Biochem. J. (1968) **107**, 63
Printed in Great Britain

Changes in the Activities of Enzymes of the Biosynthetic Pathway of the Nicotinamide Nucleotides in Rat Mammary Gland during the Lactation Cycle

By A. L. GREENBAUM and S. PINDER*
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

(*Received* 23 *August* 1967)

1. The activities of NMN pyrophosphorylase, NMN adenylyltransferase and NAD kinase in the mammary glands of rats at different stages of pregnancy, lactation and involution were measured. 2. NMN pyrophosphorylase has a low activity early in pregnancy, but its activity increases at parturition and in early lactation to reach a maximum at the tenth day of lactation, after which it remains constant until it declines abruptly in involution. 3. NMN adenylyltransferase is already quite active by the tenth day of pregnancy and its activity does not rise further in the second half of gestation. After a sharp rise in activity at parturition, the activity of the enzyme declines slowly throughout the period of lactation and, more sharply, in involution. 4. NAD kinase has a low activity for most of pregnancy, but its activity rises at parturition to a value at 2 days of lactation that is maintained until the tenth day. Between the tenth and fifteenth days of lactation the activity almost doubles, but falls sharply in mammary involution. 5. The relation of the activities of these enzymes to the rates of synthesis of NAD and NADP is discussed.

The very considerable changes in physiological activity and metabolic characteristics of the mammary gland during the lactation cycle have made this tissue an admirable experimental material for the study of the interaction of biochemical change and physiological function. There are now extensive studies of many aspects of carbohydrate, fat and protein metabolism (see Munford, 1964) in relation to the rate of production of milk.

One of the biochemical aspects that does not appear to have received adequate attention is the biosynthesis of the nicotinamide nucleotides in this tissue. This question assumes a position of considerable importance in mammary tissue, not only because of the important role that these nucleotides play in the metabolism of the gland in general, but also because of their special function, particularly that of NADP, in a tissue geared to the production of large quantities of fat (Glock & McLean, 1953; Abraham, Cady & Chaikoff, 1957; McLean, 1958, 1960). Changes in the tissue content of NAD and NADP in the mammary glands taken from rats during pregnancy, lactation and involution have been studied by McLean (1958), who found that NAD and NADP contents were both low in pregnancy, increased abruptly at parturition, rose steadily through lactation up to the thirteenth day, remained high until the end of lactation and then plunged to low values again in early involution, a pattern of change that approximates very closely to the shape of the milk-yield curve of rats.

It was therefore decided to investigate the changes in activity of the biosynthetic pathways for the two nucleotides in relation to the change in content of these nucleotides and the rate of milk formation.

The time-sequence and magnitude of changes in the pathways are important in this context in that the timing of the changes in amount of the nucleotides (sevenfold for NAD, 12-fold for NADP) may regulate the potential activity of the enzymes present. In this paper, changes in the activity of the enzymes concerned with the synthesis of the nicotinamide nucleotides are considered.

METHODS

Animals. Albino rats, undergoing their first pregnancy or lactation, were used. They were given a stock diet (41B; Bruce & Parkes, 1949) *ad lib.* In this study the number of pups was restricted to six to eight and involution was produced by removing the pups on the nineteenth day of lactation. Rats were killed by cervical dislocation at the various stages of pregnancy, lactation and mammary involution as described below.

* Present address: Department of Pharmacology, Medical College of St Bartholomew's Hospital, London, E.C. 1.

Reagents and tissue extracts. All materials and mammary-gland extracts were as described in the preceding paper (Greenbaum & Pinder, 1968).

Enzymic procedures. NMN pyrophosphorylase (NMN–pyrophosphate phosphoribosyltransferase, EC 2.4.2.12) and NMN adenylyltransferase (ATP–NMN adenylyltransferase, EC 2.7.7.1) activities were determined by the procedures described in the preceding paper (Greenbaum & Pinder, 1968). For the determination of the pyrophosphorylase activity the supernatant fraction (diluted to 10 times the original tissue volume) was used; the whole homogenate was assayed for the transferase activity.

