Biochem. J. (1988) 250, 395–399 (Printed in Great Britain)

Enzymes of the pathway of purine synthesis in the rat mammary gland

Changes in the lactation cycle and the effects of diabetes

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Measurements were made of the activities of the enzymes of the '*de novo*' and salvage pathways of purine synthesis [phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14), adenine phosphoribosyltransferase (EC 2.4.2.7) and hypoxanthine phosphoribosyltranferase (EC 2.4.2.8)] at different stages of the lactation cycle, and the effects of diabetes on the activity of these enzymes in lactation were studied. A distinctive pattern of enzyme change was observed, in which the '*de novo*' pathway enzyme phosphoribosyl pyrophosphate amidotransferase increased sharply between days 10 and 14 of pregnancy, and then remained sensibly constant until the height of lactation, whereas the enzymes of the salvage pathway increased later in pregnancy and continued to rise during lactation. Diabetes severely depressed the activity of the enzymes of the salvage pathway, but appeared to be without effect on the '*de novo*' pathway enzyme. These results are discussed in relation to the provision of purine precursors from tissues outside the mammary gland.

INTRODUCTION

The protein-synthesis activity of the mammary gland varies widely over the lactation cycle, and these changes are largely reflected by the parallel changes which occur in the nucleic acid content. The DNA content increases 10-fold from the virgin state to the height of lactation, and RNA some 50-fold over the same period [1,2]. Similar increases, although on a slightly more modest scale, have been reported in the soluble nucleotides of the gland from late pregnancy to late lactation [3].

The synthesis of nucleotides can occur by either of two pathways, the '*de novo*' and salvage pathways, both of which require *PP*Rib*P* as substrate, and both of which can be operative in the mammary gland. *PP*Rib*P* is also an activator of the first enzyme of the '*de novo*' purine- and pyrimidine-biosynthesis pathways (see [4]). Previous studies on the rat mammary gland during the lactation cycle have shown a rising tissue concentration and total tissue content of *PP*Rib*P* and *PP*Rib*P* synthetase (EC 2.7.6.1) during pregnancy and lactation [5]; a role of insulin in the regulation of *PP*Rib*P* formation has also been identified [5].

There is at present no evidence available to show the relation of changes in these two biosynthetic pathways at different stages of the lactation cycle to changes in *PP*Rib*P* formation and, in particular, whether they change in parallel, or differentially, with growth during pregnancy, with milk secretion and in mammary involution. The relative dependence of the enzymes of the '*de novo*' and salvage pathways of purine synthesis on insulin in the lactating mammary gland is also important, in view of the marked effect of diabetes on *PP*Rib*P* formation and on lactation.

In an attempt to evaluate a possible differential contribution of the two pathways, the activities of the rate-limiting enzyme of the purine '*de novo*' pathway, phosphoribosyl pyrophosphate amidotransferase (*PP*-Rib*P*-At, EC 2.4.2.14) and of the two major enzymes of the salvage pathway, adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) and hypoxanthine (guanine) phosphoribosyltransferase (HPRT, EC 2.4.2.8) have been measured at different stages of the lactation cycle. Further, in view of the dependence of lactation on the presence of insulin, and in order to trace further the role of insulin in the regulation of nucleic acid synthesis in the mammary gland, these same parameters have also been measured in the glands of rats made diabetic on day 7 of lactation and killed 3 days later.

METHODS

Albino rats of the Wistar strain, undergoing their first pregnancy and weighing 210–230 g before mating, were used. Rats were taken at the stated times in pregnancy and lactation and killed by cervical dislocation. The three abdominal glands of one side were taken for the preparation of tissue extracts. The group of animals required for the 'diabetic' experiment were taken on day 7 of lactation and treated intravenously with streptozotocin (60 mg/kg body wt.) and killed 3 days later.

The tissue suspensions were 1:10 (w/v) homogenates of mammary tissue in either 0.29 M-sucrose/0.01 M-Tris/HCl, pH 7.0 (*PP*Rib*P*-At), or 0.25 M-sucrose/0.1 mM-EDTA, pH 7.4 (APRT and HPRT). These homogenates were centrifuged at 12000 *g* for 30 min and the super-

Abbreviations used: *PP*Rib*P*, phosphoribosyl pyrophosphate; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); *PP*Rib*P*-At, phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14).

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natants dialysed against the appropriate homogenization medium for 1 h before use in the enzyme assays.

