Interrelationship of Carbohydrate and Fat Metabolism in the Involuting Mammary Gland

By PATRICIA McLEAN
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Though there have been many studies on the interrelationship of carbohydrate and fat metabolism in lactating rat mammary gland (Folley & McNaught, 1958; Folley & Greenbaum, 1960; Abraham, Hirsch & Chaikoff, 1954; Abraham, Cady & Chaikoff, 1957; Abraham & Chaikoff, 1959; McLean, 1962; Lowenstein, 1961) and it is known that the oxidation of glucose and the incorporation of glucose carbon into lipid falls sharply during mammary involution (Abraham & Chaikoff, 1959; Folley & French, 1949; Balmain & Folley, 1951), little attention has been paid to the early changes and to the sequence of events occurring in the involuting mammary gland.

It is possible to study the metabolic changes taking place in the mammary gland during involution by artificially blocking the ducts of one side of the abdominal mammary glands with collodion and leaving the other free, thus allowing lactation to continue. In this way the glands from the same animal may be used for comparison of glucose utilization in lactation and involution, and many of the difficulties in experiments employing mammary gland, a tissue that may have a very wide range of activity even among a closely matched control group (see Abraham & Chaikoff, 1959), may be eliminated.

By using such a preparation measurements have been made of the utilization of specifically labelled glucose and acetate as well as the activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate–NADP oxidoreductase (decarboxylating), EC 1.1.1.44] in an attempt to determine the sequence of events in the fall in glucose utilization and fat synthesis that occurs with the onset of involution. Particular attention has been paid to the comparison of the rate of [1-^{14}C]glucose oxidation by the tissue slices in the presence and absence of the artificial electron acceptor phenazine methosulphate, since this gives an approximate estimate of the actual and potential rate of the pentose phosphate pathway (McLean, 1960). Comparison of these rates clearly demonstrates the close relationship between the pentose phosphate pathway of glucose oxidation and fatty acid synthesis.

EXPERIMENTAL

Animals. Adult female primiparous rats of approx. 200 g. were used. Litters were in all cases reduced to eight pups. Only the abdominal mammary glands were used. Involution of one abdominal mammary gland was enforced by blocking the teats with collodion applied externally to the body wall. (I am grateful to Dr H. R. Levy, University of Chicago, for information on this method.) Animals were taken at the mid-lactation stage (12–16 days' lactation), and after one side of the abdominal mammary glands had been blocked the rats were returned to their litters. Experimental animals were killed 19, 23, 28, 48 and 72 hr. after treatment for assay of enzyme activities and for preparation of tissue slices used in the study of utilization of specifically labelled glucose and acetate.

Materials. Glucose 6-phosphate, 6-phosphogluconate and NADP were commercial products of Boehringer und Soehne G.m.b.H., Mannheim, Germany. Phenazine methosulphate was a commercial product of Sigma Chemical Co., St Louis, Mo., U.S.A. [1-^{14}C]Glucose, [6-^{14}C]glucose and [1-^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks.

METHODS

Assay of enzymes. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphoglucose isomerase (D-glucose 6-phosphate ketol isomerase, EC 5.3.1.9) were assayed as described by McLean (1958).

Glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities were expressed in terms of μmoles of NADP reduced/hr. at 37°. The temperature coefficient of 1·7 for an increase in temperature of 10° was used to convert values into activities at 37°. Phosphoglucose-isomerase activity was expressed in units which are defined as the quantity of enzyme producing 1 μmole of fructose 6-phosphate/hr. at 37°. These units were chosen so that direct comparison could be made between the activities of these enzymes and the rate of utilization of specifically labelled glucose which is expressed in terms of μmoles of [^{14}C]glucose utilized/hr. at 37°.

Since the involuting mammary gland contains a large amount of retained milk the weight of the involuting gland removed at 19, 23 and 28 hr. after the induction of involution is considerably greater than the contralateral control gland where lactation and suckling have continued normally (see Table 1). For this reason the results are expressed in terms of substrate utilized/hr./total abdominal mammary glands of one side.

