Phosphoribosyl pyrophosphate and phosphoribosyl pyrophosphate synthetase in rat mammary gland

Changes in the lactation cycle and effects of diabetes, insulin and phenazine methosulphate

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Changes in the tissue content of phosphoribosyl pyrophosphate (*PPRibP*), glucose 6-phosphate, ribose 5-phosphate (*Rib5P*), RNA and DNA, of the activity of *PPRibP* synthetase (EC 2.7.6.1) and the conversion of [$1-^{14}\text{C}$]- and [$6-^{14}\text{C}$]-glucose into $^{14}\text{CO}_2$ were measured at mid-lactation in the normal and diabetic rat and in pregnancy, lactation and mammary involution in the normal rat. The *PPRibP*, glucose 6-phosphate and *Rib5P* contents increase during pregnancy and early lactation to reach a plateau value at mid-lactation, before falling sharply during weaning. The *PPRibP* content, *PPRibP* synthetase activity and flux of glucose through the oxidative pentose phosphate pathway (PPP) all change in parallel during the lactation cycle. Similarly, after 3 and 5 days duration of streptozotocin-induced diabetes, ending on day 10 of lactation, there were parallel declines in *PPRibP* content, *PPRibP* synthetase and PPP activity. The effect of streptozotocin was prevented by pretreatment with nicotinamide and partially reversed by insulin administration. Addition of insulin to lactating rat mammary-gland slices incubated *in vitro* significantly raised the *PPRibP* content (+47%) and the activity of the PPP (+40%); phenazine methosulphate, which gives a 2-fold increase in PPP activity, raised the *PPRibP* content of lactating mammary gland slices by approx. 3-fold. It is concluded that *Rib5P*, generated in the oxidative segment of the PPP, is an important determinant of *PPRibP* synthesis in the lactating rat mammary gland and that insulin plays a central role in the regulation of the bioavailability of this precursor of nucleotide and nucleic acid synthesis.

INTRODUCTION

The rising level of biosynthetic activity in the rat mammary gland through the lactation cycle is accompanied by increases in the tissue content of RNA and soluble nucleotides, including nicotinamide nucleotides (Greenbaum & Slater, 1957; McLean, 1958; Wang & Greenbaum, 1960; Johke, 1978; Mercier & Gaye, 1983), of the enzymes of, and the flux of glucose through, the pentose phosphate pathway (PPP), and sharply rising rates of lipogenesis (Gumaa *et al.*, 1971; Robinson & Williamson, 1977; Robinson *et al.*, 1978; Kuhn, 1983). These changes are linked via the PPP, which (i) provides the NADPH required for lipogenesis; (ii) lipogenesis causes the re-oxidation of NADPH, thus increasing the flux of glucose through the PPP, and (iii) the provision of ribose 5-phosphate (*Rib5P*), which is the precursor of phosphoribosyl pyrophosphate (*PPRibP*). *PPRibP* is an essential factor in the increased biosynthetic activity of the gland, since it serves as a primary substrate in both the 'de novo' pathway and the salvage pathway of nucleotide synthesis, as well as being an activator of carbamoyl-phosphate synthase (glutamine-hydrolysing) (EC 6.3.5.5) and amidophosphoribosyltransferase (EC 2.4.2.14) (Becker *et al.*, 1979; Tatibana *et al.*, 1982; Pilz *et al.*, 1984), key enzymes of the 'de novo' pathway of synthesis. Indeed, it has been suggested that *PPRibP* regulates the 'de novo' pathway (McKeran & Watts, 1978). Further there is evidence (see Becker *et al.*, 1979) that the rate of *PPRibP* formation is modulated by the intracellular concentration of *Rib5P* and, hence, of the

activity of PPP, and that increased concentrations of *Rib5P* in some culture cells lead to excessive production of *PPRibP* and overproduction of uric acid (Becker, 1976). Becker *et al.* (1979) have pointed out that the regulatory role of *Rib5P* in *PPRibP* formation is not fully resolved and may well be dependent on changes in the P_i concentration.

In the mammary gland, lactation, glucose utilization, lipogenesis, RNA and protein synthesis and the induction of some key enzymes require, in addition to prolactin and other hormones, the presence of insulin (Topper, 1970; Topper & Green, 1971; Kuhn, 1983), and in diabetes there is a rapid switch-off of milk production, with a parallel decrease in activity of the PPP and of lipogenesis (Walters & McLean, 1968; Robinson & Williamson, 1977; Robinson *et al.*, 1978). In insulin insufficiency there is also an accompanying fall in the RNA content (Walters & McLean, 1968; Topper & Green, 1971). The present study examines some factors regulating the supply of *PPRibP* in the mammary gland during the lactation cycle, with particular emphasis on the effects of diabetes and the role of insulin and of re-oxidation of NADPH on this process.