Enzyme measurements

(i) *PP*Rib*P*-At. The activity of this enzyme was measured by the method of Wood & Seegmiller [6]. The product of this reaction, [^{14}C]glutamate, was separated from the substrate, [^{14}C]glutamine, by adsorption on DEAE-cellulose discs as described by Martin [7].

(ii) APRT. The activity of this enzyme was measured by the procedure of Kizaki & Sakurada [8], except for the addition of 3 mM-TTP, as described by Allsop & Watts [9].

(iii) HPRT. This enzyme was also measured by the method of Kizaki & Sakurada [8], again with the addition of 3 mM-TPP to inhibit the action of 5′-nucleotidase (EC 3.1.3.5), as described by Gutensohn & Guroff [10].

The radioactive product of each of these assays was counted in a Beckman LS 7500 scintillation counter, and the results are expressed per g wet wt. of tissue (corrected for the presence of retained milk as described by Gumaa *et al.* [11]).

Measurement of RNA and DNA

These were measured as previously described [5], with purified tRNA and calf thymus DNA (Sigma) as standards.

RESULTS

Enzyme measurements

The results of the assays for the three enzymes of purine synthesis are given in Table 1. Although all three enzymes increase during the course of pregnancy and lactation, different patterns are apparent in their development. The two enzymes of the salvage pathway increase in parallel throughout the lactation cycle, with APRT having an activity always some 10-fold greater than that of HPRT. For these two enzymes, there is essentially no change in activity until the last few days of pregnancy, after which their activity increases markedly, and continuously, up to the point of maximum milk yield, approx. 16–18 days in this colony. The rate-limiting enzyme of the '*de novo*' pathway, however, increases from the earliest point measured, with an 8-fold jump in activity between days 10 and 14 of pregnancy. There is a further increase in the activity of this enzyme in the last few days of pregnancy, and then, again, in the first 5 days of lactation, but no further increase occurs over the remainder of the lactation period, i.e. at a time when the activity of the salvage-pathway enzymes is rising most strongly. This difference in developmental pattern is most clearly seen when the ratio HPRT/*PP*Rib*P*-At activity is considered. This ratio is very large (approx. 60) early in pregnancy, but falls (to 5–12) late in pregnancy and in the first half of lactation. The ratio rises again in late lactation.

The contrast between the '*de novo*' and salvage pathways is even more striking in the response to diabetes (Table 1). Whereas diabetes leads to a very substantial fall in the activity of the two enzymes of the salvage pathway (to 54% for APRT and to 26% for HPRT), the '*de novo*' pathway enzyme is completely unaffected by the lesion.

DISCUSSION

The pitfalls in extrapolating from the activities of the enzymes of purine synthesis, as measured *in vitro*, to the activities of alternative pathways operating in the environment of the intact cell have been reviewed by Henderson *et al.* [12]. For the reasons quoted in that review, the present study considers, primarily, the changes in the activity of the '*de novo*' and salvage pathways, rather than their absolute activity.

Changes in the synthesis of purines in the lactation cycle

There are several transition points in the lactation cycle where changes in components contributing to nucleotide and nucleic acid synthesis are of particular interest. Among these are: (1) the period of cell division and growth in the first half of pregnancy; (2) the perinatal period, where cell division occurs and protein biosynthesis (colostrum and milk) is initiated; (3) the period at mid-lactation, when the rate of cell division is low but the amount and turnover of RNA are high as the gland manufactures protein for export; and (4) the switch-off of biosynthetic activity during mammary involution. Studies are available for the tissue content of RNA and DNA, the mitotic index and the tissue content of free nucleotides [1,3], as well as the tissue *PP*Rib*P* content [5]. The present work adds to these studies complementary data on the enzymic capacity for nucleotide biosynthesis at these same periods.

Examination of the sequence of changes during the lactation cycle reveals a distinctive pattern. During the period of tissue growth in pregnancy, the earliest change observed (5–10 days) is the 5-fold increase in the activity of *PP*Rib*P* synthetase [5], followed (days 10–14) by an 8-fold rise in *PP*Rib*P*-At. In the latter half of pregnancy (days 14–18) it is the salvage-pathway enzymes which increase (APRT 3-fold and HPRT 2-fold). Again, in the second period of cell division, immediately preceding and following parturition, it is the '*de novo*' pathway enzyme which shows an increase, with the salvage-pathway enzymes unchanged, whereas in lactation the reverse is true, both APRT and HPRT increasing substantially while *PP*Rib*P*-At remains unchanged. It should be noted that, in lactation, APRT and HPRT activities follow closely the course of increase in *PP*Rib*P*, as reported by Kunjara *et al.* [5], but that of *PP*Rib*P*-At bears little relationship to either *PP*Rib*P* synthetase activity or *PP*Rib*P* content.

