The Activity of Phosphoenolpyruvate Carboxykinase throughout the Lactation Cycle of the Guinea Pig Mammary Gland

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Abstract. **The enzyme phosphoenolpyruvate carboxykinase (PEPCK) has been measured in the guinea pig mammary gland throughout the pregnancy-lactation cycle. This is of interest since the primary importance of PEPCK is thought to be its role in gluconeogenesis and it is questionable whether or not gluconeogenesis occurs in the mammary gland. The enzyme activity, present in both the cytosol and mitochondria, was shown to follow the lactation profile. During the transition into lactation, cytosolic PEPCK activity increases 11-fold and mitochondrial PEPCK activity 43-fold while tissue weight increases 4-fold. Fructose 1,6-bisphosphatase was found to increase at a rate only slightly greater than that of the tissue weight. The increase in mitochondrial PEPCK activity is thus about 10 times greater than that of general tissue expansion, whereas the cytosolic PEPCK activity increase is only 2-fold greater. The activity of fructose 1,6-bisphosphatase appears to be merely keeping pace with general tissue expansion. The mitochondrial enzyme constitutes 59 ± 3% of the total gland PEPCK activity in the prepartum state and 86 ± 2% at midlactation. Therefore, mitochondrial PEPCK is the isoenzyme undergoing the greater increase during the transition into lactation in the guinea pig mammary gland and thus would appear to play the more important role in the conversion of oxalacetate to phosphoenolpyruvate in this tissue.**

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Gluconeogenesis, the generation of glucose from noncarbohydrate sources, is primarily a function of the liver and, to a lesser extent, of the kidney. The pathway has been observed, at minor levels, in some other tissues but is not believed to be of major importance in the mammary gland (1). It was of considerable interest, therefore, when Garcia-Ruiz *et al.* (2) reported the presence of phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) in the lactating rat mammary gland since this enzyme catalyzes one of the most important steps in the gluconeogenic pathway (3). This confirms an earlier observation of Baird (4) who was able to measure the enzyme in lactating bovine mammary gland. The study of PEPCK in the mammary gland is interesting since the enzyme appears to be serving some function other than gluconeogenesis in this tissue.

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In the rat, cytosolic PEPCK constitutes over 90% of the total activity and thus the distribution differs from that of humans in which it has been reported to be about 40% cytosolic and 60% mitochondrial (3). In studying PEPCK in the mammary gland, it is of particular interest to select a species which is similar to humans in the liver enzyme activity and distribution. The guinea pig is thought to be such a species (3). Therefore, we have measured PEPCK in the guinea pig mammary gland, determined the cellular distribution, and followed the activity changes throughout the pregnancy-lactation cycle. The following are the results of that study.

Materials and Methods

Chemicals. Fructose 1,6-bisphosphate, phosphoenolpyruvate (PEP), inosine 5′-diphosphate, reduced glutathione (GSH), NADH, NADP, malate dehydrogenase (EC 1.1.1.37), cytochrome *c*, and sodium deoxycholate were purchased from Sigma Chemical Co., St. Louis, MO. Omniflor premixed LSC powder and sodium [^{14}C] bicarbonate were purchased from New England Nuclear Research Products, Boston, MA. Triton X-100,

scintillation grade, was purchased from Amersham Corp., Arlington Heights, IL. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glucose-6-phosphate isomerase (EC 5.3.1.9) were purchased from Boehringer Mannheim Co., Indianapolis, IN.

Animals. Guinea pigs (Hartley strain) were purchased from Charles River Laboratories, Wilmington, MA. Nulliparous females, approximately 3 months old (500 g), were bred and then later sacrificed at the prepartum stage or at the various stages of the lactation cycle. The prepartum group included both virgin and pregnant animals. Litter size for each lactating animal was maintained at two pups by adding to or removing from the litters. Staging of pregnant animals was based on fetal length and weight characteristics as described by Draper (5).

Preparation of Tissue Homogenates. Animals were sacrificed by decapitation, after ether anesthesia, and the mammary glands dissected. The organs were washed in cold sucrose-EDTA-GSH buffer (0.25 *M* sucrose, 0.5 m*M* EDTA, 1 m*M* GSH, pH 7.4), blotted, weighed, placed in 5 volumes of the buffer, chopped into small pieces and then homogenized (on ice) using a Potter-Elvehjem homogenizer (Teflon/glass).