Isotope experiments. Mammary-gland slices (500 mg.) were incubated with 4·5 ml. of Krebs–Ringer bicarbonate

medium (Umbreit, Burris & Stauffer, 1957) with one of the following substrate mixtures: *A*, 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose; *B*, 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose and phenazine methosulphate (final concn. 0·1 mM); *C*, 100 μmoles of glucose containing 0·4 μc of [6-^{14}C]glucose; *D*, 100 μmoles of glucose and 100 μmoles of acetate containing 0·4 μc of [1-^{14}C]acetate. In each case the gas phase was O_2+CO_2 (95:5). At the end of the incubation period (1 hr.) 0·5 ml. of 5N-HCl was introduced into the outer compartment of the flask and 1·0 ml. of 5N-KOH into the centre well by injection through the rubber cap. The procedure with the ^{14}C-labelled substrates and determination of $^{14}CO_2$ used were those described by McLean (1960), except that the total lipid was extracted by the method of Bligh & Dyer (1959) and plated on lens tissue on 1 cm.^{2} disks and this lipid was also counted by the 'infinitely-thick'-layer technique. The results are expressed as μmoles of substrate utilized/hr./total gland.

The lactating and involuting glands from each rat are compared with each other, each rat thus serving to provide an experimental and control value. For this reason the statistical analysis of the results is based on the comparison of corresponding pairs and in Table 1 the differences between the pairs of glands are given together with the values for Fisher's *P*. The differences are considered significant if *P* is no greater than 0·05, and values greater than 0·1 are quoted as not significant (N.S.).

RESULTS

The earliest changes found in the involuting mammary gland, in the present experiments, is a decrease in the incorporation of ^{14}C from [6-^{14}C]-glucose into ^{14}C-labelled lipid and an increase in the formation of $^{14}CO_2$ from [6-^{14}C]glucose which occur 19 hr. after the induction of involution (see Table 1). The inhibition of fat synthesis at this early time is also shown by the incorporation of ^{14}C from [1-^{14}C]acetate into lipid and here again there is a parallel rise in the rate of oxidation of acetate (Fig. 1). At this time there is some depression in the oxidation of C-1 of glucose and in glucose 6-phosphate-dehydrogenase activity, but these differences are not quite significant at the 5 % level.

By 23 hr. after the induction of involution the depression in lipid synthesis is accentuated and at this time-interval there is a highly significant fall in both the oxidation of C-1 of glucose and in the activity of glucose 6-phosphate dehydrogenase, although at this time 6-phosphogluconate dehydrogenase, the rate-limiting enzyme in the formation of $^{14}CO_2$ from [1-^{14}C]glucose, has still not changed in activity (Table 1 and Fig. 2).

Table 1. *Effect of artificially induced involution on the metabolism of glucose by lactating-rat mammary gland*

Lactating rats (12–16 days' lactation) were used. One abdominal mammary gland was blocked with collodion (involuting gland) and the metabolism of glucose by this and the contralateral lactating gland were compared. In the groups in which the gland was blocked for 19 and 23 hr. (five and four animals respectively) the results are expressed as means ± S.E.M. and the differences between corresponding pairs of results (blocked and normal glands from the same animal) are given together with the Fisher's *P* values; N.S. indicates that the values are not significantly different. The values at 28, 48 and 72 hr. after induction of involution are each the mean of two pairs of values. Tissue slices (500 mg.) were incubated in 4·5 ml. of Krebs–Ringer bicarbonate medium with 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose or [6-^{14}C]glucose; the gas phase was O_2+CO_2 (95:5), and the time of incubation was 1 hr. Isotopic results are expressed as μmoles of [^{14}C]glucose converted into $^{14}CO_2$ or into ^{14}C-labelled lipid/hr./total gland at 37° and the enzyme activities as units that are directly comparable with the isotopic results, i.e. μmoles of NADP reduced/hr./total gland corrected to the rate at 37°. Details are given in the Methods section.

Functional stage	Wt. of gland (g.)	Incorporation of ^{14}C from [^{14}C]glucose into $^{14}CO_2$ (μmoles/hr./total gland) From [1-^{14}C]glucose	From [6-^{14}C]glucose	$^{14}CO_2$ from [1-^{14}C]glucose / $^{14}CO_2$ from [6-^{14}C]glucose
Lactating	3·85 ± 0·39	126 ± 13	8·4 ± 2·6	15
Involuting for 19 hr.	9·65 ± 1·16	89 ± 17	15·0 ± 2·5	5·9
Difference	—	−37 ± 17	+6·6 ± 1·5	—
Fisher's *P*	—	0·090	0·013	—
Lactating	3·87 ± 0·54	162 ± 20	7·4 ± 1·6	22
Involuting for 23 hr.	7·52 ± 0·87	50 ± 6	11·8 ± 1·0	4·2
Difference	—	−112 ± 22	+4·4 ± 1·4	—
Fisher's *P*	—	0·014	0·05	—
Lactating	4·16	210	10·0	21
Involuting for 28 hr.	6·23	27	8·2	3·3
Lactating	4·68	172	10·2	17
Involuting for 48 hr.	5·81	22	5·8	3·8
Lactating	3·97	191	6·4	30
Involuting for 72 hr.	2·88	12	2·5	4·8