Thus there appears to be a distinctive sequence of change in the enzyme activity profile which is consistent with the view that, whereas the 'de novo' pathway seems to be more associated with periods of tissue growth, lactation involves a shift from the '*de novo*' to the salvage pathway of nucleotide synthesis.

This view receives support from a second criterion, which may be taken to reflect the relative importance of the '*de novo*' and salvage pathways. This criterion is the HPRT/*PP*Rib*P*-At activity ratio, as used by Allsop & Watts [9], although some caution must apply, in view of the complex regulation of both the enzymes involved. In the present experiments, this ratio is high (60) in early pregnancy, low (10) in late pregnancy and rising (to 27) with the advance of lactation, implying that the '*de novo*'

Table 1. (*a*) Activities of the enzymes of the pathways of purine synthesis in the mammary gland at different stages of the lactation cycle and (*b*) effect of streptozocin-diabetes on the activities of these enzymes and the incorporation of uridine and thymidine into nucleic acids

Values are means ± S.E.M.; the number of animals in each group was six. Fisher's *P* values are shown by: $*P<0.05$; $**P<0.01$; $***P<0.001$.

Stage of lactation cycle	Maternal body wt. (g)	Mammary-gland wt. (g)†	Activity (μmol/h per g‡ at 37 °C) APRT	HPRT	*PP*Rib*P*-At	HPRT / *PP*Rib*P*-At	Nucleic acid (mg/total gland) RNA	DNA
(a) Changes during the lactation cycle								
Pregnancy								
5 days	238±9	0.710±0.027	20.2±5.3	1.92±0.83	0.031±0.007	62.0±5.7	2.76±0.05	1.86±0.40
10 days	240±8	2.82±0.38	20.8±2.4	2.90±0.80	0.051±0.011	57.3±8.6	5.08±0.53	2.82±0.31
14 days	265±5	3.18±0.32	18.5±3.8	2.11±0.61	0.408±0.056	5.4±0.6	11.3±1.2	4.01±0.46
18 days	299±7	3.78±0.40	54.5±8.4	4.02±0.94	0.335±0.062	12.0±1.46	17.2±1.4	3.67±0.53
21 days	318±15	4.29±0.47	56.1±8.4	4.30±0.55	0.548±0.131	7.9±0.9	20.3±4.7	5.58±0.50
Lactation								
1 day	277±13	3.39±0.42	64.1±11.8	7.41±0.61	0.561±0.078	13.2±1.4	25.9±1.2	6.02±0.16
5 days	269±10	4.74±0.31	98.2±20.5	7.81±0.60	0.705±0.095	11.1±1.9	25.4±3.4	5.69±0.33
10 days	314±21	6.16±0.63	85.7±14.4	8.96±1.64	0.682±0.103	13.1±1.9	46.0±4.1	6.87±0.50
15 days	284±6	4.59±0.43	143±28	15.3±1.3	0.704±0.111	17.7±3.0	62.5±5.8	7.04±0.52
21 days	284±11	4.27±0.49	125±26	10.8±2.2	0.398±0.082	27.1±3.8	42.2±4.4	6.90±0.34
Involution								
3 days	279±12	4.44±0.29	73.4±9.6	6.13±1.06	0.314±0.069	19.5±2.7	18.7±3.6	3.71±0.51
(*b*) Effect of diabetes on lactating mammary gland								
Normal lactating, 10 days (C)	314±21	6.16±0.63	85.7±14.4	8.96±1.64	0.682±0.103	13.1±1.9	46.0±4.1	6.87±0.50
Diabetic lactating, 10 days (D) [diabetic 3 days (days 7–10)]	230±10	2.79±0.18	46.7±3.0	2.32±0.28	0.708±0.128	3.3±0.2	16.5±1.7	4.46±0.32
D/C (%)	73**	45***	54*	26**	104	25***	36***	65**

† The weight of the three abdominal glands from one side. Each litter was restricted to eight pups after birth.
‡ Results for the enzyme assays are corrected for the presence of retained milk as described by Gumaa *et al.* [11].

pathway assumes a greater role in nucleotide synthesis in the perinatal period and a lesser role in lactation.