Preparation of Fractions for Enzyme Assay. The method employed to measure cytosolic and mitochondrial PEPCK activity was an adaption of a procedure developed by Söling *et al.* (6) in guinea pig liver. The mammary gland homogenate, obtained as described above, was divided into two equal portions and the first centrifuged at 100,000*g* for 40 min to obtain the cytosolic fraction. To the second homogenate portion, sodium deoxycholate was added to 0.4%, the mixture allowed to stand on ice for at least 30 min, and then centrifuged at 100,000*g*. This supernatant was designated "total" enzyme fraction since it now contains both the cytosolic and mitochondrial PEPCK activity. The PEPCK activity was assayed in both the cytosolic and total fractions and the mitochondrial PEPCK value calculated by subtracting the cytosolic from the total activity. The amount of sodium deoxycholate needed for optimal release of mitochondrial PEPCK activity was determined by Söling *et al.* (7).

Determination of PEPCK Activity. The activity of PEPCK was determined in the various fractions by an adaption of the method of Chang and Lane (8) as modified by Ballard and Hanson (9). The 1.0-ml reaction mixture contained the following constituents: imidazole buffer (pH 6.6), 100 m*M*; PEP, 1.25 m*M*; IDP, 1.25 m*M*; $MnCl_2$, 1.0 m*M*; GSH, 2.0 m*M*; NADH, 2.5 m*M*; malate dehydrogenase, 5 units; $KH^{14}CO_3$ (approximately 10^5 cpm/μmol), 50 m*M*; and PEPCK (up to 0.004 units). After initiation by addition of PEP, the reaction mixture was incubated at 37°C for times which varied from 5 to 20 min, depending on the activity. The reaction was terminated by the addition of 1.0 ml of 8% trichloroacetic acid and the unreacted $H^{14}CO_3^-$ removed by bubbling CO_2 gas through the mixture for 3 min. To a scintillation vial was added 0.5 ml of the reaction mixture, 0.5 ml H_2O, and 10 ml of scintillation fluid (Omniflor premixed LSC). The samples were then counted on a scintillation counter to determine the amount of radioactivity incorporated into acid stable malic acid via oxalacetate (OAA). One unit of PEPCK activity is defined as the amount of enzyme which catalyzes the carboxylation of 1.0 μmol of PEP/min at 37°C.

Determination of Cytochrome *c* Oxidase Activity. Cytochrome *c* oxidase (EC 1.9.3.1) activity was determined in aliquots of the total mammary gland homogenate and in both the pellet and supernatant after centrifugation at 100,000*g* for 40 min. This was accomplished by measuring the oxidation of ferrocytochrome *c* at 550 nm (25°C) according to the procedure of Cooperstein and Lazarow (10). A unit of enzyme activity was taken to be that defined by Wharton and Tzagoloff (11) (micromoles of cytochrome *c* oxidized per minute at infinite concentration).

Determination of Fructose 1,6-Bisphosphatase Activity. The activity of fructose 1,6-bisphosphatase (EC 3.1.3.11) was determined in mammary gland cytosolic fractions using the method of Pontremoli and Melloni (12). A unit of enzyme activity is defined as the amount which catalyzes the formation of 1 μmol of fructose 6-phosphate/min at pH 7.5.

Statistics. Data were analyzed by analysis of variance and Duncan's multiple range test. All statistical analyses were carried out using a statistical computer program (Statistical Analysis Software, PC ANOVA, Human Systems Dynamics, Northridge, CA) with a PC's Limited computer. Data points were expressed as mean ± SE and the level of significance was determined at $P < 0.01$.

Results

Guinea pigs in the various stages of the pregnancy-lactation cycle were sacrificed and the mammary glands dissected, blotted, and weighed. Homogenates were prepared and the various fractions obtained as outlined in Materials and Methods. For each of the animals, the following parameters were obtained: total gland wet weight, total gland cytosolic and mitochondrial PEPCK activity, and total gland fructose 1,6-bisphosphatase (cytosolic) activity.