METHODS

Animals

The animals were of the Wistar strain and were taken at the intervals stated in pregnancy, lactation and involution. Pregnancy averaged 22 days in this colony.

Abbreviations used: *PPRibP*, phosphoribosyl pyrophosphate; *Rib5P*, ribose 5-phosphate; PPP, pentose phosphate pathway.

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After parturition, the litters of all rats were decreased to eight pups. Lactation was allowed until day 21 *post partum*, after which the pups were removed from the mother. Involution was timed from the removal of the pups.

For the induction of diabetes, lactating rats were injected with 70 mg of streptozotocin (intravenously)/kg body wt. on either day 5 or 7 of lactation. The pups were withdrawn immediately before the injection and returned 2 h later. Control rats were given the same volume of saline by the same route. The possible toxic effects of streptozotocin were evaluated by treating a third group of rats with streptozotocin on day 7 of lactation and then maintaining them on an insulin regime comprising 2 units of Monotard MC insulin (Novo, Copenhagen, Denmark) administered twice a day. This regime maintained partial normoglycaemia, the kidney threshold being exceeded 8–10 h after each insulin injection. The last insulin injection was given 3 h before the experiment commenced. Separation of the diabetogenic and 'toxic' effects of streptozotocin was also attempted by pretreatment of the lactating rats with nicotinamide, with a dose of 500 mg/kg body wt. given 10 min before the streptozotocin. This treatment is reported to prevent the diabetogenic effects of streptozotocin (Rerup, 1970).

The lactational performance of all groups of lactating rats was determined by weighing the litters daily (Brody & Nisbet, 1938; Walters & McLean, 1968).

Determinations

(i) **PPRibP synthetase.** A portion of the abdominal mammary glands was removed and homogenized in 10 mM-potassium phosphate/1 mM-mercaptoethanol buffer, pH 7.4, as described by Lipstein *et al.* (1978), to give a 1:30 (w/v) tissue suspension. The high-speed supernatant obtained from this suspension after centrifugation was dialysed against the same buffer for 2 h and then used for the estimation of PPRibP synthetase (EC 2.7.6.1) activity essentially by the procedure of Roth *et al.* (1978), except for the addition of 0.30 mM-dithiothreitol to the incubation mixture and the substitution of an acceptor system based on the conversion of labelled orotic acid into orotidine 5'-phosphate and the decarboxylation of this product to yield $^{14}\text{CO}_2$, which was counted for radioactivity. The assay system contained: orotic acid, 0.6 mM; 0.15 μCi of [*carboxy*- ^{14}C]orotic acid (52 Ci/mol) (New England Nuclear/Dupont U.K. Ltd., Southampton, U.K.); dithiothreitol, 0.3 mM; potassium phosphate, pH 7.6, 60 mM; MgSO_4 , 17 mM; Na_2EDTA , 0.5 mM; 3 mg of mixed enzyme preparation containing orotidine 5'-phosphate pyrophosphorylase (EC 2.4.2.10) and orotidine 5'-phosphate decarboxylase (EC 4.1.1.23) [Sigma (London) Chemical Co., Poole, Dorset, U.K.], all in a total volume of 215 μl . To this mixture was added 20 μl of tissue extract, and the volume was then made up to 700 μl . The reaction was started by the addition of 40 μl of a mixture containing 25 mM-Rib5P and 25 mM-ATP. The reaction was allowed to continue, in a closed vessel, for 15 min at 37 °C and was then stopped by the injection of 0.5 ml of 1 M- H_2SO_4 through the rubber seal. The evolved $^{14}\text{CO}_2$ was collected in Hyamine and counted for radioactivity in a Beckman model LS 7500 scintillation counter.

(ii) **PPRibP.** A second portion of the abdominal mammary gland (approx. 0.5 g) was rapidly removed and

immediately dropped into 5 ml of 100 mM-potassium phosphate/1 mM-EDTA buffer, pH 7.4, maintained in a boiling-water bath at 100 °C. After 20 s the tissue was homogenized with an Ultra-Turrax blender (Janke und Kunkel, Staufen/Breisgau, W. Germany) for 30 s and then returned to the boiling-water bath for a total time of 5 min. The time of boiling allowed here is appreciably longer than that used by Reem & Friend (1976) for liver, but was found necessary to achieve full inactivation of the high phosphatase activity found in mammary gland. The heated extracts were chilled on ice for 2 min and then centrifuged for 1 min in an Eppendorf 3200 centrifuge. The supernatant of this was used directly for the assay of PPRibP essentially as described by Buchanan *et al.* (1982), except that carrier orotic acid (0.2 mM) was added and the amount of the orotidine 5'-phosphate pyrophosphorylase/decarboxylase mixture was increased to 3 mg per assay tube. The reaction time was 10 min at 37 °C, and the $^{14}\text{CO}_2$ evolved was collected in Hyamine and counted for radioactivity as above.

