefore be about 2 $\mu\text{moles/g./hr.}$, so that mammary gland is by far the most active tissue so far studied [the values quoted by Dietrich *et al.* (1966) range from 18 $\mu\text{moles/g./hr.}$ for lung to 165 $\mu\text{moles/g./hr.}$ for liver].

Two considerations make it unlikely that the incorporation of nicotinamide into NMN was indirect, proceeding via an exchange reaction between free labelled nicotinamide with NAD, catalysed by NAD glycohydrolase, and the subsequent breakdown of the labelled NAD to labelled NMN, catalysed by NMN adenylyltransferase.

Table 1. *Activities of enzymes involved in the synthesis of NAD, and the K_m values for their substrates*

Pathway	Enzyme	Liver		Mammary gland	
		Activity	K_m^* (M)	Activity†	K_m^* (M)
Tryptophan to nicotinic acid mononucleotide	3-Hydroxyanthranilate oxidase	(a) 450	(b) 2.1×10^{-5}	‡	§
	Picolinate carboxylase	(a) 1.570	§	‡	§
	Quinolinate transphosphoribosylase	(a) 0.083	(c) 6×10^{-5}	‡	§
Nicotinic acid to NAD	Nicotinate mononucleotide pyrophosphorylase	(d) 0.336	(e) 1.85×10^{-6}	(k) 0.040	(k) 1.2×10^{-5}
	NAD synthetase	(f) 1.14	(f) 1.4×10^{-4}	(k) < 0.008	§
Nicotinamide to NAD	NMN pyrophosphorylase	(g) 0.163	(g) 2.96×10^{-6}	(k) 0.980	(k) 3.5×10^{-5}
	NMN adenylyltransferase	(h) 4.5	(i) 1.2×10^{-4}	(k) 1.63	(k) 1.4×10^{-4}
Nicotinamide to nicotinic acid	Nicotinamide deamidase	(j) 0.624	(j) 0.177	(k) < 0.025	§

* K_m refers to the substrate involved as an intermediate in the pathway.

† Values not corrected for presence of retained milk.

‡ Activity too low to be measurable.

§ Values not available.

First, the glycohydrolase is a particulate enzyme (Jacobson & Kaplan, 1957) and presumably had been removed in the preparation of the supernatants used in this experiment, as had been NMN adenylyltransferase, which is located in the nucleus. Secondly, duplicate incubations carried out in the presence of concentrations of 5-methylnicotinamide that inhibit liver glycohydrolase by 99% (Grunicke *et al.* 1966) showed only 50% inhibition, which would be expected from the properties of the pyrophosphorylase reported by these workers.

NMN adenylyltransferase. Values obtained for the activity of this enzyme in liver were in good agreement with those reported by Greenbaum, Clark & McLean (1965b). The mammary-gland enzyme required slightly different conditions from those reported for liver, as described above. The pH-activity curve gives a very sharp peak at pH 7.6. The enzyme is remarkably stable and even frozen whole homogenates retain their full activity over a period of weeks. The measured K_m values for NMN for liver and mammary gland were identical, 0.15mM, although mammary gland has a higher K_m for ATP, 6mM, than has liver, 0.46mM. The activity of NMN adenylyltransferase is very high in mammary gland, about 3 μ moles/g./hr. (corrected for retained milk) compared with about 4.5 μ moles/g./hr. for liver (Greenbaum *et al.* 1965b).

Table I summarizes the above data on enzyme activities together with the K_m values for the substrates. From a consideration of Table I it is apparent that liver could synthesize NAD from any, or all, of the three pathways studied at rates that are not very different. It is also clear, however, that mammary gland cannot use the tryptophan pathway and, almost certainly, cannot synthesize NAD from nicotinic acid. On the other hand, mammary gland has a high potential activity of the enzymes of the nicotinamide pathway.

Thus, in terms of available enzyme activity, the preferred pathway in rat mammary gland would appear to be that via NMN, a view supported by the K_m values for the substrates, which are low for both nicotinamide and NMN. Further support for the suggestion that mammary gland makes its NAD via the nicotinamide route comes from the experiments *in vivo* of Pinder & Greenbaum (1967). In these it was shown that, whereas there was a time-lag in the incorporation of nicotinamide into liver NAD, no such lag existed in its incorporation into mammary-gland NAD. On the other hand, liver incorporated nicotinic acid into NAD freely and without time-lag, whereas mammary gland did not incorporate this precursor at all.

On the basis of these experiments, it is proposed that the pathway of NAD synthesis in rat mammary gland is via nicotinamide and NMN and that the tryptophan and nicotinic acid pathways are either

absent or of minor importance. This tissue therefore appears to be unique among those studied so far in that it lacks NAD synthetase and has only one route for NAD synthesis.

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