Table 1. (*cont.*)

Functional stage	Wt. of gland (g.)	Incorporation of ^{14}C from [^{14}C]glucose into ^{14}C-labelled lipid (μmoles/hr./total gland): From [1-^{14}C]glucose	From [6-^{14}C]glucose	^{14}C-labelled lipid from [1-^{14}C]glucose / ^{14}C-labelled lipid from [6-^{14}C]glucose $\times 100$* (%)
Lactating	3·85±0·39	92±8	193±24	48
Involuting for 19 hr.	9·65±1·16	76±14	133±18	57
Difference	—	−16±13	−60±22	—
Fisher's *P*	—	N.S.	0·043	—
Lactating	3·87±0·54	99±11	210±20	47
Involuting for 23 hr.	7·52±0·87	43±7	52±6	83
Difference	—	−56±13	−158±25	—
Fisher's *P*	—	0·022	0·01	—
Lactating	4·16	131	280	47
Involuting for 28 hr.	6·23	23	24	96
Lactating	4·68	110	235	47
Involuting for 48 hr.	5·81	17	17	100
Lactating	3·97	123	226	54
Involuting for 72 hr.	2·88	7	12	58

Functional stage	Wt. of gland (g.)	Dehydrogenase activities (μmoles of NADP reduced/hr./total gland): Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase: At pH 9·0	At pH 7·6
Lactating	3·85±0·39	14 500±2 610	3 580±640	1 700±350
Involuting for 19 hr.	9·65±1·16	10 660±3 020	3 800±800	1 800±460
Difference	—	−3 840±1 700	+220±210	+100±145
Fisher's *P*	—	0·079	N.S.	N.S.
Lactating	3·87±0·54	12 530±1 800	2 900±280	1 630±250
Involuting for 23 hr.	7·52±0·87	6 220±1 750	2 620±400	1 670±280
Difference	—	−6 310±780	−280±200	+40±45
Fisher's *P*	—	<0·001	N.S.	N.S.
Lactating	4·16	13 100	5 200	2 600
Involuting for 28 hr.	6·23	4 300	2 900	1 450
Lactating	4·68	13 600	3 420	1 820
Involuting for 48 hr.	5·81	2 290	1 150	600
Lactating	3·97	14 300	3 800	2 140
Involuting for 72 hr.	2·88	860	680	510

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

The pattern of change with respect to the decline in lipid synthesis both from glucose and acetate and the fall in glucose 6-phosphate-dehydrogenase activity continues at 28 hr. and becomes even more pronounced at 48 hr. The change in the activity of the pentose phosphate pathway is reflected in the decrease in the C-1/C-6 quotient for $^{14}CO_2$ formation which falls from a value of 21 in the lactating gland to about 3·5 at 28 hr. and 48 hr. after the induction of involution. Though there are many difficulties in the interpretation of this quotient (see Wood, 1955; Racker, 1957; Katz & Wood, 1963) it does, nevertheless, give an indication of the magnitude of the change in the overall pattern of metabolism. At these time-intervals almost all the lipid is derived from the glycolytic route of glucose metabolism since the quotient [(^{14}C-labelled lipid from [1-^{14}C]glucose)/(^{14}C-labelled lipid from [6-^{14}C]glucose) × 100] is approx. 100% (see Table 1 and Abraham & Chaikoff, 1959). There is a sharp decline in the activity of 6-phosphogluconate dehydrogenase when involution has continued for 28 hr. or longer.

There is some differentiation between the oxidation of [6-^{14}C]glucose and of [1-^{14}C]acetate. In the former case the rate of formation of $^{14}CO_2$ has returned to normal at 28 hr. and shows a progressive decline in the involuting gland at 48 and 72 hr. In contrast with this the oxidation of [1-^{14}C]acetate is maintained at an increased rate up

to 48 hr. after the gland has been blocked and only falls after milk secretion has stopped for 72 hr. This suggests that in the gland 28 and 48 hr. after induction of involution there is a block in the metabolism of glucose at a stage before the formation of the C_2 unit, and that oxidation by way of the tricarboxylic acid cycle can continue at an increased rate provided that there is a supply of substrate.