NAD kinase (EC 2.7.1.23) activity was determined by a procedure based on the method of Wang & Kaplan (1954) as modified by Greenbaum, Clark & McLean (1965*b*). To obtain maximal activity with mammary-gland preparations, the concentrations of ATP, $MgCl_2$ and NAD were changed, all other conditions remaining the same. As in the experiments of Greenbaum *et al.* (1965*b*), it was found necessary to add glucose 6-phosphate to ensure that all the NADP was in the reduced form, since in the absence of this addition 85–90% of the NADP formed was in the reduced form and the remaining 10–15% in the oxidized, in contrast with liver where the corresponding proportions were 25% and 75%. The final incubation mixture contained: NAD, 4·6mM; ATP, 5·2mM; nicotinamide, 10mM; $MgCl_2$, 13mM; triethanolamine, pH7·6, 0·1M; glucose 6-phosphate, 2·5mM; enzyme, 0·2ml. of a 1:5 (w/v) mammary-gland supernatant; 0·05ml. of 0·9M-NaF was added immediately after the enzyme. The final volume was 1·0ml. All reactants were adjusted to pH7·6 before addition. After the addition of the NaF the mixture was incubated for 15min. at 37° and the reaction stopped by the addition of 0·1ml. of N-NaOH. The mixture was heated for 2min. at 100°, cooled in ice and 0·1ml. of 0·25M-glycylglycine–KOH buffer, pH7·6, added. The whole was then neutralized with 0·1ml. of N-HCl. The tubes were then centrifuged in a bench centrifuge in the cold and the supernatant from this was taken for the determination of the NADPH formed by the method of Greenbaum, Clark & McLean (1965*a*). Under these conditions, and with fresh extracts, the assay was linear with time for at least 45min. and proportional to the enzyme concentration at least up to 80mg. of tissue. It was essential that this assay was performed with fresh extracts since, not only did the extracts lose activity on storage at −15° (30% overnight), but the assay became non-linear with time when such stored extracts were used. Wang & Kaplan (1954) reported that the conversion of NAD^+ into $NADP^+$ is inhibited by NADH and it was important to establish that such an inhibition was not interfering with the assay. Determination of the NADH present at the end of an incubation revealed that the amount of NADH present did not exceed 20μg., i.e. a 0·67% conversion of NAD^+ into NADH. It can be calculated from the data of Wang & Kaplan (1954) that the NAD^+/NADH ratio, 150:1, that exists during the incubation of mammary-gland extracts under the above conditions would give an inhibition of less than 1%.

RESULTS

The results of the determinations of the maximum potential activities of the enzymes involved in NAD synthesis are shown in Fig. 1 and those for NAD kinase in Fig. 2.

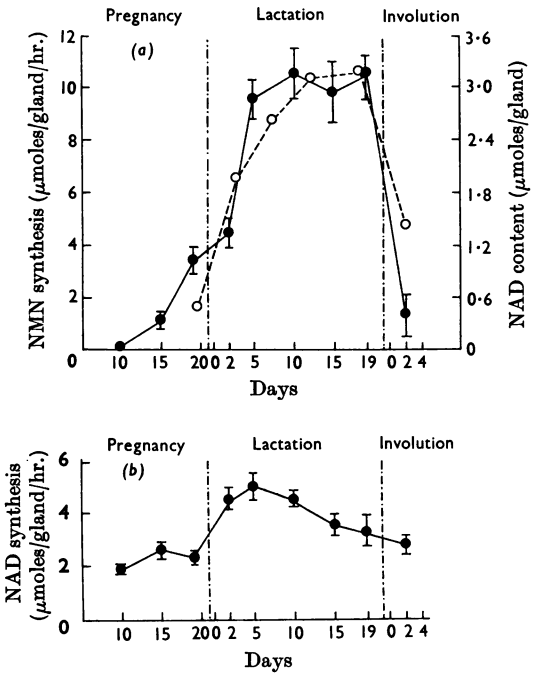

Fig. 1. Changes in the activities of NMN pyrophosphorylase (*a*) and NMN adenylyltransferase (*b*) in the mammary glands of rats during the lactation cycle. The activities were measured as described in the Methods section. Each point is the mean of six rats and the vertical bars represent twice the S.E.M. The broken line in (*a*) represents the changes in the tissue content of NAD calculated from the values of McLean (1958).

NMN pyrophosphorylase. The activity of this enzyme is extremely low on the tenth day of pregnancy, but increases fairly rapidly through the latter half of pregnancy and then sharply at parturition. It attains its maximum activity by the fifth day of lactation and then remains at this high value throughout the remaining period of lactation. The enzyme activity declines precipitously in the first 2 days of mammary involution (Fig. 1*a*).

