Effect of Thyroidectomy on Pathways of Glucose Metabolism in Lactating Rat Mammary Gland

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1. Assessment of the overall metabolic changes in lactating mammary gland after thyroidectomy has been made by measurement of the incorporation of ^{14}C from specifically labelled glucose, pyruvate and acetate into $^{14}\text{CO}_2$ and ^{14}C -labelled lipid in the experimental rats and in sham-operated control animals. 2. Thyroidectomy depressed the oxidation of ^{14}C -labelled substrates, an effect still apparent when the control rats were pair-fed with thyroidectomized rats; however, the ratio of oxidation of $[1-^{14}\text{C}]\text{glucose/oxidation of } [6-^{14}\text{C}]\text{glucose}$ was unaltered. In parallel with these studies it was revealed that the activities of hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-linked isocitrate dehydrogenase were all lower in the thyroidectomized group than in the pair-fed control group. 3. Thyroidectomy also lowered the incorporation of ^{14}C -labelled substrates into ^{14}C -labelled lipid, an effect further studied by measurement of the activities of citrate-cleavage enzyme and acetate thiokinase. Restricting the food intake of the control rats to that of the thyroidectomized group lowered the activity of citrate-cleavage enzyme, but no further depression was observed on thyroidectomy. The oxidized and reduced nicotinamide nucleotide content of mammary tissue was shown to be decreased in the thyroidectomized rats compared with the control rats.

It is well established that the biosynthetic processes of lactation are under hormonal control. There is extensive evidence that pituitary hormones, as well as insulin, thyroxine, parathyroid hormones and adrenal-cortical hormones, are needed in the initiation and maintenance of lactation (see Folley, 1952; Folley & Malpress, 1948; Cowie, 1961). The pentose phosphate pathway of glucose metabolism is very active in lactating rat mammary-gland tissue and there appears to be a close correlation between lactational ability and the extent of operation of this pathway (Glock & McLean, 1953a, 1958; Abraham, Hirsch & Chaikoff, 1954; Peeters, Debackere & Sierens, 1957; McLean, 1958; Abraham & Chaikoff, 1959). Further, in this tissue there appears to be a close integration between the pentose phosphate pathway and the synthesis of lipid, mediated by the supply of NADP⁺ and NADPH (McLean, 1960; see Folley & Greenbaum, 1960). Abraham, Cady & Chaikoff (1960) have studied the effects of hypophysectomy and of certain pituitary hormones and hydrocortisone on the pattern of carbohydrate metabolism in the rat mammary gland. Willmer (1960) and Greenbaum & Darby (1964) have shown that adrenal hormones are of importance in controlling enzymes of the pentose phosphate pathway; the

latter authors have made a detailed study of alternative pathways of glucose metabolism with differentially labelled glucose, pyruvate and acetate and have shown that a block exists in both the pentose phosphate pathway and the Embden-Meyerhof pathway of glucose utilization.

The growth rate of litters has been widely used as an index of lactational performance, and on this basis there is evidence that both the thyroid and parathyroid hormones have a marked effect on lactation (Folley, 1938; Karnovsky, 1942; Cowie & Folley, 1945; Toverud & Munson, 1956; Munson, 1955; Cowie, 1961). However, there has been relatively little work on the action of these hormones on the enzymic pattern of the tissue and on the distribution of glucose among the various pathways of metabolism.

In the present work the overall metabolic changes in the lactating mammary gland after thyroidectomy were assessed by measurement of the incorporation of carbon from specifically labelled glucose, pyruvate and acetate into $^{14}\text{CO}_2$ and ^{14}C -labelled lipid in thyroidectomized lactating rats and in sham-operated control animals. In parallel with these studies measurements were also made of the activities of hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate

dehydrogenase and NADP-linked isocitrate dehydrogenase. The depression in lipid synthesis from ^{14}C -labelled precursors observed in thyroidectomized lactating rats was further studied by measurements of the activities of citrate-cleavage enzyme and acetate thiokinase.

METHODS

Materials. Commercial reagents were used with the exception of 6-phosphogluconate dehydrogenase, which was a partially purified preparation from liver prepared as described by Glock & McLean (1953b) and used in the assay of hexokinase. Glucose 6-phosphate dehydrogenase and NADP⁺ were obtained from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, Germany). Mercaptoethanol was a product of L. Light and Co. Ltd. (Colnbrook, Bucks.).

