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Received July 7, 1966. P.S.E.B.M., 1966, v123.

# Activity Patterns of Several Enzymes of Liver, Adipose Tissue, and Mammary Gland of Virgin, Pregnant, and Lactating Mice.* (31573)

J. C. BARTLEY,† S. ABRAHAM,‡ AND I. L. CHAIKOFF§
*Department of Physiology, University of California, Berkeley*

The specific activities of many enzymes of mammary gland are higher in the lactating than in the non-lactating gland(1-8). Superficially, the response of the glucose-ATP phosphotransferases‖ appeared to be analogous to that of other mammary gland enzymes(3,6-8). Closer examination, however, revealed that the increase in glucose-ATP phosphotransferase activity in the mammary gland during lactation resulted from an increase in the proportion of hexokinase of high specific activity associated with the particulate fraction(9). It was suggested that this change in intracellular distribution of hexokinase during lactation is probably due neither to a change in intracellular site of the enzyme nor to an increase in enzyme synthesis, but to an increase in the proportion of secretory tissue in which hexokinase is particle-bound and a decrease in the proportion of adipose tissue in the lactating gland(9). If this is the true explanation, the intracellular distribution of hexokinase in adipose tissue should be unaffected by lactation.

The present study was undertaken, in part, to compare the intracellular distribution of glucokinase and hexokinase activities in adipose tissue devoid of mammary gland parenchyma with that in mammary glands of the same virgin, pregnant, and lactating mice.

It is clear that the activities of hepatic glucokinase(9), glucose-6-phosphate dehydrogenase (glucose-6-$PO_4$ dehydrogenase), and 6-phosphogluconate dehydrogenase(3,4) are also higher in lactating mice than in virgin and pregnant mice. The response of these hepatic enzymes to the transition from preg-

* Aided by a grant from Nat. Science Foundation.

† Special Research Fellow of Nat. Inst. of Arthritis & Metab. Dis. (2-F3-AM-11,212-03). Present address: Dept. of Clinical Sciences, School of Veterinary Med., Univ. of California, Davis.

‡ Present address: Bruce Lyon Memorial Research Laboratory, Children's Hospital Med. Center of Northern California, Oakland.

§ This study was in progress at the time of Dr. Chaikoff's death, 25 January 1966.

‖ The total activity of the enzymes catalyzing the phosphorylation of glucose at the 6 position is referred to as glucose-ATP phosphotransferase activity. Under the conditions of the assay of these enzymes as described by Viñuela *et al*(23), enzymatic activity that has a high Michaelis constant ($K_m$) for glucose and is inhibited by N-acetylglucosamine is referred to as glucokinase, and that with a low $K_m$ and unaffected by N-acetylglucosamine is referred to as hexokinase.

nancy to lactation is similar to their response to the transition from fasting to the refeeding of a high-carbohydrate diet(10-14). The activities of the glucose-ATP phosphotransferases(15,16), glucose-6-$PO_4$ dehydrogenase (17), malic enzyme(17), and citrate-cleavage enzyme(17) of rat adipose tissue have also been shown to be affected by similar dietary changes. Furthermore, the activity of glucose-6-$PO_4$ dehydrogenase of adipose tissue increased 50% within one hour after oral administration of glucose, whereas that of liver was unchanged during this period(18).

Therefore, at the same time that glucose-ATP phosphotransferase activity was assayed, the specific activities of glucose-6-$PO_4$ dehydrogenase, malic enzyme, and citrate-cleavage enzyme of liver and adipose tissue of the same mice were determined, to test whether these enzymes respond to lactation in a manner analogous to that reported for hepatic glucose-6-$PO_4$ dehydrogenase, 6-phosphogluconate dehydrogenase(3,4), and glucokinase (9).