NMN adenylyltransferase. The changes in the activity of this enzyme (Fig. 1*b*) follow a most unusual course for a mammary-gland enzyme. It is already at a high level of activity by mid-pregnancy and only rises slightly during the remainder of gestation. The rise that occurs at parturition is only by the factor 2–2·5, rather small by comparison with many other enzymes, e.g. cytochrome oxidase (Smith & Richter, 1958), succinate dehydrogenase (Greenbaum & Slater, 1957) and hexokinase, phosphoglucose isomerase and glucose 6-phosphate

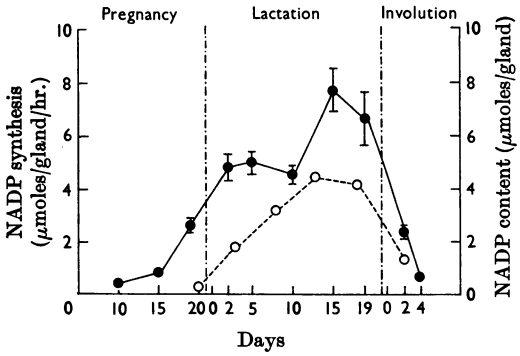

Fig. 2. Changes in the activity of NAD kinase in the mammary glands of rats during the lactation cycle. The activity was measured as described in the Methods section. Each point is the mean of six rats and the vertical bars represent twice the S.E.M. The broken line represents the changes in the tissue content of NADP calculated from the values of McLean (1958).

dehydrogenase (McLean, 1958). It is also unusual in that it decreases in activity over practically the whole of lactation, i.e. over the period of increasing milk formation. The maximum is achieved as early as the fifth day of lactation. The decline in activity during mammary involution is, again, unusually slow.

NAD kinase. The presence of this enzyme in mammary gland has been reported by Abraham, Matthes & Chaikoff (1961) and Matthes, Abraham & Chaikoff (1963), but changes in the activity of the enzyme with physiological function have not previously been described. The activity of the enzyme on the tenth day of pregnancy is very low, but it rises fairly rapidly in the second half of this period (Fig. 2). It increases twofold at parturition and remains constant at this higher value for some 10 days. There is a sharp increase in activity in the second half of lactation and a very sharp fall during early involution. This pattern of a slow rise in activity in early lactation followed by a sharp rise in later lactation is not uncommon among mammary-gland enzymes, and a number of enzymes have been shown to follow it, e.g. glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Glock & McLean, 1953), arginase (Folley & Greenbaum, 1947) and glutamate dehydrogenase (Greenbaum & Greenwood, 1954).

It is of interest to compare the maximum activities of the three enzymes in liver and mammary gland. NAD kinase performs the same function in both tissues and the values are of a comparable order (liver 3000 and mammary gland 1800 mμmoles/g./hr.). NMN adenylyltransferase also occurs in both tissues, but probably performs different functions. In mammary gland it adenylates NMN to form NAD, whereas in liver it most probably adenylates nicotinic acid mononucleotide to form deamido-NAD. The activity of the enzyme in liver is 5000 and in mammary gland 1500 mμmoles/g./hr. [The value for liver quoted here is probably too low in the sense that the activity was measured with NMN as substrate. Preiss & Handler (1958) have reported that the activity is almost doubled if the liver enzyme is used with nicotinic acid mononucleotide as substrate and have quoted a value of 7900 mμmoles/g./hr.] The third enzyme, NMN pyrophosphorylase, is on the main pathway for NAD synthesis in mammary gland (Greenbaum & Pinder, 1968) and has an activity of 2600 mμmoles/g./hr., but it is probably not on the main pathway in liver and in this latter tissue has an activity of only 160 mμmoles/g./hr. (Dietrich, Fuller, Yero & Martinez, 1966). In quoting values for mammary gland above, the values have been corrected for the presence of retained milk (Slater, 1957).

DISCUSSION

Two aspects of the observed changes in the activities of the enzymes of nicotinamide nucleotide synthesis have to be considered. They are, first, the question whether this synthesis is rate-limited by any of the enzymes measured and, second, the question whether the sequence of changes is consistent with the changed contents and rates of synthesis in the gland.