Animals. Primiparous albino rats of the Wistar strain were used; the litters were reduced to eight pups. The rats were thyroidectomized or submitted to sham operations (for the control groups) on the fourth day of lactation; ether anaesthesia was used. The animals were divided into three groups carefully matched for body weight: 1, thyroidectomized rats, allowed food *ad lib.*; 2, sham-operated control rats, pair-fed with the thyroidectomized rats; 3, sham-operated control rats, allowed food *ad lib.*

There was a considerable reduction in food intake in the thyroidectomized lactating rats compared with the free-fed control group and this posed a serious problem since it is known that restricted food intake causes a depression in milk secretion (see Fig. 1 and Table 1). In an attempt to overcome this problem the two groups of control rats were used.

Since the removal of the thyroid gland in rats also entails the removal of the parathyroids embedded in them, calcium gluconate was given in the drinking water (1%, w/v) to alleviate the effects of parathyroidectomy. The blood calcium concentrations of the thyroidectomized and control rats were 4.07 and 4.10 mg./100 ml. of blood respectively. We are grateful to Mr R. Warren for these estimations.

To check that the thyroid glands had been removed completely, pairs of rats were taken, one thyroidectomized rat and one sham-operated control rat, and on the tenth day after operation a dose of $1\mu\text{C}$ of ^{131}I iodide was given intraperitoneally; 48 hr. later the rats were killed and the neck region was removed and placed in a scintillation counter for measurement of the ^{131}I . The control rats had 3183 ± 434 counts/min. and the thyroidectomized rats 16 ± 6 counts/min.

The litter weights were used as an index of milk secretion; the rate of growth of the litters decreased after thyroidectomy. Typical growth curves are shown in Fig. 1. The average increases in weight of a litter of eight pups in the last 4 days before killing were $13 \pm 2\text{g.}$, $8 \pm 0.9\text{g.}$ and $4 \pm 0.7\text{g.}$ for free-fed controls, pair-fed controls and thyroidectomized rats respectively. Administration of $10\mu\text{g.}$ of thyroxine/day to thyroidectomized rats maintained the growth rate of the litters at control values.

The rats were killed on the eleventh or twelfth day of lactation, 7 or 8 days after thyroidectomy. Only the abdominal mammary glands were used.

Radioactive labelling experiments. Mammary gland slices (250 mg.) were incubated with 4.5 ml. of Krebs-Ringer

Fig. 1. Typical growth curves of litters from rats: thyroidectomized (■); control pair-fed (▲); control fed *ad lib.* (●). Each litter contained eight pups. The mothers were either thyroidectomized or sham-operated upon on the fourth day of lactation.

bicarbonate medium (Umbreit, Burris & Stauffer, 1949) together with one of the following substrate mixtures: A, 100 μmoles of glucose containing $0.4\mu\text{C}$ of $[1-^{14}\text{C}]\text{glucose}$, $[2-^{14}\text{C}]\text{glucose}$ or $[6-^{14}\text{C}]\text{glucose}$; B, 100 μmoles of glucose containing $0.4\mu\text{C}$ of $[1-^{14}\text{C}]\text{glucose}$ and phenazine methosulphate (final concn. 0.1mM); C, 100 μmoles of acetate containing $0.4\mu\text{C}$ of $[1-^{14}\text{C}]\text{acetate}$; D, 100 μmoles of pyruvate containing either $0.2\mu\text{C}$ of $[1-^{14}\text{C}]\text{pyruvate}$ or $0.2\mu\text{C}$ of $[2-^{14}\text{C}]\text{pyruvate}$.

In each case the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). At the end of the 60 min. incubation period at 37° , 1 ml. of 5N-HCl was introduced into the outer compartment and 1 ml. of 5N-NaOH into the centre well by injection through the rubber cap. A further 2 hr. was allowed before the flasks were removed. The $^{14}\text{CO}_2$ was collected as BaCO_3 and plated at infinite thickness on 1 cm.² disks and counted in a Nuclear-Chicago gas-flow counter (counting efficiency 30% for ^{14}C). The total lipid was extracted by the method of Bligh & Dyer (1959) and counted in a Nuclear-Chicago scintillation counter (counting efficiency 85–90% for ^{14}C).

The results are expressed as μmoles of substrate utilized/hr./total gland at 37° .

Preparation of tissue extracts. Portions of mammary gland were homogenized with a Potter homogenizer in 20 vol. of ice-cold medium containing 150 mM-KCl, 5 mM-MgCl₂, 5 mM-EDTA and 10 mM-mercaptoethanol, adjusted to pH 7.4 with KHCO_3 . The homogenate was centrifuged at 13000g for 45 min. at 0° and the supernatant dialysed against the same extracting medium for 1 hr. in the cold.