(iii) **RNA, DNA and metabolic intermediates.** A third portion of mammary tissue was taken and freeze-clamped in Wollenberger tongs precooled in liquid N_2 . The frozen tissue was extracted with 0.6 M- HClO_4 and homogenized. The resultant homogenate was centrifuged at 10000 g for 10 min. Glucose 6-phosphate and Rib5P were measured in the neutralized HClO_4 extract as described by Bergmeyer (1974). RNA and DNA were measured as previously described (Walters & McLean, 1968), with purified tRNA and calf thymus DNA (Sigma) as standards. The tissue content of the intermediates was corrected to the milk- and fat-free tissue weight as described by Gumaa *et al.* (1971).

Incubation procedures

(i) **Measurement of metabolic flux.** Tissue slices were prepared from lactating mammary glands of 10-day-lactating animals with a Stadie-Riggs tissue slicer. Approx. 300 mg of slices were incubated in 4.5 ml of Krebs-Ringer bicarbonate medium containing 20 mM-glucose, 0.5 μCi of [^{14}C]glucose or 0.5 μCi of [^{14}C]glucose (The Radiochemical Centre, Amersham, Bucks., U.K.), with or without the following additions: (i) 1 unit of insulin (Novo; zinc-free; 24 units/mg) or (ii) phenazine methosulphate (0.1 mM final concn). After gassing with O_2/CO_2 (19:1) for 10 min, the flasks were incubated for 1 h at 37 °C and the reaction was stopped by the addition of 1 ml of 5 M-HCl. The evolved $^{14}\text{CO}_2$ was collected in 0.5 ml of Hyamine in the centre well. After 1 h had been allowed for absorption, the Hyamine was transferred to a scintillation vial and counted for radioactivity as above.

(ii) **Effect of addition of insulin on PPRibP synthetase activity.** Tissue slices, (500 mg), prepared as above, were incubated in 4.5 ml of Krebs-Ringer bicarbonate medium containing 20 mM-glucose with or without 1 unit of insulin (Novo; zinc-free). The flasks were gassed and incubated as above for 1 h at 37 °C. At the end of this time the slices were collected for the preparation of tissue homogenates for the determination of PPRibP synthetase as described above.

Table 1. Changes in the activity of *PPRibP* synthetase and the tissue contents of *PPRibP*, glucose 6-phosphate and ribose 5-phosphate in the mammary glands of rats at different stages of lactation cycle

The values are given as means $\pm$ S.E.M. of four values in each group. Results for the enzyme and intermediate phosphorylated compounds are corrected for the presence of retained milk as described by Gurnaa *et al.* (1971). The approximate activity of the pentose phosphate pathway was calculated from the difference in the yields of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ (C-1—C-6).