*Experimental.* Female mice of the $C_3H$ strain that had been raised and maintained on a nutritionally adequate diet (Purina Lab Chow) were used. At least one month prior to the experiments, the inguinal fat pads were cleared of mammary gland tissue as described previously(19). Livers, mammary gland-free fat pads, abdominal adipose tissue, and mammary glands were excised from virgin, pregnant (16-18th day of gestation), and lactating mice (11-14th day of lactation). The mice (3 to 5 months old) were killed by cervical fracture, and the tissues were rapidly excised and placed in an ice-cold 0.25 M sucrose solution. All preparative procedures were carried out at 2-4°.

The tissues were sliced 0.4 mm thick with a McIlwain-Buddle tissue chopper(20). The slices of mammary gland obtained from lactating mice were washed 5 times with the sucrose solution to remove preformed milk. The tissues were minced and homogenized. Homogenates were fractionated by centrifugation as described previously(21). The supernatant layer that formed after the homogenate was spun for 10 min at 350 $\times$ *g* is designated Fraction I (it contained cytoplasmic fluid, mitochondria, and microsomes), and that resulting from centrifugation at 100,000 $\times$ *g* for 60 min is designated Fraction III (particle-free supernatant fraction). Mitochondria were separately isolated as described previously(22).

Glucokinase and hexokinase activities were measured spectrophotometrically by the methof described by Viñuela *et al*(23). Glucose-6-$PO_4$ dehydrogenase(24), malic enzyme(25), and citrate-cleavage enzyme(26) were measured spectrophotometrically in the particle-free supernatant fraction. Mitochondrial protein was estimated by the biuret method of Gornall *et al*(27). The protein concentrations of Fractions I and III were calculated from the absorbancy at 260 and 280 m$\mu$(28). Enzyme activities are expressed as m$\mu$moles of pyridine nucleotide reduced or oxidized/mg protein/min (specific activity).

*Results.* Examination of the intracellular distribution of hexokinase and glucokinase activities revealed, as reported previously(9), that between 80 and 90% of the total glucose-ATP phosphotransferase activity in Fraction I of the mammary gland of virgin and pregnant mice was recovered in the particle-free supernatant fraction (Table I). The presence of small amounts of activity in the isolated mitochondria confirmed this observation. In contrast, less than 40% of the total glucose-ATP phosphotransferase activity was found in Fraction III prepared from the mammary glands of lactating mice, the remainder being bound to the mitochondria. More than 82% of the total activity detected in Fraction I from adipose tissue (either abdominal or inguinal fat pads cleared of glandular elements) was recovered in Fraction III (Table I). Similar observations have been reported for rat adipose tissue(21). This indication that some of the glucose-ATP phosphotransferase activity was particle-bound in adipose tissue was not supported by the results of direct analysis of isolated, washed mitochondria. Almost no activity (less than 0.5 m$\mu$mole/mg/min) could be detected in mitochondria isolated from adipose tissue. Lactation had no effect on the intracellular distribution of glucose-ATP phosphotransferase activity in adipose tissue. No such activity was detected in

TABLE I. Intracellular Distribution of Glucose-ATP Phosphotransferase in Liver, Mammary Gland, and Adipose Tissue of Virgin, Pregnant and Lactating Mice. Pregnant mice were 3-5 days prepartum. Lactating mice were 11-14 days postpartum. Each value is the average of results of assays on 2 or more tissue samples. See text for other experimental details.

| Tissue | State of mouse | % of glucose-ATP phosphotransferase in: Particulate fraction* | Soluble fraction |
|---|---|---|---|
| Liver | Lactating | 0 | 100 |
| Mammary gland | Virgin | 15 | 85 |
| | Pregnant | 17 | 83 |
| | Lactating | 64 | 36 |
| Abdominal adipose | Virgin | 15 | 85 |
| | Pregnant | 12 | 88 |
| | Lactating | 18 | 82 |
| Mammary gland-free, inguinal adipose† | Virgin | 11 | 89 |
| | Pregnant | 10 | 90 |
| | Lactating | 8 | 92 |

* Calculated as the difference in activity/g tissue between Fractions I and III.
† Inguinal fat pads cleared of mammary gland secretory tissues(19).

mitochondrial preparations from the livers of the lactating mice, and all the activity in Fraction I could be accounted for in Fraction III.