This preparation was used for the estimation of all the enzymes.

ATP-glucose phosphotransferase activity. The ATP-glucose phosphotransferase activity, henceforth called hexokinase (EC 2.7.1.1) since it has a low K_m for glucose, was estimated essentially according to the method of Sharma, Manjeshwar & Weinhouse (1963), with modifications as described by McLean & Brown (1966). The final glucose concentration used was 1 mM. A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μ mole of glucose 6-phosphate/hr. at 25°.

Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-linked isocitrate dehydrogenase. The activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate-NADP oxidoreductase, EC 1.1.1.44) and the NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell [L-isocitrate-NADP oxidoreductase (decarboxylating), EC 1.1.1.42] were measured by the methods of Glock & McLean (1953b) and Ochoa (1955) as adapted by McLean & Brown (1966). A unit of enzyme is defined as the amount of enzyme catalysing the formation of 1 μ mole of NADPH/hr. at 25°.

The rate of reduction of NADP⁺ was measured in a Unicam SP.800 recording spectrophotometer with a constant-temperature cell-housing and scale-expansion accessory.

Citrate-cleavage enzyme and acetate thiokinase. The activities of citrate-cleavage enzyme [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] and acetate thiokinase [acetate-CoA ligase (AMP), EC 6.2.1.1] were estimated by the colorimetric method described by Kornacker & Lowenstein (1965a). A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μ mole of acetyl-CoA/hr. at 37°.

Nicotinamide nucleotides. The oxidized forms of the nucleotides were extracted in 0.1N-HCl and the reduced forms in 0.1N-NaOH according to the method of Glock & McLean (1955a). The nicotinamide nucleotides were assayed by the method of Greenbaum, Clark & McLean (1965a).

Nucleic acids. DNA and RNA were determined as

described previously (Glock & McLean, 1955b) but with the modified diphenylamine reagent described by Burton (1956) for DNA.

Statistical analysis. The mean values are given together with their s.e.m. values. The statistical analysis of the results is based on the comparison of corresponding pairs (the sham-operated rat pair-fed with the thyroidectomized rat). The differences between these pairs are given together with the values for Fisher's *P*. The differences are considered significant if *P* is no greater than 0.05. Values greater than 0.1 are quoted as not significant.

RESULTS

The difference in food intake between the thyroidectomized and the control rats necessitated the use of two control groups, one fed *ad lib.* and one restricted to the food intake of the thyroidectomized rats. This posed the problem of the best method of expressing the results. The results given in Table 1 show that the total weight of the two abdominal glands was decreased in both the thyroidectomized rats and the pair-fed control group, compared with the control group fed *ad lib.* Measurement of the DNA content of the glands showed that this was virtually unchanged when expressed as DNA content/total weight of the two abdominal mammary glands; in contrast with this, the RNA of the thyroidectomized rats had fallen from 51.4 ± 4.7 to 39.2 ± 3.7 mg./total gland. For this reason all the results have been expressed as activity or units/total gland. This has the advantage of giving a value related to the total capacity of the mammary gland for milk production and, since the DNA remains constant also, a value proportional to the activity or content/cell.

Oxidation of glucose, pyruvate and acetate. The incorporation of ¹⁴C from specifically labelled glucose, pyruvate and acetate into ¹⁴CO₂ by mammary tissue from the three groups of rats is

Table 1. *Nucleic acid content of mammary glands from control and thyroidectomized rats*

Results are given as means $\pm$ s.e.m. For details of nucleic acid estimations see the Methods section. N.S., Not significant.

	Control groups		Thyroidectomized group	Fisher's <i>P</i> (2 versus 3)
	Free-fed	Pair-fed		
Group no.	1	2	3	
No. of animals	6	6	6	
Body wt. (g.)	219 $\pm$ 7	222 $\pm$ 11	215 $\pm$ 12	
Average daily food intake (g.)	81 $\pm$ 5	40 $\pm$ 4	39 $\pm$ 4	
Litter wt. (g.)	179 $\pm$ 5	140 $\pm$ 8	97 $\pm$ 7	0.015
Wt. of mammary glands (g.)	7.70 $\pm$ 0.5	5.87 $\pm$ 0.32	4.58 $\pm$ 0.16	0.046
Nucleic acid content				
DNA P (mg./g. of tissue)	2.58 $\pm$ 0.16	3.07 $\pm$ 0.3	3.47 $\pm$ 0.21	N.S.
RNA P (mg./g. of tissue)	12.1 $\pm$ 1.1	8.42 $\pm$ 0.1	7.57 $\pm$ 0.66	N.S.
DNA P (mg./total gland)	20.1 $\pm$ 1.1	18.5 $\pm$ 0.8	18.2 $\pm$ 1.4	N.S.
RNA P (mg./total gland)	92.8 $\pm$ 8.2	51.4 $\pm$ 4.7	39.2 $\pm$ 3.7	0.067