In the first case an estimate of the rate of synthesis of the nicotinamide nucleotides can be obtained from the total amount present in the gland and the turnover rate of the nucleotide. Considering NAD first, the content of the gland is available from the work of McLean (1958) and an approximation to the turnover may be deduced from the data of Narrod, Langan, Kaplan & Goldin (1959) and Bonasera, Mangione & Bonavita (1963) on mouse liver. From these sources it would appear that the turnover time of hepatic NAD is about 2hr. Assuming a similar rate for mammary gland, the following calculation can be made. The tissue content of NAD rises from 0·68 to 2·56 μmoles/gland over a period of 15 days, i.e. at a rate of 0·125 μmole/gland/day. If the turnover rate is 2hr. then the gland must synthesize 8·5 μmoles/day on the second day of lactation and 32 μmoles/day on the fifteenth day to maintain the observed concentrations. From Fig. 1 it can be calculated that the NMN pyrophosphorylase can synthesize 72 μmoles/gland/day at the nineteenth day of pregnancy rising to 224 μmoles/gland/day at the fifteenth day of lactation. The enzyme is therefore present in an eight- to ten-fold excess. The NMN adenylyltransferase activity would permit the

synthesis of 65 and 103 μmoles/gland/day at the nineteenth day of pregnancy and the fifteenth day of lactation respectively, i.e. an excess falling from eight- to three-fold. Thus, though the enzyme appears to be present in amounts greater than those required, the excess is very modest and may be marginal when it is considered that the rates measured are in the presence of excess of substrate.

For NADP it can be calculated, in the same manner as above for NAD, from the data of McLean (1958) on tissue content and from those of Slater & Sawyer (1966) on turnover time (with similar assumptions to those already employed) that the rates of synthesis of NADP in mammary gland are 0·16 μmole/gland/day at the nineteenth day of pregnancy rising to 2·76 μmoles/gland/day at the fifteenth day of lactation. The results in Fig. 2 permit the calculation of the maximum potential biosynthetic activity of NAD kinase as 70 μmoles/gland/day in pregnancy and 156 μmoles/gland/day in lactation, both values in great excess over the rates of synthesis necessary to attain the NADP contents found. Thus the conclusion emerges that neither NAD nor NADP synthesis is enzyme-limited at any stage of the lactation cycle. It therefore becomes a reasonable hypothesis to presume that the rate of synthesis is substrate-limited. Until methods become available for the determination of all the substrates and intermediates this point cannot be established for NAD synthesis, but a first approximation can be made for NADP synthesis, i.e. NAD kinase activity.

Assuming that the intracellular distribution of NAD in mammary gland is the same as that found by Glock & McLean (1956) in liver, i.e. about 90% in the cytoplasm, it can be calculated from the data of McLean (1958) that the cytoplasmic NAD concentration is 0·12mM on the nineteenth day of pregnancy and 0·83mM on the thirteenth day of lactation. The total cell ATP concentration is approx. 3mM at both stages (R. Sobers & A. L. Greenbaum, unpublished work). Assuming again that the distribution is the same as that in liver, i.e. about 40% in the cytoplasm (see Slater & Sawyer, 1966), a value of 1·2mM in the cytoplasm would seem appropriate.

From the measured rates and the respective K_m values (1·5mM for NAD and 2mM for ATP) it can be calculated that the expected activity of the enzyme at the substrate concentrations occurring in the tissue would be 0·29 and 14·0 μmoles of NADP formed/gland/day for the nineteenth day of pregnancy and the fifteenth day of lactation respectively. These values would give turnover times for NADP of 2½hr. at late pregnancy and about 1hr. in late lactation compared with a turnover time of 5hr. found by Slater & Sawyer (1966) for liver NADP.

Turning to the second point, the time-correspondence of the changes in the concentrations of the nucleotides and the activities of the enzymes that make them, it may be noted that all three enzymes are active before the increase in nicotinamide nucleotides occurs and that two of them, NMN pyrophosphorylase and NAD kinase, follow time-courses that generally reflect the increasing NAD and NADP contents. Indeed, the changes of NMN pyrophosphorylase activity present a close parallel with the changes in NAD content. NAD kinase activity exhibits a two-stage rise, the first, at parturition, to give a value that is maintained through the first half of lactation, followed by a secondary rise, almost doubling the activity, in the latter half of lactation. Although this pattern does in some degree reflect the increase in the amount of NADP present in the gland, it does not follow it closely. It may be that the secondary rise in the activity of the enzyme reflects an increased turnover of NADP consequent on the rises in activity of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase that occur over the same time-period as the secondary rise of NAD kinase (Glock & McLean, 1953, 1954).