Stage of lactation cycle	Maternal body (g)	Litter wt. (g)*	Mammary-gland wt. (g)†	<i>PPRibP</i> synthetase ($\mu\text{mol/h per g}$)	<i>PPRibP</i> (nmol/g)	Glucose 6-phosphate ($\mu\text{mol/g}$)	Rib5P ($\mu\text{mol/g}$)	$^{14}\text{CO}_2$ yield from $[1-^{14}\text{C}]\text{glucose}$ ($\mu\text{mol/h per g}$)	$^{14}\text{CO}_2$ yield from $[6-^{14}\text{C}]\text{glucose}$ ($\mu\text{mol/h per g}$)	PPP activity (C-1—C-6) ($\mu\text{mol/h per g}$)
Pregnancy										
5 days	202 $\pm$ 5	—	0.51 $\pm$ 0.08	1.42 $\pm$ 0.25	0.028 $\pm$ 0.006	0.016 $\pm$ 0.002	—	1.04 $\pm$ 0.11	0.25 $\pm$ 0.02	0.80 $\pm$ 0.12
10 days	219 $\pm$ 6	13 $\pm$ 1	1.97 $\pm$ 0.06	6.75 $\pm$ 0.82	1.35 $\pm$ 0.52	0.027 $\pm$ 0.004	0.107 $\pm$ 0.004	2.24 $\pm$ 0.19	1.05 $\pm$ 0.15	1.19 $\pm$ 0.26
20 days	364 $\pm$ 10	40 $\pm$ 4	3.88 $\pm$ 0.62	17.1 $\pm$ 1.5	3.36 $\pm$ 0.17	0.062 $\pm$ 0.005	0.143 $\pm$ 0.008	2.72 $\pm$ 0.33	1.58 $\pm$ 0.10	1.13 $\pm$ 0.36
Lactation										
1 day	266 $\pm$ 5	52 $\pm$ 4	2.99 $\pm$ 0.34	15.9 $\pm$ 1.9	4.00 $\pm$ 0.96	0.035 $\pm$ 0.006	0.118 $\pm$ 0.048	18.2 $\pm$ 1.4	2.95 $\pm$ 0.22	15.3 $\pm$ 1.5
5 days	276 $\pm$ 6	124 $\pm$ 11	3.69 $\pm$ 0.48	16.7 $\pm$ 1.8	5.83 $\pm$ 0.51	0.095 $\pm$ 0.008	0.265 $\pm$ 0.067	26.1 $\pm$ 1.9	2.24 $\pm$ 0.30	23.9 $\pm$ 2.9
10 days	289 $\pm$ 5	204 $\pm$ 17	4.72 $\pm$ 1.01	63.2 $\pm$ 6.7	10.2 $\pm$ 1.8	0.092 $\pm$ 0.006	0.285 $\pm$ 0.029	31.3 $\pm$ 1.4	2.33 $\pm$ 0.15	29.0 $\pm$ 2.9
20 days	287 $\pm$ 12	361 $\pm$ 22	4.21 $\pm$ 0.24	46.1 $\pm$ 5.8	7.57 $\pm$ 0.89	0.112 $\pm$ 0.005	0.281 $\pm$ 0.042	32.6 $\pm$ 1.8	2.10 $\pm$ 0.30	30.4 $\pm$ 2.9
Involution										
2 days	258 $\pm$ 6	—	4.64 $\pm$ 0.37	15.0 $\pm$ 2.2	3.35 $\pm$ 1.98	0.080 $\pm$ 0.008	—	3.68 $\pm$ 0.24	0.57 $\pm$ 0.06	3.10 $\pm$ 0.39
4 days	251 $\pm$ 6	—	1.41 $\pm$ 0.38	4.27 $\pm$ 0.71	2.12 $\pm$ 0.20	0.032 $\pm$ 0.005	0.144 $\pm$ 0.009	3.20 $\pm$ 0.42	0.38 $\pm$ 0.02	2.82 $\pm$ 0.17

* Each litter was restricted to eight pups after birth.

† The weight of three abdominal glands from one side.

Fig. 1. Effect of streptozotocin-diabetes, with and without treatment with insulin or pretreatment with nicotinamide, on the growth curves of litters as a measure of lactational performance

In each case the litters were restricted to eight pups. The daily weights of the litters are shown as means $\pm$ S.E.M.; the numbers of values per group are as in Table 2. The symbols are: normal control, $\circ$; streptozotocin-diabetic (STZ), 3 days, $\ominus$, 5 days, $\bullet$; streptozotocin-diabetic rat treated with insulin (STZ+I), Δ ; the group treated with nicotinamide followed by streptozotocin (NIC+STZ) is shown by $\blacktriangle$. The litter-growth curve of normal rats treated with nicotinamide was not significantly different from that of untreated normal control animals.

RESULTS

Changes in *PPRibP* and *PPRibP* synthetase during the lactation cycle

The changes in *PPRibP* concentration during pregnancy, lactation and mammary involution are shown in Table 1. The concentration of *PPRibP* increases approx. 6-fold between day 10 of pregnancy, when tissue growth is almost at a maximum but the secretion of the milk has not yet been initiated, and day 10 of lactation, when tissue growth is minimal but milk secretion is proceeding at a high rate. The *PPRibP* concentration falls slightly in the last period of lactation, as the pups become more independent of the maternal milk supply, and then sharply during mammary involution.

PPRibP synthetase activity shows an analogous pattern, rising some 9-fold from mid-pregnancy to mid-lactation, falling by 30% in the last week of lactation and by 90% after 4 days of involution. The transition from pregnancy to lactation is not accompanied by any sharp changes in the developmental pattern of either *PPRibP* or *PPRibP* synthetase, and the increment of both these quantities during lactation is linked to the increased stress of milk production.

The precursors of *PPRibP* are glucose 6-phosphate

Table 2. Effect of diabetes on the activity of *PPRibP* synthetase and the tissue contents of *PPRibP*, glucose 6-phosphate and ribose 5-phosphate of the lactating rat mammary gland

The values are given as means $\pm$ S.E.M., for day 10 of lactation. Insulin (2 units of Monotard MC insulin) was given twice daily. Nicotinamide (500 mg/kg body wt.) was given intraperitoneally 10 min before streptozotocin. Results for the enzyme and intermediate phosphorylated compounds are corrected for the presence of retained milk as described by Gumaar *et al.* (1971).