Table 2. *Effect of thyroidectomy on the oxidation of [^{14}C]glucose, [^{14}C]pyruvate and [^{14}C]acetate by lactating rat mammary gland slices*

Mammary gland slices (250 mg.) were incubated for 1 hr. at 37° in 4.5 ml. of Krebs-Ringer bicarbonate solution containing: A, 100 μmoles of glucose with 0.4 μC of [^{14}C]glucose, [^{14}C]glucose or [^{14}C]glucose; B, 100 μmoles of glucose with 0.4 μC of [^{14}C]glucose and phenazine methosulphate (final concn. 0.1 mM); C, 100 μmoles of pyruvate with 0.2 μC of [^{14}C]pyruvate or [^{14}C]pyruvate; D, 100 μmoles of acetate with 0.4 μC of [^{14}C]acetate. Values are given as means $\pm$ S.E.M. Fisher's *P* values are given for the comparison of corresponding pairs of the thyroidectomized rats with the pair-fed control animals.

	Incorporation of ^{14}C from ^{14}C -labelled substrates into $^{14}\text{CO}_2$ ($\mu\text{moles/hr./total gland}$)			Fisher's <i>P</i> (values for corresponding pairs, 2 versus 3)	
	Control groups		Thyroid- ectomized group		
	Free-fed	Pair-fed			
Group no.	1	2	3		
No. of animals	8	8	8		
Wt. of mammary glands (g.)	7.70 $\pm$ 0.50	5.87 $\pm$ 0.32	4.58 $\pm$ 0.16		
Substrates					
[1- ^{14}C]Glucose	183 $\pm$ 15	165 $\pm$ 23	101 $\pm$ 31	64 $\pm$ 18	0.012
[2- ^{14}C]Glucose	106 $\pm$ 9	138 $\pm$ 21	86 $\pm$ 10	62 $\pm$ 15	0.027
[6- ^{14}C]Glucose	11.7 $\pm$ 1.8	12.2 $\pm$ 1.6	5.8 $\pm$ 0.8	6.4 $\pm$ 0.78	0.001
[1- ^{14}C]Glucose	15.6	13.6	17.4		
[6- ^{14}C]Glucose					
[1- ^{14}C]Glucose + phenazine methosulphate	352 $\pm$ 24	293 $\pm$ 31	189 $\pm$ 26	104 $\pm$ 19	0.005
[1- ^{14}C]Pyruvate	1270 $\pm$ 120	1210 $\pm$ 36	777 $\pm$ 57	433 $\pm$ 61	0.001
[2- ^{14}C]Pyruvate	77 $\pm$ 7	52 $\pm$ 5.5	31 $\pm$ 4	22 $\pm$ 6	0.025
[1- ^{14}C]Acetate	33 $\pm$ 3.5	29 $\pm$ 4	20 $\pm$ 3	9.6 $\pm$ 2.5	0.011

shown in Table 2. The incorporation of ^{14}C -labelled substrates into $^{14}\text{CO}_2$ was in each case significantly less in mammary tissue from thyroidectomized rats than in the tissue from the pair-fed controls, when comparison was made between corresponding pairs of rats. This method of calculation was necessary because of the differences in food intake and the correspondingly large variation in the pair-fed control group of animals.

The effect of phenazine methosulphate on the rate of formation of $^{14}\text{CO}_2$ from [^{14}C]glucose is one means of measuring how far the pentose phosphate pathway is limited by hydrogen-acceptor systems such as lipid synthesis. Phenazine methosulphate caused an almost identical percentage increase in the three groups of animals; in each case the rate of $^{14}\text{CO}_2$ formation was almost doubled. This raised the value in the thyroidectomized rats to the basal control values but not to the value found in control tissues in the presence of the artificial electron acceptor.

In addition to the block in pathways of glucose metabolism there was a decreased rate of conversion of pyruvate into acetate as shown by the rate of $^{14}\text{CO}_2$ formation from [^{14}C]pyruvate, and evidence of a decreased rate of oxidation of C_2 units by way of the tricarboxylic acid cycle.