The relative contributions of hexokinase and glucokinase to the total glucose-ATP phosphotransferase activity in Fraction III of liver and the increase in glucokinase activity during lactation have been reported previously (9). The finding(9) that glucokinase activity contributes only about 10% to the total glucose-ATP phosphotransferase activity of the particle-free supernatant fluid from the mammary glands of virgin and pregnant mice has been reaffirmed here. The small amount of activity attributed to glucokinase in Fraction III of mammary gland of lactating mice (Table II) may have been due to the adipose tissue content of the gland since other workers(6,9) have been unable to detect glucokinase activity in lactating mammary gland. However, according to a recent study of the tissue distribution of the isozymes of hexokinase(29), no evidence for a true glucokinase was found in adipose tissue of rats. The three isozymes of hexokinase have $K_m$ values of about $10^{-6}$, $10^{-5}$, and $10^{-4}$ M glucose, and the $K_m$ value of glucokinase is $10^{-2}$ M(29). Therefore, the apparent glucokinase activity in adipose tissue observed here (Table II) and elsewhere(16) was probably due to the isozyme of hexokinase with a $K_m$ for glucose of $10^{-4}$ M(29). Hence, because of this evidence, and because of lack of confidence in low values for glucokinase(6,

TABLE II. Enzymic Activity in the High-Speed Supernatant Fluid of Liver, Mammary Gland, and Adipose Tissue of Virgin, Pregnant and Lactating Mice. Pregnant mice were 3-5 days prepartum. Lactating mice were 11-14 days postpartum. Enzyme specific activity is expressed as mμmoles pyridine nucleotide reduced or oxidized/mg particle-free supernatant protein/min. Enzyme activities are presented as average ± S.E. See text for other experimental details.

| Tissue | State of mouse | n* | Specific activity of: Hexokinase | Glucokinase | Glucose-6-$PO_4$ dehydrogenase | Malic enzyme | Citrate-cleavage enzyme |
|---|---|---|---|---|---|---|---|
| Liver | Virgin | 2 | 1.6 | 8.2 | 14.4 | 40.3 | 8.6 |
| | Pregnant | 5 | .6 ± .1 | 4.6 ± .7 | 14.8 ± 2.8 | 30.8 ± 4.6 | 6.6 ± 1.5 |
| | Lactating | 7 | 1.6 ± .2 | 9.3 ± .8 | 30.9 ± 2.5 | 72.1 ± 5.4 | 14.4 ± 1.0 |
| Mammary gland | Virgin | 2† | 11.9 | 3.2 | 176 | 155 | 37.8 |
| | Pregnant | 5 | 10.7 ± .8 | 1.2 ± .3 | 100.8 ± 8.6 | 50.0 ± 5.4 | 11.8 ± 2.4 |
| | Lactating | 3 | 7.3 ± .6 | 2.0 ± .2 | 243 ±16 | 139 ± 1 | 49.3 ± 3.8 |
| Abdominal adipose† | Virgin | 2 | 9.6 | 2.6 | 322 | 65.6 | 26.0 |
| | Pregnant | 3 | 7.4 ± 1.0 | 1.9 ±1.0 | 147 ± 5 | 16.9 ± 3.2 | 9.0 ± 3.5 |
| | Lactating | 3 | 6.7 ± 1.5 | 2.3 ± .5 | 80.1 ± 3.0 | 166 ±15 | 17.7 ± 5.4 |
| Mammary gland-free,‡ inguinal adipose† | Virgin | 2 | 8.0 | 2.9 | 196 | 146 | 32.2 |
| | Pregnant | 3 | 3.0 ± 1.6 | .6 ± .3 | 143 ±28 | 76.4 ±16.6 | 9.3 ± 3.1 |
| | Lactating | 2 | 10.4 | 4.2 | 108 | 142 | 18.6 |

* No. of tissue samples assayed.
† Tissue from 3 or more mice were pooled for each determination.
‡ Inguinal fat pads cleared of mammary gland secretory tissues(19).

