Rosevear, J. W. & Smith, E. L. (1958). *J. Amer. chem. Soc.* **80**, 250.
Sanger, F. (1949). *Biochem. J.* **45**, 563.
Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 353.
Schwimmer, S. & Bevenue, A. (1956). *Science*, **123**, 543.
Steven, F. S. & Tristram, G. R. (1958). *Biochem. J.* **70**, 179.
Tracey, M. V. (1952). *Biochem. J.* **52**, 265.
Warner, R. C. (1954). In *The Proteins*, vol. 2A, p. 435. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
White, T. (1940). *J. chem. Soc.* p. 428.
Wieland, T. & Fischer, E. (1948). *Naturwissenschaften*, **35**, 29.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
Yanari, S. (1956). *J. biol. Chem.* **220**, 683.

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Quantitative Studies on Mammary-Gland Enzymes Involved in Lactose Synthesis

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The steps by which lactose is synthesized from glucose in the mammary gland have been tentatively elucidated, and although for some of the stages the evidence is merely the demonstration of a theoretically suitable enzyme in the tissue, a scheme of synthesis has been proposed which probably accounts for about 70% of the lactose formed under normal conditions. The participation of alternative pathways responsible for the remaining 30% of synthesis has been indicated by work in which the glucose and galactose moieties of lactose formed from [1-^{14}C]glucose or from labelled acetate showed a differential labelling; this could not have occurred if all the synthesis had followed the proposed major route. These problems of the biosynthesis of lactose have been discussed elsewhere (Malpress, 1958).

It seemed possible that some confirmation of the mechanism of synthesis might be obtained by studying the variation in the activities of the postulated lactose-synthesizing enzymes in glands removed at different stages of lactation. In common with other enzymes involved in the synthesis of milk constituents (Moore & Nelson, 1952; Greenbaum & Greenwood, 1954) their activities might be expected to vary directly with the amount of milk produced, and in particular to increase for a period after parturition and to fall during a period of lactational decline. Hexokinase, which is the first enzyme required in the synthesis of lactose, has already been investigated in this way for the rat (McLean, 1958) and a clear parallelism has been shown between its activity in the gland and the lactational performance of the animal.

This paper reports similar studies with mammary-gland homogenates on other enzymes of importance in lactose synthesis: phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase and inorganic pyrophosphatase.

METHODS

Animals. Rats, mainly primiparous and weighing 240–280 g., were killed at 0, 6 and 15 days after parturition; a further group was weaned from the litters 6 days after parturition and killed on the eighth day. Values for guinea-pig glands were obtained for 6- and 15-day intervals after parturition.

After slaughter, the abdominal mammary glands were dissected, weighed and homogenized in a Nelco homogenizer for 2 min. with 10 vol. of 0·1M-2-amino-2-hydroxymethyl-propane-1:3-diol (tris)–HCl buffer, pH 7·4. This homogenate was strained through muslin and the unhomogenized residue was blotted on filter paper and weighed. The amount of tissue homogenized under these conditions varied with the lactation status of the animal, approaching 100% for individual guinea-pig glands in full lactation and falling to a mean value of 63% for rat glands during involution. Inspection of the unhomogenized residues showed that they consisted almost entirely of non-secretory tissue.

Lactose in mammary-gland homogenates and correction for 'milk error'. Lactose was determined by the method of Malpress & Morrison (1949) on samples of the homogenate deproteinized with 0·3N-$Ba(OH)_2$ and 5% $ZnSO_4$. The lactose contents of rat and guinea-pig milks were derived from the equations of the regression lines of lactose on deoxyribonucleic acid phosphorus (DNA-P) for a series of glands at each lactation interval studied, as suggested by Greenbaum & Slater (1957*a*). True tissue weights, free from 'milk error', were then calculated in the way outlined by these authors and form the basis on which all results in this paper are expressed.