The activities of mammary gland hexokinase and

of the two dehydrogenases of the pentose phosphate cycle are shown in Fig. 2. The activities of these three enzymes in thyroidectomized rats are significantly lower than the corresponding pair-fed control values. The dependence of the enzyme activities on food intake is illustrated by the marked difference between lactating rats receiving food *ad lib.* and those limited to the food intake of the thyroidectomized groups (see Figs. 2 and 3). Indeed, with citrate-cleavage enzyme the fall in activity in the thyroidectomized rats can be almost completely ascribed to the lowered food intake. In contrast with these results, the activity of cytoplasmic NADP-linked isocitrate dehydrogenase did not change markedly in the pair-fed control group but there is a significant fall in the thyroidectomized group.

Measurements of the concentrations of the oxidized and reduced nicotinamide nucleotides in the control rats fed *ad lib.* and the thyroidectomized animals showed that there was a striking fall in NADPH; the NADP $^+$ values were too low to be measured. The NAD $^+$ and NADH concentrations were also lower in the thyroidectomized group; the NAD $^+$ /NADH quotient rose from 2.7 for the control group to 5.6 for the thyroidectomized group, showing the more marked loss of the reduced form of the coenzyme. The lower concentration of the

Fig. 2. Activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and hexokinase in mammary gland tissue from control rats fed *ad lib.* (□), control rats pair-fed (▨) and thyroidectomized rats (■). Mean values of eight rats are given $\pm$ s.e.m. For details of estimation of each enzyme see the Methods section. Fisher's *P* values for the thyroidectomized rats versus pair-fed controls are given below the Figure.

Fig. 3. Activities of citrate-cleavage enzyme, acetate thiokinase and NADP-linked isocitrate dehydrogenase in mammary gland tissue from controls fed *ad lib.* (□), controls pair-fed (▨) and thyroidectomized rats (■). Mean values of eight rats are given $\pm$ s.e.m. For details of estimation of each enzyme see the Methods section. Fisher's *P* values for the thyroidectomized rats versus pair-fed controls are given below the Figure; N.S., not significant.

reduced form of the nicotinamide nucleotides may be related to the decreased activity of the dehydrogenases of the pentose phosphate cycle and to the depressed rate of fatty acid synthesis. Preliminary experiments, in which thyroxine (10 μ g./day) was administered to thyroidectomized rats for 5 days before the end of the experiment, have shown that the growth rate of the litters returns to control values, and that oxidation of labelled substrates and enzyme and nucleotide concentrations approach control values.

Lipid synthesis from glucose, pyruvate and acetate. The pattern of change with regard to the incorporation of the labelled substrates into lipids is in accord with the changes seen in the oxidation of these substrates. The most marked difference

between the free-fed and pair-fed control groups is the fall in the incorporation of [$1-^{14}$ C]glucose, [$6-^{14}$ C]glucose and [$2-^{14}$ C]pyruvate into lipid (Table 3). The decreased citrate-cleavage enzyme activity in control rats on restricted food intake may be of importance in this depressed rate of lipid synthesis (Fig. 3). The incorporation of [$1-^{14}$ C]acetate into lipid is unchanged and here again there is agreement between this finding and the observation that acetate thiokinase is not altered by the paired feeding regime.

Comparison of the thyroidectomized rats with the corresponding pair-fed control group showed that there was a highly significant fall in the incorporation of 14 C from all three labelled substrates into lipid. Neither citrate-cleavage enzyme

Table 3. *Effect of thyroidectomy on the incorporation of ^{14}C from $[^{14}\text{C}]\text{glucose}$, $[^{14}\text{C}]\text{pyruvate}$ and $[^{14}\text{C}]\text{acetate}$ into total lipids by mammary gland slices*

Mammary gland slices (250 mg.) were incubated for 1 hr. at 37° in 4.5 ml. of Krebs-Ringer bicarbonate solution containing: A, 100 μmoles of glucose with 0.4 μC of $[1\text{-}^{14}\text{C}]\text{glucose}$, $[2\text{-}^{14}\text{C}]\text{glucose}$ or $[6\text{-}^{14}\text{C}]\text{glucose}$; B, 100 μmoles of pyruvate with 0.2 μC of $[2\text{-}^{14}\text{C}]\text{pyruvate}$; C, 100 μmoles of acetate with 0.4 μC of $[1\text{-}^{14}\text{C}]\text{acetate}$. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). Values are given as means $\pm$ S.E.M. Fisher's *P* values are given for the comparison of the thyroidectomized rats with the pair-fed control animals.