At 72 hr. after induction of involution the oxidation of C-1 of glucose, the synthesis of lipid from both glucose and acetate and the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are all decreased to

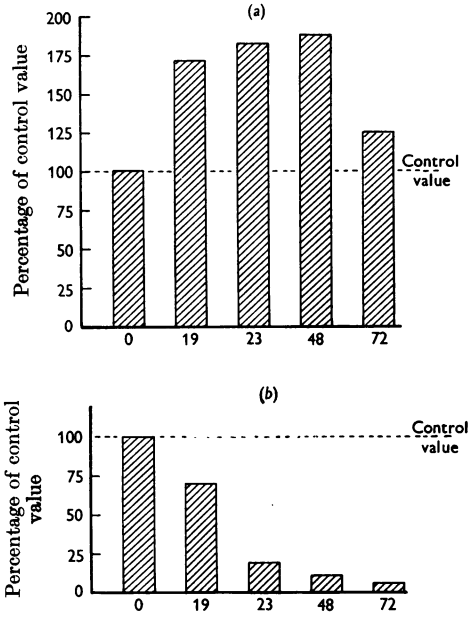

Fig. 1. Effect of artificially induced involution on the metabolism of [1-^{14}C]acetate. (*a*) The incorporation of [1-^{14}C]acetate into $^{14}CO_2$. (*b*) The incorporation of [1-^{14}C]-acetate into ^{14}C-labelled lipid. The incubation system contained mammary-gland slices (500 mg.) in 4·5 ml. of Krebs–Ringer bicarbonate medium, 100 μmoles of acetate containing 0·4 μc of [1-^{14}C]acetate, and 100 μmoles of glucose. The gas phase was $O_2 + CO_2$ (95:5), and the incubation time was 1 hr. The results (as μmoles/hr./total gland) for the involuting gland are expressed as percentages of the values for the control lactating gland (the contralateral gland). The columns represent values for animals obtained 19, 23, 48 and 72 hr. after the induction of mammary-gland involution and contain 5, 4, 2 and 2 pairs of results respectively. The mean values for all control glands in terms of μmoles of [1-^{14}C]acetate utilized/hr./total gland at 37° are 9·94 $\pm$ 1·65 μmoles converted into $^{14}CO_2$ and 155 $\pm$ 17 μmoles converted into ^{14}C-labelled lipid. The $^{14}CO_2$ formation from [1-^{14}C]acetate is significantly increased at 19 hr. after the induction of involution (difference between corresponding pairs, 7·5 $\pm$ 2·5; P = 0·016); the fall in lipid formation from [1-^{14}C]acetate is significant at the 5% level (difference between corresponding pairs, 45 $\pm$ 15; P = 0·05).

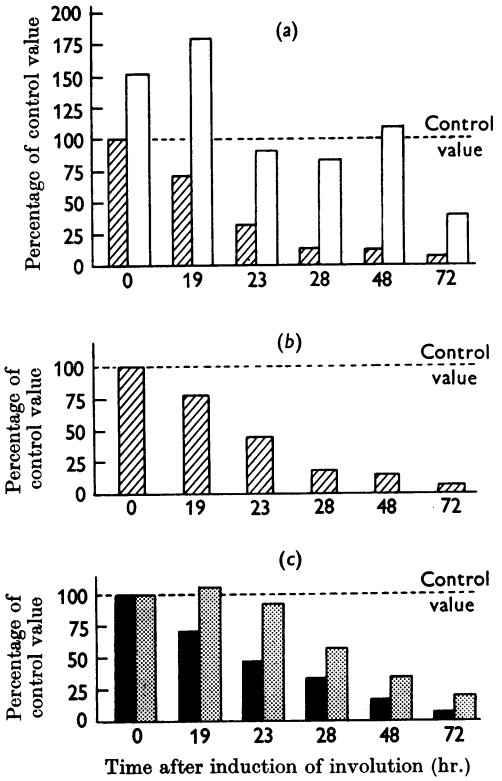

Fig. 2. Effect of artificially induced involution on the metabolism of [1-^{14}C]glucose and on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. (*a*) The incorporation of [1-^{14}C]glucose into $^{14}CO_2$ in the presence (□) and absence (▨) of phenazine methosulphate (final concn. 0·1 mM). (*b*) The incorporation of [1-^{14}C]glucose into ^{14}C-labelled lipid in the absence (▨) of phenazine methosulphate (final concn. 0·1 mM). (*c*) The activity of glucose 6-phosphate dehydrogenase (■) and 6-phosphogluconate dehydrogenase (▦). The results (as μmoles/hr./total gland) for the involuting gland are expressed as percentages of the values for the control lactating gland of the contralateral side. The columns represent values for animals at 19, 23, 28, 48 and 72 hr. after the induction of mammary-gland involution, by blocking one abdominal mammary gland of the lactating rat (12–16 days' lactation) with collodion, and contain 5, 4, 2, 2 and 2 pairs of results respectively. The incubation system contained mammary-gland slices (500 mg.) in 4·5 ml. of Krebs–Ringer bicarbonate medium and 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose. The gas phase was $O_2 + CO_2$ (95:5), and the incubation time was 1 hr. The mean value for all the control glands in terms of μmoles of [1-^{14}C]glucose utilized/hr./total gland at 37° are 156 $\pm$ 12 for $^{14}CO_2$ formation and 102 $\pm$ 6 for ^{14}C-labelled lipid synthesized.