Rats	Insulin status	No. of animals	Maternal body wt. (g)	Litter wt. (g)*	Mammary-gland wt. (g)†	Blood glucose (mm)	<i>PPRibP</i> synthetase (μ mol/h per g)	<i>PPRibP</i> (nmol/g)	Glucose 6-phosphate (μ mol/g)	Rib5P (μ mol/g)	Nucleic acid (mg/total gland)		^{14}C CO ₂ yield from [1- ^{14}C]glucose (μ mol/h per g)	^{14}C CO ₂ yield from [6- ^{14}C]glucose (μ mol/h per g)	PPP (C-1-C-6) (μ mol/h per g)
											RNA	DNA			
Normal lactating	Normal	9	300 $\pm$ 6	210 $\pm$ 5	4.47 $\pm$ 0.44	6.2 $\pm$ 0.6	46.8 $\pm$ 5.3	9.74 $\pm$ 1.04	0.084 $\pm$ 0.008	0.275 $\pm$ 0.030	44.6 $\pm$ 5.8	8.0 $\pm$ 1.2	34.4 $\pm$ 2.8	2.54 $\pm$ 0.23	31.9 $\pm$ 2.6
Diabetic lactating	Diabetic 3 days (days 7-10)	5	245 $\pm$ 12	156 $\pm$ 7	1.75 $\pm$ 0.19	25.3 $\pm$ 1.8	15.4 $\pm$ 0.81	2.78 $\pm$ 0.34	0.096 $\pm$ 0.013	0.122 $\pm$ 0.012	17.5 $\pm$ 1.2	4.58 $\pm$ 0.38	6.86 $\pm$ 0.78	2.39 $\pm$ 0.31	4.47 $\pm$ 0.52
	Diabetic 5 days (days 5-10)	4	217 $\pm$ 13	132 $\pm$ 3	1.51 $\pm$ 0.18	28.8 $\pm$ 2.2	18.6 $\pm$ 1.7	2.85 $\pm$ 0.97	0.123 $\pm$ 0.002	0.112 $\pm$ 0.013	19.1 $\pm$ 1.4	4.6 $\pm$ 0.40	5.81 $\pm$ 0.69	2.50 $\pm$ 0.42	3.31 $\pm$ 0.67
Insulin-treated diabetic lactating	'Diabetic' 3 days, insulin 3 days (days 7-10)	4	316 $\pm$ 14	198 $\pm$ 6	2.91 $\pm$ 0.36	8.6 $\pm$ 1.9	22.1 $\pm$ 1.5	8.02 $\pm$ 0.51	0.067 $\pm$ 0.003	0.156 $\pm$ 0.036	35.6 $\pm$ 4.2	4.11 $\pm$ 0.29	37.0 $\pm$ 2.1	1.40 $\pm$ 0.18	35.6 $\pm$ 3.8
Nicotinamide-treated	Normal	4	300 $\pm$ 13	199 $\pm$ 13	4.72 $\pm$ 0.73	5.5 $\pm$ 0.4	35.9 $\pm$ 5.9	7.90 $\pm$ 1.17	—	0.306 $\pm$ 0.071	42.9 $\pm$ 4.4	7.08 $\pm$ 1.0	29.9 $\pm$ 1.4	2.00 $\pm$ 0.28	27.9 $\pm$ 1.6
	Diabetic 3 days, nicotinamide + streptozotocin (days 7-10)	4	286 $\pm$ 14	173 $\pm$ 3	3.72 $\pm$ 0.09	6.6 $\pm$ 0.6	26.2 $\pm$ 2.4	6.11 $\pm$ 0.60	—	0.247 $\pm$ 0.016	37.3 $\pm$ 2.8	7.76 $\pm$ 0.76	26.9 $\pm$ 0.9	2.24 $\pm$ 0.09	24.7 $\pm$ 0.8

* Each litter was restricted to eight pups after birth.

† The weight of three abdominal glands from one side.

Table 3. Effect of insulin and phenazine methosulphate on the *PPRibP* content of 10-day-lactating rat mammary-gland slices incubated *in vitro*

The values are given as means $\pm$ S.E.M.. Fisher's *P* values are given for the comparison with the control group. The numbers of separate determinations are shown in parentheses. The incubation medium contained 4.5 ml of Krebs–Ringer bicarbonate medium and 20 mM-glucose with approx. 200 mg of mammary-gland slices prepared from the abdominal glands of 10-day-lactating rats. Insulin (1 unit/flask) or phenazine methosulphate (0.1 mM final concn.) was added to the flask as indicated. *PPRibP* synthetase activity was measured on mammary-gland slices at the end of an incubation period of 1 h in Krebs–Ringer bicarbonate medium/20 mM-glucose with and without added insulin. The PPP activity was estimated in separate flasks containing, in addition to the above components, 0.5 μ Ci of [1- 14 C]glucose or [6- 14 C]glucose. The gas phase was O₂/CO₂ (19:1); the incubation period was 1 h. The approximate activity of the PPP was taken as the difference in radiochemical yield of 14 CO₂ from [1- 14 C]glucose and [6- 14 C]glucose, (C-1 – C-6). The initial mammary-gland content of *PPRibP*, determined on the intact tissue from the contralateral gland, was 5.5 ± 0.7 nmol/g (*n* = 7). No correction was made for the retained milk in the gland or mammary-gland slices.