	Incorporation of ^{14}C from ^{14}C -labelled substrates into ^{14}C -labelled lipid ($\mu\text{moles/hr.}/\text{total gland}$)			Fisher's <i>P</i> (values for corresponding pairs, 2 versus 3)
	Control groups		Thyroid- ectomized group	
	Free-fed	Pair-fed		
Group no.	1	2	3	
No. of animals	8	8	8	
Wt. of mammary glands (g.)	7.70 $\pm$ 0.50	5.87 $\pm$ 0.32	4.58 $\pm$ 0.16	
Substrates				
[1- ^{14}C]Glucose	230 $\pm$ 19	184 $\pm$ 18	102 $\pm$ 12	82 $\pm$ 17 0.007
[2- ^{14}C]Glucose	404 $\pm$ 45	420 $\pm$ 49	190 $\pm$ 16	230 $\pm$ 45 0.002
[6- ^{14}C]Glucose	557 $\pm$ 61	380 $\pm$ 38	218 $\pm$ 18	162 $\pm$ 37 0.005
$\left(\frac{[1\text{-}^{14}\text{C}]\text{Glucose}}{[6\text{-}^{14}\text{C}]\text{Glucose}}\right) \times 100\%*$	41	48	47	
[2- ^{14}C]Pyruvate	114 $\pm$ 9	57 $\pm$ 6	32 $\pm$ 6	25 $\pm$ 5 0.006
[1- ^{14}C]Acetate	12.0 $\pm$ 1.6	13.8 $\pm$ 2.2	7.5 $\pm$ 0.8	6.3 $\pm$ 1.8 0.013

* This quotient given an approximate estimate of the percentage of ^{14}C -labelled lipid derived from glucose via the glycolytic route (for discussion of this quotient see the text).

nor acetate thiokinase activity showed any decrease below the pair-fed control value in the thyroidectomized rats, so that the block in lipid synthesis from glucose or pyruvate could be located at the level of formation of acetyl-CoA from pyruvate. Evidence for this comes from the finding that the amount of decarboxylation of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ is decreased in thyroidectomized rats. The amount of incorporation of $[1\text{-}^{14}\text{C}]\text{acetate}$ into lipid is also lower in thyroidectomized rats, suggesting perhaps that stages in lipid synthesis after the formation of acetyl-CoA may be depressed.

The quotient (^{14}C -labelled lipid from $[1\text{-}^{14}\text{C}]\text{glucose}/^{14}\text{C}$ -labelled lipid from $[6\text{-}^{14}\text{C}]\text{glucose}) \times 100$ has been used to estimate the contribution of the pentose phosphate pathway and glycolytic route of carbohydrate metabolism to lipid synthesis in mammary gland (Abraham & Chaikoff, 1959). Similar calculations made in the present work show that there is almost no difference in the value of this quotient between the thyroidectomized rats and controls (Table 3).

DISCUSSION

Pattern of glucose metabolism in tissue slices. While it is not possible to make a quantitative assessment of the activity of the pentose phosphate pathway relative to the glycolytic route from the

quotient ($^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{glucose}/^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]\text{glucose})$ in view of the cogent criticisms of Katz & Wood (1960), Katz (1961) and Wood, Katz & Landau (1963), this quotient has been shown nevertheless to parallel changes in the glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities and to undergo a striking increase during lactation (Abraham *et al.* 1954; Glock, McLean & Whitehead, 1956; McLean, 1958). Most of these studies have shown that the quotient increases from a value of 1 during pregnancy to approximately 16 at the height of lactation. The present finding that this quotient does not differ in lactating mammary glands from thyroidectomized rats compared with controls, despite the marked fall in glucose oxidation, supports the view that the relative proportions of glucose metabolized by the two alternative routes is unchanged.

Additional evidence in favour of this view comes from the examination of the relative amounts of glucose carbon atoms incorporated into lipid from the $[1\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]\text{glucose}$. It has been proposed that the quotient $100 \times (^{14}\text{C}\text{-labelled lipid from } [1\text{-}^{14}\text{C}]\text{glucose})/(^{14}\text{C}\text{-labelled lipid from } [6\text{-}^{14}\text{C}]\text{glucose})$ represents the proportion of lipid arising via the glycolytic route, since C-1 of glucose is lost in the first oxidative steps of the pentose phosphate cycle and a correlation has been found

between this quotient and the extent of the operation of the pentose phosphate pathway at different stages of the lactation cycle (Abraham *et al.* 1954; Abraham, Cady & Chaikoff, 1957, 1960; Abraham & Chaikoff, 1959). In the present experiments the proportion of lipid arising from the two pathways remained unchanged (see Table 3). Thus on the basis of these two criteria it would seem that there was little change in the distribution of glucose between these two pathways of metabolism in thyroidectomized rats.