In Figure 1, the total mammary gland wet weights (grams) are plotted against various stages of the pregnancy-lactation cycle. From the graph, it can be observed that the average gland weight is 4.5 ± 0.31 g during the prepartum stage and this increases about 4-fold ($P < 0.01$) early in lactation, reaching a peak of 18.6 ± 1.0 g by Days 5–8 of lactation. The gland weight remains high throughout the remainder of lactation and is on the decline by Days 17–24. This graph, which

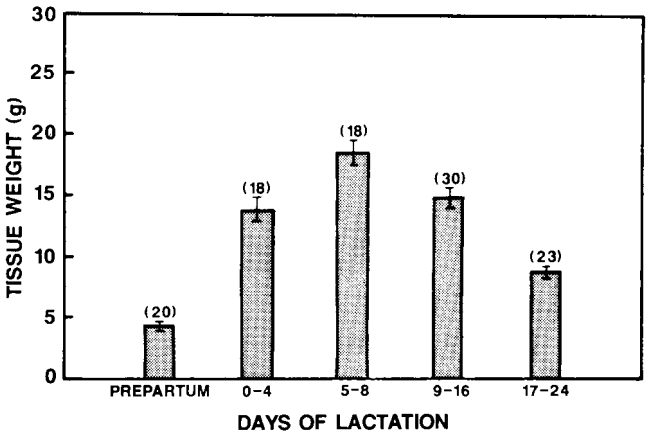

Figure 1. Gram wet weight of mammary glands versus periods of pregnancy-lactation. The average gram wet weights of the mammary glands were obtained immediately after dissection and blotting. Each point represents the mean ± SE for the number of animals in parentheses. The designated periods of pregnancy-lactation are 4 days each for the periods in early lactation and 8 days each for those in mid to late lactation. The early lactation periods are shorter since changes are more rapid during these times.

confirms results obtained previously by Ciaccio (13), approximates the lactation profile for the guinea pig.

For the determination of cytosolic and mitochondrial PEPCK activities, we initially employed standard centrifugation techniques to isolate the fractions (14). The mitochondrial fraction was sonicated to release the latent enzymatic activity and the PEPCK activity determined in the two fractions. A major problem arises with this method in that mitochondrial yields are usually only about 50–75% and are quite variable. In addition, cross-contamination of fractions occurs which must be assessed by measuring other marker enzymes. A method has been developed in guinea pig liver by Söling et al. (6) which eliminates these problems. This procedure, which employs deoxycholate solubilization of the mitochondrial membranes, is described in Materials and Methods.

First, it is important to establish that there is not a problem of cross-contamination between cytosolic and mitochondrial PEPCK activities when this method is employed with the mammary gland. Cytochrome *c* oxidase activity was measured in both the pellet and supernatant after homogenizing the tissue and centrifuging at 100,000*g* for 40 min. The percentage of the total cytochrome *c* oxidase activity in the cytosolic fraction (determined in 18 mammary gland preparations throughout the entire pregnancy-lactation cycle) ranges from 0 to 1. This demonstrates that there is negligible mitochondrial contamination of the cytosolic fraction with this procedure. In a study of PEPCK activity in guinea liver by Hamada and Matsumoto (15) using the Söling *et al.* (6) method, it was found that mitochondrial contamination of the cytosolic fraction was about 4%.

In addition, we have demonstrated that most of the mitochondrial PEPCK is solubilized by the deoxycholate treatment and is present in the supernatant after solubilization and centrifugation at 100,000g for 40 min. The average percentage of the total PEPCK activity in the supernatant was found to be 97 ± 0.28 based on determinations in five representative separations. Thus, it is reasonable to assume that the separation of cytosolic and mitochondrial PEPCK is essentially complete within experimental error.

The mitochondrial and cytosolic PEPCK activities plotted together against the various stages of the pregnancy-lactation cycle are shown in Figure 2. Values are presented as total units per whole mammary gland and have not been corrected for individual gland size differences. The reason for this is that mammary gland weight, total protein, and total DNA increase greatly after parturition (13) along with the various enzyme activities and, thus, if these parameters were used for correction, the magnitude of the enzyme activity increases would not be fully demonstrated. The mitochondrial PEPCK activity which is 0.24 ± 0.02 total units in the prepartum state increases by about 43-fold ($P < 0.01$) during early lactation, reaching a peak of 10.4 ± 0.95 total units by Days 5–8 of lactation. It remains high through midlactation and is on the decline by Days 17–24. With cytosolic PEPCK activity, the average value is 0.20 ± 0.04 total units during the prepartum state and this increases about 11-fold during early lactation, reaching an average peak value of 2.25 ± 0.27 total units by Days 5–8 of lactation.