less than 10 % of the values for the lactating gland from the same animal; the oxidation of [1-^{14}C]-acetate, however, is not altered.

Since the rate of formation of $^{14}CO_2$ from [1-^{14}C]-glucose in mammary gland is dependent on the rate of reoxidation of $NADPH_2$ (McLean, 1960) and there is evidence for a close correlation between the rate of formation of lipid utilizing $NADPH_2$ and the oxidation of C-1 of glucose in the lactating mammary gland, it was decided to study both the potential rate of oxidation of C-1 of glucose in the presence of phenazine methosulphate as well as the actual rate observed without artificial stimulation of the rate of reoxidation of $NADPH_2$. The results of this experiment (Fig. 2) show that, despite the decrease in glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities at 23, 28 and 48 hr. after the induction of mammary involution, the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose can be restored to normal by the addition of phenazine methosulphate. Even 72 hr. after the ducts of the mammary gland had been blocked a large measure of activity could be restored by the addition of phenazine methosulphate.

The activities of the dehydrogenases of the pentose phosphate pathway may be compared with the rate of oxidation by this pathway as shown by the formation of $^{14}CO_2$ from [1-^{14}C]glucose (Table 1). 6-Phosphogluconate-dehydrogenase activity (at pH 7·6) is present in tenfold excess in the lactating gland and approximately fivefold excess even when the rate of oxidation is stimulated with phenazine methosulphate.

The activity of phosphoglucose isomerase was also measured in certain of these experiments since this enzyme plays an important role both as the second step in the glycolytic route and as the final stage in the recycling of the pentose phosphate pathway. In lactating mammary gland the activity of this enzyme closely approaches that of glucose 6-phosphate dehydrogenase: the average units of enzyme activity are 10600 for phosphoglucose isomerase compared with 14000 for glucose 6-phosphate dehydrogenase, both enzymes being expressed as content in the abdominal mammary glands from one side only (weight, not corrected for milk content, approx. 4·0 g.; see Table 1). At 23 hr. after the induction of involution there was a tendency for phosphoglucose-isomerase activity to be increased, the mean values for lactating and involuting glands (four animals) being 10600 ± 1000 and 14200 ± 310 respectively; the differences of corresponding pairs are $+3600 \pm 1030$ ($P = 0{\cdot}04$). After the mammary gland had been blocked with collodion for 72 hr. there was a marked fall in the activity of this enzyme to approx. 30 % of that of the lactating gland.

DISCUSSION

The aim of the present experiments was to determine the pattern of change in the metabolism of glucose and the synthesis of lipid and, if possible, the actual sequence of events after the induction of mammary involution by blocking the gland with collodion. The use of the same rat for experimental and control values greatly facilitates this study since variations among experimental animals are reduced to a minimum and both glands are subject to the same hormonal and neural influences. In the present work it would seem that there is a very close correlation between the oxidation of C-1 of glucose and the rate of lipid synthesis, as measured with specifically labelled glucose and acetate; moreover, there is some evidence that it is lipid synthesis that shows the earliest change (Table 1 and Fig. 1).

Table 1 shows that of the two enzymes 6-phosphogluconate dehydrogenase is rate-limiting in the formation of $^{14}CO_2$ from [1-^{14}C]glucose, and this enzyme shows no significant alteration until 28 hr. after induction of involution, some 9 hr. after alterations were observed in lipid synthesis; it would seem therefore that the changes in fat synthesis precede those of the pentose phosphate pathway and, because of the close 'gearing' of these two systems, is largely responsible for the fall in $^{14}CO_2$ formation from [1-^{14}C]glucose. Particularly striking from this point of view is the fact that phenazine methosulphate restores the low rate of $^{14}CO_2$ formation to normal, suggesting that it is probably the rate of reoxidation of $NADPH_2$ that is rate-limiting. Phenazine methosulphate has a very similar effect in stimulating the low rate of $^{14}CO_2$ formation from [1-^{14}C]glucose by mammary-gland slices from pregnant rats and may in fact raise it to the rate found in the fully lactating gland (McLean, 1960).