Abraham *et al.* (1960) have studied the distribution of glucose between the pathways of metabolism in hypophysectomized rats and hypophysectomized rats in which lactation was hormonally induced. In the hypophysectomized rats almost all the ^{14}C for fatty acid synthesis was derived from the glycolytic pathway and the quotient ($^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{glucose}$)/($^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]\text{glucose}$) was close to unity. These authors found that lactation could be induced by injections of prolactin and Δ^1 -hydrocortin acetate with a resultant lactational performance of 50–60% of normal milk yield, and that here half or more of the glucose-derived fatty acids arose via the pentose phosphate pathway. Treatment with growth hormone caused no additional effect on the metabolic pattern of the lactating gland. Since full lactational performance was not reached with the replacement hormones used in this study, it is possible, in view of the observed effects of thyroidectomy on milk yield, that thyrotrophic hormone or thyroxine would have caused more complete restoration of the milk yield.

Although there is a certain similarity in the effects of hypophysectomy and thyroidectomy on mammary gland metabolism, the removal of the adrenal glands produces a different pattern of metabolism (Greenbaum & Darby, 1964). These authors found clear evidence of a differential effect of the treatment on the relative activities of the two alternative pathways of glucose metabolism, the activity of the pentose phosphate pathway showing the more marked fall.

Hexokinase and enzymes of the pentose phosphate pathway. The marked fall in hexokinase activity of mammary gland extracts could, by substrate limitation, have the effect of lowering the rate of operation of both pathways of glucose metabolism in thyroidectomized rats. Hormonal control of hexokinase activity has been shown in hypophysectomized rats, where it was found that adipose tissue, adrenal gland and testis all had a much lower activity than in the control animals (Brown, McLean & Greenbaum, 1966). Adipose-tissue hexokinase activity could be restored by small physiological doses of thyroxine, whereas the hexokinase activities of testis and of adrenal gland of hypophysectomized rats was increased by lutein-

izing hormone and adrenocorticotrophic hormone but not by thyroxine alone (Brown & McLean, 1965; Brown *et al.* 1966). Thus there is a certain similarity in the response of adipose tissue and mammary gland to thyroxine, a similarity also seen in the response of these two tissues to insulin *in vitro*.

Both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are lower in the mammary glands of thyroidectomized rats compared with the pair-fed control animals. Similar effects of lack of thyroid hormone have been reported for liver (Glock & McLean, 1955b; Huggin & Yao, 1959). It is not at present clear if this is a direct hormonal effect or if it is secondary to the fall in hexokinase that could result in a decrease in the rate of formation of the substrate glucose 6-phosphate. A correlation has been found between hexokinase activity and glucose 6-phosphate dehydrogenase activity in diabetes (Brown & McLean, 1967) and has been suggested in the increased glucose 6-phosphate dehydrogenase activity of tumours (McLean & Brown, 1966).

Lipid synthesis. There is considerable evidence that lack of thyroxine leads to a depression in lipid synthesis (see Masoro, 1962; Hoch, 1962), and it has been shown that thyroxine will restore lipogenesis to normal in both hypophysectomized rats and thyroidectomized animals (Bates, Zomzely & Mayer, 1955; Dayton, Dayton, Drimmer & Kendall, 1960; Fain & Wilhelmi, 1962). The fall in amount of lipid synthesis in mammary gland was seen when either $[^{14}\text{C}]\text{glucose}$ or $[2\text{-}^{14}\text{C}]\text{pyruvate}$ was used as substrate. There was evidence for a block in the formation of acetyl-CoA from pyruvate, since the rate of decarboxylation of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ was slower in the thyroidectomized animals. The conversion of pyruvate into acetyl-CoA has been shown to be inhibited by acetyl-CoA (Garland & Randle, 1964) and it is possible that this product inhibition is of importance in control in the mammary gland, particularly since rates of some reactions removing acetyl-CoA are decreased, namely, the incorporation into lipid and the oxidation by the Krebs cycle. In addition to the inhibition of total lipid synthesis there is also a difference in the pattern of fatty acid synthesized; thyroidectomy decreases the incorporation of $[6\text{-}^{14}\text{C}]\text{glucose}$ into short-chain fatty acids and increases the incorporation into longer-chain acids (Greenbaum, Walters & McLean, 1967).