30) as measured by the method of Viñuela *et al*(23), we suggest that neither mammary gland nor adipose tissue contains any true glucokinase activity.

The specific activity of hexokinase and the apparent specific activity of glucokinase in adipose tissue, and their relative contributions to the total glucose-ATP phosphotransferase activity of lactating mice, were similar to those found in mammary glands of virgin mice (Table II and ref. 9). This enzymatic activity in the soluble portion of both liver and adipose tissue of pregnant and lactating mice was unchanged from that in virgin mice.

Only Fraction III was examined for glucose-6-$PO_4$ dehydrogenase(3), malic enzyme (31), and citrate-cleavage enzyme(26) since these enzymes are known to be located almost exclusively in this particle-free supernatant fraction.

The increase in the specific activity of hepatic glucose-6-$PO_4$ dehydrogenase during lactation (Table II) confirms previous reports by other workers(3,4). The increment of this increase was similar to that observed for the enzyme performing the same role in mammary gland tissue (Table II and ref. 8). In sharp contrast to the activity of the enzyme in liver and mammary gland, the specific activity of glucose-6-$PO_4$ dehydrogenase in adipose tissue decreased successively during the transition from the virgin to the pregnant to the full lactating state (Table II).

The specific activities of malic enzyme and citrate-cleavage enzyme responded to pregnancy and lactation similarly in all 3 tissues (Table II). The lowest specific activity observed in all 3 tissues was during pregnancy. The specific activities of malic enzyme and citrate-cleavage enzyme in livers of lactating mice were twice those in livers of pregnant mice (Table II). During lactation the specific activity of both malic enzyme and citrate-cleavage enzyme in mammary gland was 3- to 4-fold higher than that observed in glands from pregnant mice. In adipose tissue, the specific activity of malic enzyme was 10-fold higher in lactating glands than in glands from pregnant mice, while that of citrate-cleavage enzyme was only 2-fold higher in the lactating glands (Table II).

Because the amount of tissue available from the fat pad cleared of mammary gland was limited, tissue from several mice was pooled for the assays. As a consequence, individual assays and a large number of measurements were precluded. As can be readily seen in Table II, the standard errors of these measurements are quite large. For the values in Table II that can be tested for random probability (Fisher's "t" test), there are no significant differences between the results of assays of abdominal adipose tissue and those of gland-free, inguinal fat pads. It is clear that the pattern of enzymatic response to lactation by adipose tissue was the same regardless of its location in the mouse.

*Discussion.* The mammary gland enzymes that respond to lactation are the same as those in liver that respond to dietary and hormonal changes. Three examples of such enzymes were assayed in the present study: glucose-6-$PO_4$ dehydrogenase, malic enzyme, and citrate-cleavage enzyme. Recently, these same 3 enzymes in adipose tissue were shown to adapt to dietary changes in a manner similar to those in liver(17). The results of the present study demonstrate that in both liver and adipose tissue, these enzymes respond to lactation. The activity of hepatic glucokinase has also been shown to adapt during lactation (9). If lactation is viewed as a condition involving the whole animal and not just the mammary gland, changes in enzymatic activity in tissues other than mammary gland are not unexpected. Indeed, liver(32) and adipose tissue(33,34,35) both respond to a variety of hormones, and thereby may act as key tissues in the maintenance of metabolic homeostasis.

The increase in specific activity of glucose-6-$PO_4$ dehydrogenase(36,37,38), malic enzyme(38,39), and citrate-cleavage enzyme (39) of liver and adipose tissue(17,18) in response to dietary manipulations has been correlated with the rate of lipogenesis. While there is no doubt that lactating mammary gland exhibits active lipogenesis(40), it is doubtful whether or not increased fatty acid biosynthesis explains the response of these enzymes to lactation. The fact that the specific activity of glucose-6-$PO_4$ dehydrogenase increased in mammary gland and liver and decreased in adipose tissue during the transition from pregnancy to lactation, while malic enzyme and citrate-cleavage enzyme increased in all three tissues, precludes an explanation based solely on a generalized phenomenon, such as increased lipogenesis.