	Control	Insulin	Phenazine methosulphate
<i>PPRibP</i> content (nmol/g)	6.2 ± 0.4 (7)	10.1 ± 1.2 (7)	17.9 ± 1.8 (7)
<i>P</i>		<0.01	<0.001
<i>PPRibP</i> synthetase (μ mol/h per g)	31.9 ± 2.4 (3)	34.2 ± 1.9 (3)	—
PPP activity, 14 CO ₂ (C-1 – C-6) (μ mol/h per g)	28.6 ± 2.8 (9)	40.2 ± 2.5 (9)	56.3 ± 3.8 (9)
<i>P</i>		<0.01	<0.001

and, more immediately, Rib5P. For these two intermediates, the major increase in content occurs during lactation.

Effect of diabetes on *PPRibP* and *PPRibP* synthetase in lactation

The effect of diabetes on milk production, as shown by the growth rate of the pups, is given in Fig. 1 which illustrates (i) that streptozotocin treatment seriously impairs milk formation and (ii) that this impairment is not the result of streptozotocin toxicity, since maintaining near-normoglycaemia with insulin, or pre-treatment with nicotinamide, restores lactational performance. The growth curves of litters of normal rats treated with nicotinamide were not significantly different from those for untreated normal rats (results not shown).

In line with the apparent decreased biosynthetic activity of the gland is the sharply decreased gland weight and RNA and DNA contents (Table 2). The fall in *PPRibP* content, to approx. 30% of the control value, is paralleled by a decline of *PPRibP* synthetase activity. These changes appear to occur rapidly and are as great after 3 days of diabetes as they are at 5 days.

The Rib5P content is also decreased by diabetes, approximately in line with the fall in *PPRibP*, but the glucose 6-phosphate content remained unchanged or was even raised (+46% after 5 days of diabetes; Table 2). These results are also not due to any toxic effect of streptozotocin, since injection of streptozotocin-treated rats with insulin prevents, to a large degree, these changes. Such differences as do occur may probably be ascribed to a less-than-perfect insulin regimen and glycaemic control. Pre-treatment of rats with nicotinamide, which prevents the diabetogenic activity of streptozotocin but leaves toxic effects (Rerup, 1970), prevented the changes in *PPRibP* and *PPRibP* synthetase.

Influence of insulin and of phenazine methosulphate on the *PPRibP* concentration *in vitro*

In order to investigate the role of insulin in the

regulation of the *PPRibP* content of mammary gland, use was made of the known effect of this hormone *in vitro* in stimulating glucose utilization, PPP activity, lipogenesis and nucleic acid formation (Gumaa *et al.*, 1971; Topper & Green, 1971; Robinson *et al.*, 1978).

The effects of insulin and of phenazine methosulphate on the tissue content of *PPRibP* and the oxidation of glucose through the PPP, and the effect of insulin on the activity of *PPRibP* synthetase, are shown in Table 3. Both insulin and phenazine methosulphate increased the *PPRibP* content (by 63% and 190% respectively) and also the activity of the PPP (C-1 – C-6), which was significantly raised by both agents. The activity of *PPRibP* synthetase was not changed by incubation with insulin *in vitro*.

DISCUSSION

Changes in *PPRibP* in the lactation cycle

A large number of factors are known to influence the formation of *PPRibP*, including the amount of *PPRibP* synthetase, the supply of the substrates Rib5P and ATP and the presence of activators, particularly P_i, and feedback inhibition of this enzyme by nucleotides (Becker *et al.*, 1979); any or all of these may change in the mammary gland in the lactation cycle. The present experiments provide evidence for the importance of increases in *PPRibP* synthetase in the regulation of the concentration of *PPRibP* in the mammary gland, with both *PPRibP* and *PPRibP* synthetase showing parallel changes throughout the lactation cycle (Table 1). The *K_m* of *PPRibP* synthetase for Rib5P has been reported as in the range 0.03–0.29 mM (Becker *et al.*, 1979); thus the changes in Rib5P found in mammary gland (Table 1) lie within the physiologically significant range. The ATP content of mammary gland in late pregnancy and at the height of lactation is approx. 1.4 mM (Gumaa *et al.*, 1971), and is an order of magnitude greater than the reported *K_m* for *PPRibP* synthetase (Becker *et al.*, 1979). The ATP concentration would thus not appear to be a significant regulatory factor at these periods of the

lactation cycle. However, the massive decrease in ATP during weaning, to only 10% of the lactating-mammary-gland value, a change which occurs as early as 16 h of weaning (Gumaa *et al.*, 1971), suggests that ATP concentration might become a limiting factor in *PPRibP* synthesis during mammary involution. Atkinson & Fall (1967) and Fox & Kelley (1972) have drawn attention to the role of the 'energy charge' in the regulation of *PPRibP* synthetase in *Escherichia coli* and erythrocytes; this parameter could also be important in the control of *PPRibP* synthesis in the mammary gland.