Some further support derives from the experiments of Wang & Greenbaum [3], which showed that the rate of incorporation of [^{14}C]glycine into ribonucleotides, which can occur only via the '*de novo*' pathway, increased sharply from late pregnancy to early lactation, but then fell to a lower, and constant, value until late lactation. The change in activity of *PP*Rib*P*-At corresponds closely to this pattern, except for the decline in incorporation between days 2 and 10 of lactation. The discrepancy between the two types of measurement at this time interval may be due to one of two possibilities. (i) Glycine is incorporated at the phosphoribosylglycinamide synthase (EC 6.3.4.13) stage, i.e. the step after *PP*Rib*P*-At, which is the first, and most closely regulated [13], step in the pathway. It is therefore possible to differentiate between *PP*Rib*P*-At, which regulates the initial step, and glycine incorporation, which represents the remainder of the pathway. In the present experiments, *PP*Rib*P*-At was measured under $V_{max.}$ conditions, and thus was not subject to regulation by the many factors (including feedback inhibition by adenine and guanine nucleotides) which operate *in vivo* [13], whereas the glycine incorporation was measured *in vivo*. (ii) This period coincides with the hypertrophy of the liver in response to lactation [14] and possible changes in the pool size of available precursors for nucleotide synthesis [3,15]. Nevertheless, the present results show that the increase in RNA content of the gland in lactation occurred without any evidence for any corresponding rise in the contribution from the '*de novo*' pathway.

True mammary involution commences on the removal of the litter on day 22 *post partum*. However, the most significant criterion for the effect of involution is the comparison of the activities of the pathways in the involuting gland with those obtaining on day 15 of lactation, i.e. before the pups begin eating pelleted diet and become less dependent on the maternal milk supply. On this basis, the activities of enzymes of both the '*de novo*' and salvage routes decrease by day 3 after removal of the litter, to approximately one-half to one-third of the value at 15 days lactation (Table 1). Previous studies on the changes in *PP*Rib*P* synthetase and *PP*Rib*P* content [5] point to a more rapid decline of this system in mammary involution.

Effect of diabetes on pathways of purine synthesis

In the mammary gland, lactation, glucose utilization, lipogenesis, lactose synthesis and RNA and protein synthesis require, in addition to prolactin and other hormones, the presence of insulin [16–22]. The mammary-gland responsiveness to insulin is apparent during pregnancy and is retained during lactation, but is lost during mammary involution, the cells reverting to the insulin-insensitive state of the gland from the virgin rat [19,23]. In insulin insufficiency in lactation, there is a rapid switch-off of milk production, with parallel decreases in the pentose phosphate pathway, lipogenesis, RNA content and *PP*Rib*P* formation [5,18,24–26].

Diabetes of 3 days duration sharply reduces lactational performance [5,24] and the activities of APRT and HPRT, the latter declining to 26% of the age-matched control group, but has no apparent effect on *PP*Rib*P*-At activity measured under $V_{max.}$ conditions (Table 1). These changes again highlight the importance of the salvage pathway in lactational performance. It is not possible to ascribe the changes in diabetes entirely to the decrease in insulin, since Robinson *et al.* [26] have shown a 7-fold decrease in plasma prolactin 2 h after administration of streptozotocin.

A caveat to the present discussion should be added, in that the present measurements of activities of enzymes of the '*de novo*' and salvage pathways of purine synthesis are made *in vitro* under $V_{max.}$ conditions, and, of the complex controls regulating these routes (see [12,13,15]), only the concentration of *PP*Rib*P* has been measured under parallel conditions. The substrate/velocity curves for *PP*Rib*P*-At are sigmoidal, with a Hill coefficient of 2 and concentration giving half-maximal velocity, $s_{0.5}$, of 0.25 mM [6], compared with a tissue *PP*Rib*P* concentration of 10 μM at 10 days' lactation, falling to 2.8 μM at 3 days of diabetes. The control of the synthesis of nucleotides by the supply of precursors of purines from the liver [15], and in addition, possibly, other tissues, is a potentially important aspect of regulation in the mammary gland; the dependence of lactation on an integrated response from other organs has also been stressed by Williamson [16]. In the light of the results reported here, it seems reasonable to suggest that the supply of external nucleotide precursors in pregnancy, before and inter-organ response has developed, is limited, and hence the gland manufactures its requirement *de novo*. Once lactation is established and other tissues respond, particularly the hypertrophy by the liver [14], under the stress of lactation, the external source of nucleotide bases expands and fuels the more energetically favourable salvage pathway, hence the preponderance of this pathway in lactation.

This work was supported by grants from the British Diabetic Association, the Basil Samuel Charitable Trust and the Association for International Cancer Research.

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Received 28 October 1987/8 December 1987; accepted 15 December 1987