Also, there is a very close correlation between the changes in activity of glucose 6-phosphate dehydrogenase and lipid synthesis and it is possible that this enzyme is more closely linked to the process of lipid synthesis than is 6-phosphogluconate dehydrogenase and that, despite the potentially high activity of glucose 6-phosphate dehydrogenase when measured with excess of substrate and co-factors, it might still control lipid synthesis.

The possibility that the changes in the formation of $^{14}CO_2$ from [1-^{14}C]glucose are due to an early and specific lesion in fat synthesis, a situation reminiscent of the diabetic state (Abraham, Matthes & Chaikoff, 1959), must also be considered. That it is the process of lipid synthesis rather than the supply of precursors for lipid synthesis which is rate-limiting is suggested by the fact that [1-^{14}C]acetate is also more slowly incorporated into lipid 19 and

23 hr. after the induction of mammary involution. The process of activation of acetate and formation of acetyl-CoA is probably not rate-limiting since there is an increased rate of oxidation of this substrate at these time-intervals. This increased rate of oxidation of [1-^{14}C]acetate also provides evidence that the rise in the oxidation of C-6 of glucose at these time-intervals is due to an increased oxidation by way of the tricarboxylic acid cycle rather than to an alternative pathway, such as that involving the formation of glucuronate and xylulose (Utter, 1958) which could give rise to the production of more carbon dioxide from C-6 of glucose than from C-1.

It is evident from the quotient [(^{14}C-labelled lipid from [1-^{14}C]glucose)/(^{14}C-labelled lipid from [6-^{14}C]glucose) × 100] (Table 1) that the contribution of the pentose phosphate pathway and the glycolytic route of carbohydrate metabolism to lipid synthesis are widely different in the lactating and involuting gland (see also Abraham & Chaikoff, 1959). In the lactating gland a calculation similar to that of Katz & Wood (1963) may be applied by using the experimentally determined value for the formation of $^{14}CO_2$ from [2-^{14}C]glucose for an approximate estimation of the factor Q of these authors, which represents the dilution of the specific activity of the hexose 6-phosphate derived from [1-^{14}C]glucose relative to substrate glucose.

In the present experiments the rate of formation of $^{14}CO_2$ from [2-^{14}C]glucose in lactating rat mammary gland was almost exactly half the value for [1-^{14}C]glucose, a result in close agreement with those of Abraham *et al.* (1954) and Abraham, Cady & Chaikoff (1960). Since the quotient ($^{14}CO_2$ from [2-^{14}C]glucose)/($^{14}CO_2$ from [6-^{14}C]glucose) is high (approx. 10), it is assumed that the major part of the $^{14}CO_2$ from [2-^{14}C]glucose arises by way of the pentose phosphate pathway after one complete turn of the cycle. When [1-^{14}C]glucose is the substrate for metabolism the rate of formation of unlabelled hexose 6-phosphate will be equivalent to this rate of formation of $^{14}CO_2$ from [2-^{14}C]glucose. When [6-^{14}C]glucose is the substrate the resynthesized hexose will bear the label in its initial position.

Each mole of [1-^{14}C]glucose yields 1 mole of labelled triose via the glycolytic route and no labelled triose via the pentose phosphate cycle; each mole of [6-^{14}C]glucose yields 1 mole of labelled triose via the glycolytic route and $\frac{1}{3}$ mole of labelled triose via the pentose phosphate cycle. If C_1 and C_6 represent the incorporation of ^{14}C from [1-^{14}C]-glucose and [6-^{14}C]glucose into ^{14}C-labelled lipid respectively under the present experimental conditions and in terms of μmoles utilized/hr., then, after correction for the observed recycling of the pentose phosphate cycle, it follows that $(C_1 + 0{\cdot}5C_1)$ μmoles of glucose are involved in triose formation via the glycolytic route and that

$$[C_6 - (C_1 + 0{\cdot}5C_1)] \times 3 \,\mu\text{moles}$$

of glucose are involved in triose formation via the pentose phosphate cycle. By substituting the average values obtained for the incorporation of specifically labelled glucose into lipid in lactating mammary gland shown in Table 1, which are 110 and 230 μmoles for [1-^{14}C]glucose and [6-^{14}C]glucose respectively, then:

$$C_1 + 0{\cdot}5C_1 = 165$$

$$[C_6 - (C_1 + 0{\cdot}5C_1)] \times 3 = (230 - 165) \times 3 = 195$$

and the contribution of the pentose phosphate pathway is 54 %.