The results of the glucose-ATP phosphotransferase assays support the view that the increase observed previously in total activity of this group of enzymes during lactation(3,8, 9) is mainly due to an increase in the proportion of parenchymal tissue, in which hexokinase appears to be associated with the mitochondrial fraction of the cell, concomitant with a decrease in the adipose tissue content of the gland. In adipose tissue, the quantity of hexokinase associated with mitochondria is small, and is unaffected by lactation. On the other hand, the proportion of the total activity of the glucose-ATP phosphotransferases that is bound to mitochondria in the mammary gland is much higher in lactating mice than in virgin or pregnant mice. Thus, during lactation, when the proportion of parenchymal tissue to adipose tissue is about 9 to 1(41), the proportion of glucose-ATP phosphotransferase activity associated with mitochondria is also high. In mammary glands of virgin mice, in which the proportion of adipose tissue is much higher than that of parenchymal tissue(41), the percentage of total glucose-ATP phosphotransferase activity associated with mitochondria is very low.

*Summary*. Glucose-ATP phosphotransferases (glucokinase and hexokinase), glucose-6-$PO_4$ dehydrogenase, malic enzyme, and citrate-cleavage enzyme were assayed in the 100,000 $\times$ *g* supernatant fraction (Fraction III) of liver, inguinal fat pads cleared of mammary gland tissue, abdominal adipose tissue, and mammary gland excised from virgin, pregnant, and lactating mice of the $C_3H$ strain. In addition, the activity of glucose-ATP phosphotransferases in the 350 $\times$ *g* supernatant fraction (Fraction I) and that associated with mitochondria of these same tissues was measured. Mitochondria isolated from adipose tissue contained only a small quantity of glucose-ATP phosphotransferase activity. No evidence for such activity was detected in mitochondria isolated from livers of lactating mice. Mitochondria from the mammary glands of lactating mice contributed more than 60% of the total glucose-ATP phosphotransferase activity of the cell, whereas those from virgin and pregnant mice contributed less than 17%.

The specific activities of malic enzyme and citrate-cleavage enzyme were higher in mammary glands of virgin and lactating mice than in those of pregnant mice. Glucose-6-$PO_4$ dehydrogenase activity was higher in liver and mammary gland of lactating mice than in virgin and pregnant mice. In adipose tissue, glucose-6-$PO_4$ dehydrogenase was lower in lactating mice than in virgin and pregnant mice. The effect of lactation on the activity of the enzymes of adipose tissue and liver indicates that lactation involves more than just the mammary gland. The response cannot be brought about in all tissues by a generalized mechanism since the pattern of the enzymatic change was not the same in all tissues studied. The finding that the intracellular distribution of glucose-ATP phosphotransferase activity in liver and adipose tissue was unchanged during lactation adds strong support to the view that the increase in hexokinase activity associated with mitochondria of lactating mammary glands is due to an increase in the proportion of parenchymal tissue in the gland.

The authors are indebted to Mrs. Hope McGrath for preparation of the mammary gland-free inguinal fat pads and for the care and breeding of the mice.

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Received July 11, 1966. P.S.E.B.M., 1966, v123.

## Thiamine, Magnesium and Plasma Lactate Abnormalities in Alcoholic Patients. (31574)

R. L. DELANEY, H. G. LANKFORD, AND J. F. SULLIVAN
(Introduced by Donal F. Magee)

*Departments of Medicine and Pathology, Creighton University School of Medicine, and Omaha V. A. H., Omaha, Nebr.*

Thiamine and magnesium deficiency as well as increased plasma lactate levels have been independently described in chronic alcoholics by several investigators(1-7). While previous attempts to measure thiamine levels have been at best cumbersome(8,9), a more practical assay by means of the transketolase reaction is now available(9). Based on the production of glucose resulting from *in vitro* incubation of thiamine pyrophosphate with a