Both P_i and bivalent cations are absolute requirements for *PPRibP* synthetase activity, P_i having a stimulatory effect over a broad range of P_i concentrations (Becker *et al.*, 1979; Raivio *et al.*, 1981b). Measurements of P_i and bivalent cations in the mammary gland during the lactation cycle are difficult because of the high content of these components in milk and, to a lesser extent, in colostrum. Thus the effects of these activators *in vivo* have not been assessed in the present experiments.

Both the oxidative and non-oxidative segments of the PPP can contribute to the formation of Rib5P. The present results suggest that the oxidative segment of the PPP plays an increasing role in Rib5P formation as lactation progresses, the C-1 – C-6 value increasing more than 20-fold from late pregnancy to the height of lactation. The conversion of ribulose 5-phosphate into Rib5P may be a limiting factor in *PPRibP* formation; however, the activity of Rib5P isomerase is increased 4-fold in the period late-pregnancy to late-lactation and is comparable in activity with 6-phosphogluconate dehydrogenase (Novello & McLean, 1968; Gumaa *et al.*, 1971, 1973).

The relative low activity of the oxidative PPP during pregnancy, at a time of rapidly increasing *PPRibP* synthetase activity and *PPRibP* and RNA contents, strongly suggest that the non-oxidative PPP, involving transketolase and transaldolase, is the more important source of Rib5P during the growth of the gland (Greenbaum & Slater, 1957; Gumaa *et al.*, 1971, 1973), as it is in ascites cells (Gumaa & McLean, 1969) and in human fibroblast cultures (Raivio *et al.*, 1981a).

Regulation of *PPRibP* concentration by insulin

Pilz *et al.* (1984) have provided convincing evidence that the intracellular concentration of *PPRibP* is an important determinant of the rate of purine synthesis *de novo* and have shown that the *PPRibP* concentration rises in parallel with the glucose concentration in the medium and with cellular Rib5P concentrations. The present experiments *in vivo*, in which insulin treatment of streptozotocin-diabetic rats resulted in the elevation of the *PPRibP* content to normal values and normalization of the rate of glucose oxidation via the PPP, with partial restoration of *PPRibP* synthetase, point to the supply of Rib5P via the PPP, rather than the amount of synthetase protein, as the critical factor in restoring *PPRibP* under these conditions. This view is further strengthened by the effects of insulin on mammary-gland slices *in vitro* (Table 3). Here the increase in *PPRibP* is accompanied by a rise in glucose oxidation via the PPP and occurs without any stable change in the amount of *PPRibP* synthetase. These results do not exclude changes in *PPRibP* synthetase activity brought about by low- M_r effector molecules or as a result of reversible phosphorylation/dephosphorylation.

The view, presented above, that links the enhanced *PPRibP* and lactational performance to the presence of insulin is not at odds with the observation by Flint *et al.* (1979) that the serum insulin decreases in lactation. The data given by Flint *et al.* (1979) show that, although the concentration of insulin falls from its highest point at the time of parturition, the value at the end of lactation approximates to that found in the virgin animal, and is presumably sufficient to sustain adequate PPP flux and increased lactation. There is the additional possibility that the increased concentration of prolactin, which rises throughout lactation, may be involved in the regulation of the tissue content of *PPRibP*, acting either by itself or in conjunction with insulin. Indeed, the changes in rat serum prolactin reported by Flint *et al.* (1979) more closely parallel the changes in the tissue *PPRibP* than does the serum insulin and, further, Robinson *et al.* (1978) have shown a 7-fold decrease in plasma prolactin 2 h after the administration of streptozotocin. These results emphasize the desirability of a study of the relationship between prolactin, insulin and *PPRibP* content of mammary gland.