Citrate-cleavage enzyme. An extramitochondrial pathway of citrate metabolism has been described in both liver and mammary gland (D'Adamo & Haft, 1962, 1965; Madsen, Abraham & Chaikoff, 1964) in which citrate-cleavage enzyme plays a key role. The importance of citrate-cleavage enzyme as a control point in fatty acid synthesis has been

Table 4. *Oxidized and reduced nicotinamide nucleotide content of mammary tissue from control and thyroidectomized rats*

Results are given as means $\pm$ s.e.m. and Fisher's *P* values are given. NADP⁺ values were too low to be measured. For details of extraction and determination procedures see the Methods section.

	Control group	Thyroidectomized group	Fisher's <i>P</i>
No. of animals	4	4	
Concn. of nicotinamide nucleotides (μ g./total gland)			
NAD ⁺	1690 $\pm$ 127	900 $\pm$ 57	0.011
NADH	650 $\pm$ 83	160 $\pm$ 29	0.012
NAD ⁺ /NADH	2.6	5.6	
NADPH	459 $\pm$ 37	254 $\pm$ 23	0.005

widely discussed (Srere, 1965; Kornacker & Lowenstein, 1965a) and it has been shown that, in many conditions, the activity of this enzyme changes in a manner parallel to that of fatty acid synthesis (Kornacker & Lowenstein, 1965a,b; Abraham, Kopelovich & Chaikoff, 1964). The concurrent alteration in the rate of fatty acid synthesis and citrate-cleavage enzyme activity during the lactation cycle in the rat mammary gland has been demonstrated by Lowenstein, Spencer & Kornacker (1964) and by Howanitz & Levy (1965).

In the present experiments citrate-cleavage enzyme activity decreased markedly in control rats pair-fed with the thyroidectomized group, another example of the change in the activity of this enzyme with food intake (Abraham *et al.* 1964; Inone, Honjo & Takeda, 1966), but no further decrease was found in the thyroidectomized rats. It would seem that in mammary gland this enzyme is not greatly influenced by thyroxine, in contrast with the situation in liver and adipose tissue (Brown & McLean, 1965; Brown *et al.* 1966).

It has been shown by Madsen *et al.* (1964) with [2,5-¹⁴C₂]glutamate that, of the total amount of glutamate metabolized by the Krebs cycle in the presence of glucose, 20–30% proceeded by the so-called backward reaction, that is a pathway via α -oxoglutarate $\rightarrow$ isocitrate $\rightarrow$ *cis*-aconitate $\rightarrow$ citrate $\rightarrow$ acetyl-CoA + oxaloacetate. The NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell is involved in this route, and the activity of this enzyme is substantially decreased in the thyroidectomized rats, another factor that may be related to the decreased lipid synthesis.

Nicotinamide nucleotides. The pentose phosphate pathway and lipid synthesis are linked by the oxidation–reduction state of NADP. The former is limited by the supply of NADP⁺ and a supply of NADPH is essential for lipid synthesis.

In the present experiments the addition of an artificial electron acceptor, phenazine metho-

sulphate, caused a substantial rise, almost twofold, in the amount of formation of ¹⁴CO₂ from [1-¹⁴C]-glucose, revealing the extra capacity of the pentose phosphate pathway in both control and thyroidectomized rats. In view of the evidence that NADP is largely in the reduced form in mammary tissue from both control and thyroidectomized rats, and that the rate of oxidation of glucose can be stimulated by artificial electron acceptors, it would seem that lack of NADPH is not the prime factor in decreased lipid synthesis. There is evidence that hydrogen from NADH as well as from NADPH is required in fatty acid synthesis (Katz & Rognstad, 1966; Rognstad & Katz, 1966). Thus the fall in the total NAD⁺ + NADH and the marked increase in the NAD⁺/NADH quotient found in the thyroidectomized rats could be important factors in the control of lipid synthesis (Table 4).

In addition to the changes in the oxidation–reduction state of the nicotinamide nucleotides, there is also a decrease in the total amounts of them present in the tissue of the thyroidectomized rats. In this respect the mammary gland appears to be more sensitive than liver or diaphragm, since treatment with thiouracil had little effect on total NAD or NADP in either the liver or the diaphragm (Glock & McLean, 1955c). The activities of two of the enzymes concerned in the synthesis of NAD and NADP, namely nicotinamide mononucleotide adenylyltransferase and nicotinamide–adenine dinucleotide kinase, appear to be influenced by thyroid hormone; both were increased in liver after a short period of administration of thyroxine (Greenbaum, Clark & McLean, 1965b).

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