In rats killed on the eighth day after preliminary weaning, lactose values were frequently zero and no statistical value for lactose in the milk of such animals could be

obtained. These zero values were not always associated with a negligible retained fluid volume, however, and it seemed that an uncharacteristic lactose-free milk was present in some of the glands, for which lactose content was therefore a useless parameter for calculating retained fluid volumes and true tissue weights. For these animals true tissue weights were calculated instead from the mean DNA-P value of 14·3 mg./100 g. of mammary tissue obtained from a series of glands in the group which showed no sign of retained fluid. The formula used was:

$$\text{True wt. of tissue} = \text{observed wt.} \times \frac{\text{observed DNA-P}}{14{\cdot}3}\ (\text{mg./100 g. of tissue}).$$

Deoxyribonucleic acid extraction. DNA was extracted from the homogenates by the modification of the procedure of Schneider (1945), used by Greenbaum & Slater (1957*b*) for mammary-tissue suspensions, and estimated by the diphenylamine method (Dische, 1955) with a 3-min. heating time. After keeping the extract for 2 hr. in the dark at room temperature, readings were made at 595 mμ on a Unicam SP. 600 spectrophotometer.

Measurement of phosphoglucomutase activity. A solution (0·6 ml.) containing 6 mM-glucose 1-phosphate (dipotassium salt) and 2 mM-$MgSO_4$ in 0·1M-tris buffer, pH 7·4, was incubated with 0·4 ml. of the mammary-gland homogenate at 30° for 10 min. The enzyme action was stopped by adding 1 ml. of 10% trichloroacetic acid. Similar tubes, unincubated, in which the addition of the trichloroacetic acid preceded the addition of the homogenate, gave initial control values. The conversion of labile phosphate proceeded at a steady rate for at least 15 min. under these conditions and was unaffected by the addition of cysteine. The precipitated protein was centrifuged and washed twice with 1 ml. of 5% trichloroacetic acid, the original supernatant and the washings being combined and diluted to 10 ml. Labile phosphate was estimated on samples of these solutions by the method of Fiske & Subbarow (1925) after hydrolysis with N-H_2SO_4 for 10 min. at 100°.

Measurement of inorganic-pyrophosphatase activity. A solution (0·2 ml.) containing 5 mM-$Na_4P_2O_7$ in 0·1M-tris buffer adjusted to pH 7·4 was incubated for 5 min. at 30° with 0·8 ml. of the mammary-gland homogenate. Activity was stopped by the addition of 1 ml. of 10% trichloroacetic acid. Control values were obtained from similar, but unincubated, tubes in which the addition of trichloroacetic acid preceded the addition of the homogenate. The precipitated protein was washed with 2 ml. of 2·5% trichloroacetic acid, and pyrophosphate was estimated in the combined supernatants and washings after selective precipitation as manganese pyrophosphate. The rate of enzyme activity decreased after the first 5 min. and set a limit to the useful incubation period.

Uridine diphosphate glucose-pyrophosphorylase activity. This was assessed by measuring the inorganic pyrophosphate produced in 0·2 ml. of a solution containing 0·05M-glucose 1-phosphate and 5 mM-uridine triphosphate (sodium salt, Nutritional Biochemicals Corp.) in 0·1M-tris buffer, pH 7·4, incubated for 10 min. at 30° with 0·8 ml. of the mammary-gland homogenate, to 10 ml. of which 1 ml. of 0·01M-ethylenediaminetetra-acetate (EDTA), pH 7·4, had been previously added. The addition of EDTA decreased the inorganic-pyrophosphatase activity of the homogenates to approx. 20% of non-inhibited values. It has been shown by Munch-Petersen (1955) that EDTA has no effect on the rate of uridine diphosphate glucose (UDPG)-pyrophosphorylase activity, which, under the conditions used in these experiments, was constant for 20 min. Activity was stopped by the addition of 1 ml. of 10% trichloroacetic acid, control values being obtained from similar, unincubated solutions to which trichloroacetic acid was added before the addition of the homogenate. No pyrophosphate was formed in incubates containing either 0·05M-glucose 1-phosphate or 5 mM-uridine triphosphate as the sole substrate. The precipitated protein was washed with 1 ml. of 5% trichloroacetic acid, and 1 ml. of a standard sodium pyrophosphate solution, containing 31 μg. of P/ml., was added to the combined supernatant and washing before pyrophosphate was estimated by selective precipitation as manganese pyrophosphate. This addition ensured that pyrophosphate values would fall within the 90% recovery range (see next section).