Regulation of *PPRibP* concentration by phenazine methosulphate

The increase in the *PPRibP* concentration of lactating-mammary-gland slices incubated with phenazine methosulphate indicates that the supply of Rib5P may be one of the rate-limiting factors in *PPRibP* formation. The present experiments parallel those of Buchanan *et al.* (1982), who observed that compounds utilizing NADPH, e.g. proline and ascorbic acid, stimulated *PPRibP* formation in cultured 3T6 cells. It is suggested that in lactating mammary gland, one of the factors co-ordinating the regulation of a number of biosynthetic pathways is by regulation centering on the $NADP^+/NADPH$ couple. Insulin, by increasing glucose uptake and promoting lipogenesis, shifts the redox state of $NADP^+/NADPH$ so that there is an increased flux through the PPP. This leads to increased bioavailability of Rib5P and synthesis of *PPRibP*, which in turn would lead to a rise in nucleotide and nucleic acid synthesis, the basis for protein formation.

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REFERENCES

- Atkinson, D. E. & Fall, L. (1967) *J. Biol. Chem.* **242**, 3241–3242
- Becker, M. A. (1976) *J. Clin. Invest.* **57**, 308–318
- Becker, M. A., Raivio, K. O. & Seegmiller, J. E. (1979) *Adv. Enzymol.* **49**, 281–306
- Bergmeyer, H.-U. (1974) *Methods in Enzymatic Analysis*, 2nd edn., Academic Press, New York
- Brody, S. & Nisbet, R. (1938) *Res. Bull. Mo. Agric. Exp. Stn.* **285**
- Buchanan, J. M., Smith, M. L. & Smith, R. J. (1982) *Adv. Enzyme Regul.* **20**, 135–152
- Flint, D. J., Sinnott-Smith, P. A., Clegg, R. A. & Vernon, R. G. (1979) *Biochem. J.* **182**, 421–427
- Fox, I. H. & Kelley, W. N. (1972) *J. Biol. Chem.* **247**, 2126–2131

- Greenbaum, A. L. & Slater, T. F. (1957) *Biochem. J.* **66**, 155–161
- Gumaa, K. A. & McLean, P. (1969) *Biochem. Biophys. Res. Commun.* **35**, 86–93
- Gumaa, K. A., Greenbaum, A. L. & McLean, P. (1971) in *Lactation* (Falconer, I. R., ed.), pp. 197–238, Butterworths, London
- Gumaa, K. A., Greenbaum, A. L. & McLean, P. (1973) *Eur. J. Biochem.* **34**, 188–198
- Johke, T. (1978) in *Lactation IV* (Larson, B. L., ed.), pp. 513–522, Academic Press, New York and London
- Kuhn, N. J. (1983) in *Biochemistry of Lactation* (Mepharm, T. B., ed.), pp. 351–379, Elsevier, Amsterdam and New York
- Lipstein, B., Boer, P. & Sperling, O. (1978) *Biochim. Biophys. Acta* **521**, 45–54
- McKeran, R. O. & Watts, R. W. E. (1978) in *Recent Advances in Endocrinology and Metabolism* (O'Riordan, J. L. H., ed.), pp. 219–252, Churchill Livingstone, London
- McLean, P. (1958) *Biochim. Biophys. Acta* **30**, 316–324
- Mercier, J. C. & Gaye, P. (1983) in *Biochemistry of Lactation* (Mepharm, T. B., ed.), pp. 177–227, Elsevier, Amsterdam and New York
- Novello, F. & McLean, P. (1968) *Biochem. J.* **107**, 775–791
- Pilz, R. B., Willis, R. C. & Boss, G. R. (1984) *J. Biol. Chem.* **259**, 2927–2935
- Raivio, K. A., Lazar, C. S., Krumholz, H. & Becker, M. A. (1981a) *Biochim. Biophys. Acta* **678**, 51–57
- Raivio, K. A., Lazar, C. S. & Becker, M. A. (1981b) *Biochim. Biophys. Acta* **678**, 58–64
- Reem, G. & Friend, C. (1976) *J. Cell. Physiol.* **88**, 193–196
- Rerup, C. C. (1970) *Pharmacol. Rev.* **22**, 485–518
- Robinson, A. M. & Williamson, D. H. (1977) *Biochem. J.* **164**, 153–159
- Robinson, A. M., Girard, J. R. & Williamson, D. H. (1978) *Biochem. J.* **176**, 343–346
- Roth, R. G., White, C. & Deuel, T. F. (1978) *Methods Enzymol.* **51**, 12–17
- Tatibana, M., Kita, K. & Ikeda, F. (1982) *Eur. J. Biochem.* **128**, 631–636
- Topper, Y. J. (1970) *Recent Prog. Horm. Res.* **26**, 287–302
- Topper, Y. J. & Green, M. R. (1971) in *Lactation* (Falconer, I. R., ed.), pp. 239–248, Butterworths, London
- Walters, E. & McLean, P. (1968) *Biochem. J.* **109**, 407–417
- Wang, D. Y. & Greenbaum, A. L. (1960) *Biochem. J.* **77**, 552–558

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