When lactation has been artificially blocked for 28 hr. ^{14}C from C-1 and C-6 of glucose is incorporated at equal rates into ^{14}C-labelled lipid and, in agreement with the results of Abraham & Chaikoff (1959), the quotient (^{14}C-labelled lipid from [1-^{14}C]-glucose)/(^{14}C-labelled lipid from [6-^{14}C]glucose) is approximately unity, suggesting that the C_2 units for lipid formation arise exclusively from the glycolytic route. The diminishing contribution of the pentose phosphate cycle to lipid synthesis with the onset of involution is shown by the gradually increasing values for this quotient (Table 1).

It is clear that factors other than the rate of reoxidation of $NADPH_2$ must control the activity of the pentose phosphate pathway, since even in the presence of phenazine methosulphate the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose still falls far below the potential rate of glucose 6-phosphate dehydrogenase and 6-phosphogluconate-dehydrogenase activities measured spectrophotometrically. The supply of glucose 6-phosphate to the pentose phosphate pathway in the involuting gland could be limited both by rate of formation and by conversion into fructose 6-phosphate or into lactose. However, since phenazine methosulphate will restore the rate of glucose oxidation to normal in a gland that has been involuting for up to 48 hr., it would seem that in such glands the rate of formation of glucose 6-phosphate is not in itself the main limitation. The activity of phosphoglucose isomerase in the lactating rat mammary gland (twelfth day) is approximately the same as that of glucose 6-phosphate dehydrogenase, i.e. 10600 and 14000 μmoles/gland/hr. respectively. Since the Michaelis constant for mammary-gland isomerase (1 mM) is considerably higher than for glucose 6-phosphate dehydrogenase [58 μM for the crude enzyme (McLean, 1958), 30 μM for the purified mammary-gland enzyme (Levy, 1963)], it is clear that glucose 6-phosphate dehydrogenase would compete very effectively for available substrate.

With the onset of involution (23 hr.) the conditions are more favourable towards the glycolytic route since phosphoglucose-isomerase activity increases to 14200 units while that of glucose 6-phosphate dehydrogenase falls to 6200 units. Information on the change in the concentration of glucose 6-phosphate in mammary tissue with the onset of involution would be very valuable in this context.

The present results are in keeping with those of Folley & French (1949), Balmain & Folley (1951) and Abraham & Chaikoff (1959), who have previously studied the fate of glucose in involuting mammary gland although none of these authors have made measurements at so short a time as 19 hr. after the induction of involution. However, Balmain & Folley (1951) observed that the effect of insulin in increasing lipogenesis by lactating-mammary-gland slices was lost in both pregnancy and in mammary involution.

In the present experiments the oxidation of C-6 of glucose and of [1-^{14}C]acetate is increased during the early stages of mammary-gland involution, and this may indicate a degree of uncoupling of oxidative phosphorylation; the oxidation of C-6 of glucose may be stimulated by the addition of dinitrophenol to lactating-rat mammary-gland slices (McLean, 1960). Further evidence may be adduced from the experiments of Wang (1960), who found that the ATP content of involuting mammary tissue decreased slowly during the first 6 hr. of involution and then more sharply between 6 and 12 hr. after weaning; at 12 hr. this change was fully reversible. Racker (1962) and Fanestil, Hastings & Mahowald (1963) have reported that bicarbonate stimulates the adenosine-triphosphatase activity of ox-heart and ox-liver mitochondrial preparations, and in this context it is perhaps significant that carbonic anhydrase shows marked changes during lactation and mammary involution (Brown & Bialy, 1963). However, electron-microscopy studies of mammary tissue during involution would seem to indicate that mitochondrial structure was maintained up to 24 hr. after the induction of involution (Wellings & Deome, 1963).

Since glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and the malonyl-CoA pathway of lipid synthesis are located in the soluble fraction of the cell, electron-microscopy studies would not necessarily throw further light on the problem of the initial changes leading to decreased fat synthesis. However, one of the earliest changes observed by Wellings & Deome (1963) was an accumulation of fat and protein droplets and some vacuolization in the epithelial cells of the alveoli. One enzyme activity that has been shown to change strikingly during both the onset and the cessation of lactation is mammary-gland lipoprotein-lipase activity, which increases dramatically within 2 hr. of the onset of lactation and disappears extremely rapidly with involution (McBride & Korn, 1963; Robinson, 1963). The relationship of this enzyme to the process of milk secretion remains obscure but the time relationship of the changes of this enzyme and of fat production suggests that these processes may be related.