Inorganic pyrophosphate. This was estimated in the deproteinized solutions by a modification of the method of Lehninger & Smith (1949) closely resembling that used by Kenney, Colowick & Barbehenn (1957). The precipitation of the pyrophosphate was, however, made from an initial volume of 4 ml., by the addition of 0·6 ml. of 2M-sodium acetate, 0·5 ml. of 10% $MnCl_2$ and 0·7 ml. of acetone; the final hydrolysis was carried out by heating in N-H_2SO_4 for 30 min. at 100°. Recovery of standard pyrophosphate was 90 ± 1% for the range 30–80 μg. of P.

RESULTS

Lactose in retained fluids. Equations for the regression of lactose on DNA-P, the correlation coefficients *r* and the derived values for lactose in

Table 1. *Relationship between lactose and deoxyribonucleic acid phosphorus in rat and guinea-pig mammary glands*

Rat	No. of animals	Regression equation of lactose on DNA-P	*r*	DNA-P (mg./100 g. wet wt. of gland)†
Lactating (< 18 hr.)	10	Lactose = 1·22 − 0·078 (DNA-P)	−0·66	15·6
Lactating (6 days)	10	Lactose = 2·27 − 0·113 (DNA-P)	−0·60	20·1
Lactating (15 days)	10	Lactose = 2·30 − 0·105 (DNA-P)	−0·54	21·9
Lactating (6 days) / Involuting (2 days)	15	*	—	14·3*
Guinea pig				
Lactating (6 days)	10	Lactose = 2·77 − 0·095 (DNA-P)	−0·82	29·2
Lactating (15 days)	20	Lactose = 3·13 − 0·109 (DNA-P)	−0·66	28·7

* See Methods section.

† Corrected for 'milk error'.