The behaviour of NMN adenylyltransferase is difficult to explain. The progress curve showing the change of activity of this enzyme with advancing lactation bears little relation to the NAD content of the gland, though at all stages it seems to have a sufficiently high potential activity to account for the observed rate of NAD synthesis. It could be that this enzyme is more related to the rate of cell division than to the absolute amount of NAD. Morton (1958) has proposed that the concentration of NAD in the cell is the regulator of the rate of cell division, and a correlation between the NMN adenylyltransferase activity and mitosis has been reported by Branster & Morton (1956), Dawkins (1959) and Morton (1961). In the present context, there is a correlation in time between the activity of the transferase and the occurrence of cell division in the mammary gland. The very slow rise in enzyme activity between the tenth and nineteenth days of pregnancy corresponds to a period when mammary-gland cell division is virtually absent (Weatherford, 1929). The increased enzyme activity between the nineteenth day of pregnancy and the fifth day of lactation is synchronous with a burst of cell division in the gland that leads to an approximate doubling of the tissue DNA (see Munford, 1964) and the appearance of many new small alveolar cells (Weatherford, 1929), and the decrease of the activity of the enzyme during the remainder of lactation may be related to the fact that mitosis is comparatively rare during this period. The correlation between the NMN adenylyltransferase activity and the occurrence of cell

division in mammary gland may well be circumstantial, but it does fit the observed changes in the activity of the enzyme rather better than a direct correlation with the NAD content.

REFERENCES

Abraham, S., Cady, P. & Chaikoff, I. L. (1957). *J. biol. Chem.* **224**, 955.

Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1961). *Biochim. biophys. Acta*, **49**, 268.

Bonasera, N., Mangione, G. & Bonavita, V. (1963). *Biochem. Pharmacol.* **12**, 633.

Branster, M. J. & Morton, R. K. (1956). *Biochem. J.* **63**, 640.

Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 202.

Dawkins, M. J. R. (1959). *Proc. Roy. Soc.* B, **150**, 284.

Dietrich, L. S., Fuller, L., Yero, I. L. & Martinez, L. (1966). *J. biol. Chem.* **241**, 188.

Folley, S. J. & Greenbaum, A. L. (1947). *Biochem. J.* **41**, 261.

Glock, G. E. & McLean, P. (1953). *Biochim. biophys. Acta*, **12**, 590.

Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**. 171.

Glock, G. E. & McLean, P. (1956). *Exp. Cell Res.* **11**, 234.

Greenbaum, A. L., Clark, J. B. & McLean, P. (1965*a*). *Biochem. J.* **95**, 161.

Greenbaum, A. L., Clark, J. B. & McLean, P. (1965*b*). *Biochem. J.* **96**, 507.

Greenbaum, A. L. & Greenwood, F. C. (1954). *Biochem. J.* **56**, 625.

Greenbaum, A. L. & Pinder, S. (1968). *Biochem. J.* **107**, 55.

Greenbaum, A. L. & Slater, T. F. (1957). *Biochem. J.* **66**, 155.

McLean, P. (1958). *Biochim. biophys. Acta*, **30**, 316.

McLean, P. (1960). *Biochim. biophys. Acta*, **37**, 296.

Matthes, K. J., Abraham, S. & Chaikoff, I. L. (1963). *Biochim. biophys. Acta*, **71**, 568.

Morton, R. K. (1958). *Nature, Lond.*, **181**, 540.

Morton, R. K. (1961). *Aust. J. Sci.* **24**, 260.

Munford, R. E. (1964). *Dairy Sci. Abstr.* **26**, 293.

Narrod, S. A., Langan, T. A., Kaplan, N. O. & Goldin, A. (1959). *Nature, Lond.*, **183**, 1674.

Preiss, J. & Handler, P. (1958). *J. biol. Chem.* **233**, 493.

Slater, T. F. (1957). *Analyst*, **82**, 818.

Slater, T. F. & Sawyer, B. C. (1966). *Biochem. Pharmacol.* **15**, 1267.

Smith, J. C. & Richter, B. (1958). *Arch. Biochem. Biophys.* **74**, 398.

Wang, T. P. & Kaplan, N. O. (1954). *J. biol. Chem.* **206**, 311.

Weatherford, H. L. (1929). *Amer. J. Anat.* **44**, 199.