SUMMARY

1. The changes in the incorporation of ^{14}C from [1-^{14}C]glucose, [6-^{14}C]glucose and [1-^{14}C]acetate into $^{14}CO_2$ and ^{14}C-labelled lipid and in the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been studied at 19, 23, 28, 48 and 72 hr. after the initiation of mammary-gland involution.

2. The earliest change observed, at 19 hr., was a decrease in the incorporation of ^{14}C from [6-^{14}C]-glucose and [1-^{14}C]acetate into lipid and a stimulation in the formation of $^{14}CO_2$ from both these labelled substrates. This was followed at 23 hr. by a depression in the formation of $^{14}CO_2$ from [1-^{14}C]glucose and of glucose 6-phosphate-dehydrogenase activity, whereas 6-phosphogluconate-dehydrogenase activity was unchanged and phosphoglucose-isomerase activity increased by about 34%.

3. At 28 and 48 hr. after the induction of involution the fall in the oxidation of C-1 of glucose was more marked, although this could be restored to the high value of the lactating gland by the addition of phenazine methosulphate (0·1 mM); the depression in fat synthesis was also striking. 6-Phosphogluconate-dehydrogenase activity decreased in involuting glands at these time-intervals.

4. These results are discussed with reference to the time sequence of events and the interrelationship of carbohydrate and fat metabolism during mammary-gland involution.

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Heterogeneity of the Peptide Chains of γ-Globulin

BY S. COHEN AND R. R. PORTER
Department of Immunology, St Mary's Hospital Medical School, London, W. 2

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When γ-globulin is reduced in neutral aqueous solution and dialysed against acetic acid or propionic acid, the molecule dissociates into two types of peptide chain (A and B); these can be separated by gel-filtration (Fleischman, Pain & Porter, 1962) and retain several biological properties of the original molecule. The A chains comprise about three-quarters of the molecule, have a molecular weight of about 50000 (Pain, 1963), are associated with the antibody-combining sites of horse antibodies (Fleischman, Porter & Press, 1963), and carry the antigenic (Cohen, 1963*a*, *c*) and allotypic (S. Lawler, personal communication; see Cohen, 1963*a*) determinants specific for each type of γ-globulin. The B chains have a molecular weight of 20000 (Pain, 1963) and carry antigenic and allotypic determinants common to all types of γ-globulin (Cohen, 1963*a*).

The heterogeneity of both A and B chains is apparent from the antigenic determinants and antibody-combining sites that they carry. Similarly, the *N*-terminal amino acids of the chains are complex in all species that have been examined (Fleischman *et al.* 1962; Cohen, 1963*c*). Further evidence of the complexity of the A chains is described in the present paper and, in particular, the B chains from several species are shown to exist in some eight to ten electrophoretically distinct forms. A brief account of this work has been given by Cohen (1963*b*).

MATERIALS

γ-Globulin preparations. Normal 7s and γ_1M-globulins and pathological 7s, γ_1A- and γ_1M-globulins were prepared as described by Cohen (1963*c*). Subfractions of normal 7s γ-globulin were prepared by chromatography on carboxymethylcellulose according to the method described by Sober & Peterson (1958). Serum taken from a colostrum-deprived calf 19 days after birth was fractionated by precipitating twice with Na_2SO_4 (final concn. 18%, w/v). This precipitate, which contained an α_2-globulin (fetuin) and γ-globulin, was dialysed against water at 2°. The euglobulin precipitate was washed in water and dissolved in 0·2M-boric acid buffer, pH 8·2; this fraction had the mobility of γ-globulin on paper electrophoresis, and in the ultracentrifuge contained a main peak (having an S_{20} value of 6·8s) comprising about 85% of the total and three minor components having S_{20} values of 3·3s, 14s and 21s. The yield of γ-globulin was approx. 30 mg./100 ml. of serum. Samples of γ-globulin from the same colostrum-deprived calf at 40 and 67 days were prepared by chromatography on diethylaminoethylcellulose (Sober & Peterson, 1958); the yields of γ-globulin were 130 and 200 mg./100 ml. respectively. A euglobulin fraction of adult bovine γ-globulin was prepared from serum dialysed against 0·0175M-sodium phosphate buffer, pH 6·4. The euglobulin precipitate was washed in water, dissolved in 0·9% NaCl and precipitated three times with Na_2SO_4 (final concn. 18%, w/v).

Preparation of antisera. Antisera to human γ-globulin and Bence-Jones proteins were obtained by immunizing rabbits with subcutaneous injections of the antigens (1–3 mg. of protein/week for 3–6 weeks) in Freund's complete adjuvant. Antisera to ovalbumin and bovine