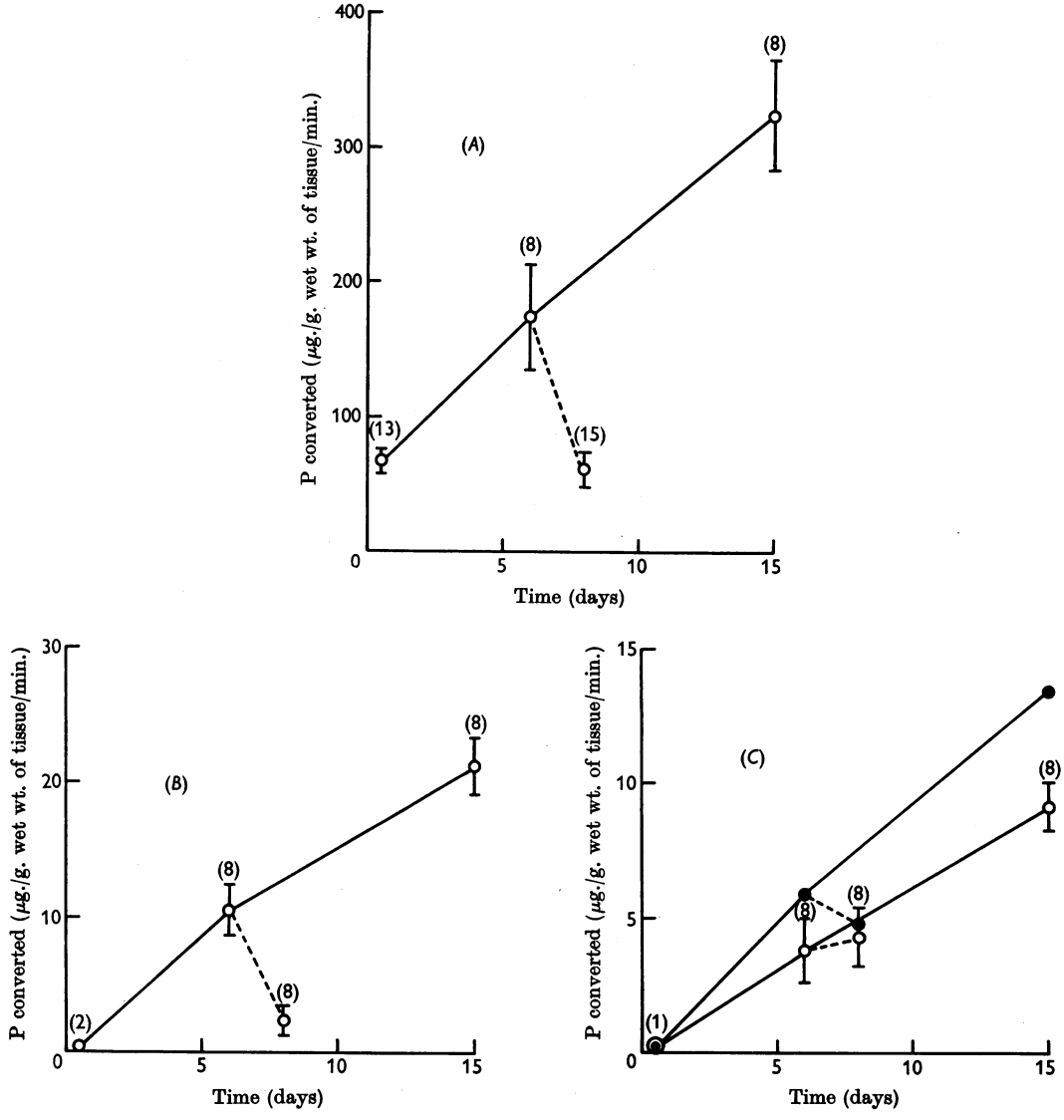

Fig. 1. Activities, calculated for milk-free tissue (see text), of phosphoglucomutase (*A*), inorganic pyrophosphatase (*B*) and UDPG-pyrophosphorylase (*C*) in the rat mammary gland, expressed as mean values ± S.E.M. Figures in parentheses give the number of animals in each group. —, Period of lactation; - - -, period of involution; ●, activities of UDPG-pyrophosphorylase corrected for residual inorganic-pyrophosphatase action.

Table 2. *Enzyme activities in guinea-pig mammary tissue*

Values given are means ± S.E.M. with numbers of animals in parentheses.

	P converted (μg./g. wet wt. of tissue/min.)	
	Lactating (6 days)	Lactating (15 days)
Phosphoglucomutase	145·7 ± 10·0 (8)	81·6 ± 8·1 (20)
Inorganic pyrophosphatase	16 (2)	36 (3)
UDPG-pyrophosphorylase	12* (2)	13* (3)

* Corrected for residual inorganic-pyrophosphatase activity.

rat and guinea-pig retained fluids are given in Table 1. The values for lactose showed close agreement with the results of direct estimations on expressed milk samples. Calculated values are also given for tissue DNA-P.

Enzyme activities. The relations of phosphoglucomutase, inorganic-pyrophosphatase and UDPG-pyrophosphorylase activities to the lactation status of the rat mammary gland are shown in Fig. 1 (*A–C*). In all cases activity is expressed as μg. of P converted/g. wet wt. of tissue/min. The UDPG-pyrophosphorylase values are given both before and after correcting for the residual inorganic-pyrophosphatase activity found in the homogenates after the addition of EDTA and estimated as 20% of the true pyrophosphatase activity. Comparable values for the guinea pig for the sixth and fifteenth days of lactation are given in Table 2.

DISCUSSION

Lactose values for the retained fluids of rat mammary glands found in this study are in satisfactory agreement with the results of Greenbaum & Slater (1957*a*) except in the involuting gland. In this work the glands after 2 days' involution were usually almost entirely free of retained fluid and gave homogenates with a negligible lactose content; in a few animals retained milky fluid was obviously present in the gland but in these cases, too, lactose values were zero or less than 1%. These results are in contrast with the value of 5·11% for lactose in retained fluid of rat glands involuting for 2 days, reported by Greenbaum & Slater (1957*a*). It seems most likely that an initial rise in the lactose content of the retained fluid may follow weaning, owing to continuing metabolic activity, but that this activity slowly ceases and destruction or resorption of lactose subsequently causes a fall in lactose values. On this basis the discrepancy between these two series of results could be explained by a difference in the extent and rate of these processes in the two colonies; in this connexion it may be noted that the mean percentage of retained fluid in the glands of rats used by Greenbaum & Slater after involution was 46 compared with the value approaching zero found in this study.

In the rat the relationships between the activity of the enzymes studied and the lactational status of the glands followed the pattern already found for hexokinase (McLean, 1958): a low activity at the start of the lactation, which increases over a period of 10 days with increasing milk production and falls sharply during a period of involution. This direct relationship suggests that all three enzymes are involved in processes of milk formation in the gland.

It is not known whether the preparation of glucose 1-phosphate used in these experiments provided sufficient glucose 1:6-diphosphate to ensure maximal phosphoglucomutase activity. In terms of conversion of phosphorus, however, the relative activities of phosphoglucomutase, inorganic pyrophosphatase and UDPG-pyrophosphorylase in these mammary homogenates were roughly 25:2:1. By the same criterion the phosphoglucomutase activity shown in these experiments was twice as great as the hexokinase activity of mammary tissue found in the experiments of McLean (1958), and about one-tenth of the activity of phosphoglucomutase in rat-liver and rat-muscle homogenates measured in the presence of optimum glucose 1:6-diphosphate concentrations (Bodansky, 1958). Guinea-pig mammary glands showed activities comparable with those of rat tissue for each of the enzymes investigated in this work.

SUMMARY

1. The variations in the activities of phosphoglucomutase, inorganic pyrophosphatase and uridine diphosphate glucose pyrophosphorylase in homogenates of rat mammary glands at different stages of lactation have been studied.

2. In each case the activity varies directly with the milk-secretory activity of the glands. The results support the postulated participation of these enzymes in lactose synthesis.

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REFERENCES

Bodansky, O. (1958). *J. biol. Chem.* **232**, 859.
Dische, Z. (1955). In *The Nucleic Acids*, p. 285. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Greenbaum, A. L. & Greenwood, F. C. (1954). *Biochem. J.* **56**, 625.
Greenbaum, A. L. & Slater, T. F. (1957*a*). *Biochem. J.* **66**, 148.
Greenbaum, A. L. & Slater, T. F. (1957*b*). *Biochem. J.* **66**, 155.
Kenney, F. T., Colowick, S. P. & Barbehenn, E. (1957). *Arch. Biochem. Biophys.* **69**, 617.
Lehninger, A. L. & Smith, S. W. (1949). *J. biol. Chem.* **181**, 415.
McLean, P. (1958). *Biochim. biophys. Acta*, **30**, 303.
Malpress, F. H. (1958). *Proc. Roy. Soc.* B, **149**, 362.
Malpress, F. H. & Morrison, A. B. (1949). *Biochem. J.* **45**, 455.
Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
Munch-Petersen, A. (1955). *Acta chem. scand.* **9**, 1523.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.