The activities of both mitochondrial and cytosolic PEPCK, from the previous figure, are expressed as units per gram of tissue and presented along with values for the percentage of total activity as mitochondrial (Table I). There is about a 10-fold increase ($P < 0.01$) in the mitochondrial PEPCK activity per gram of tissue from

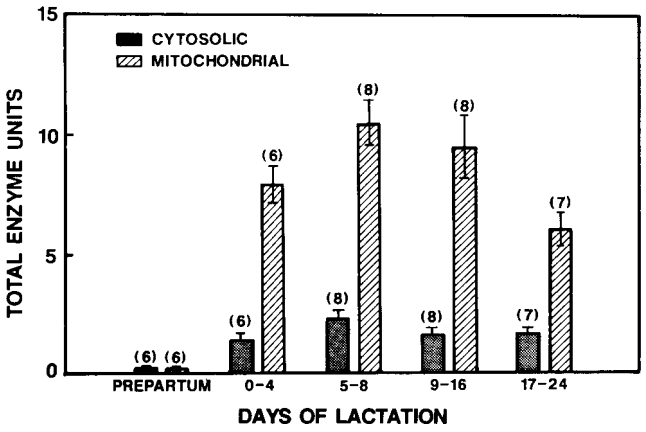

Figure 2. Total gland cytosolic and mitochondrial PEPCK activity throughout the pregnancy-lactation cycle. The average total gland cytosolic and mitochondrial PEPCK activities (enzyme units) were determined as outlined in Materials and Methods in each of the periods of pregnancy-lactation. Each point represents the mean ± SE for the number of animals in parentheses. The periods of pregnancy-lactation are the same as those in Figure 1.

Table I. PEPCK Units per Gram of Tissue and the Percentage of Total PEPCK as Mitochondrial versus Periods of Pregnancy-Lactation

Periods of lactation cycle	PEPCK (units/g tissue)		% as mitochondrial
	Cytosolic	Mitochondrial	
Prepartum	0.05 ± 0.01 (6)	0.06 ± 0.01 (6)	59 ± 3 (6)
0–4 days lactating	0.08 ± 0.01 (6)	0.47 ± 0.04 (6)	86 ± 2 (6)
5–8 days lactating	0.11 ± 0.01 (8)	0.52 ± 0.05 (8)	82 ± 2 (8)
9–16 days lactating	0.12 ± 0.03 (8)	0.67 ± 0.04 (8)	86 ± 3 (8)
17–24 days lactating	0.18 ± 0.03 (7)	0.58 ± 0.04 (7)	77 ± 3 (7)

Note. The average mammary gland cytosolic and mitochondrial PEPCK activities per gram of tissue (enzyme units) and the percentage as mitochondrial PEPCK were determined as outlined in Materials and Methods for each of the periods of pregnancy-lactation. Each value represents the mean ± SE for the number of animals in parentheses. The periods of pregnancy-lactation are the same as those in Figure 1.

prepartum to midlactation, but only a 2-fold increase ($P < 0.01$) in the cytosolic PEPCK activity per gram of tissue over the same time period. The mitochondrial PEPCK is 59 ± 3% of total activity in the prepartum gland and this increases to 86 ± 2% by Days 0–4 of lactation ($P < 0.01$).

Since PEPCK is generally considered a gluconeogenic enzyme, it is useful to determine whether other key gluconeogenic enzymes such as fructose 1,6-bisphosphatase increase in a parallel fashion during the transition into lactation in this tissue. It has previously been assumed that fructose 1,6-bisphosphatase is absent or is of very low activity in the mammary gland (4, 16). The fructose 1,6-bisphosphatase activity was determined in the mammary gland cytosolic fraction and the average, total mammary gland activities are plotted against the various stages of the cycle (Fig. 3). The values are plotted on the same scale as the PEPCK values in Figure 2 for comparison.

As can be observed, the enzyme is present at relatively low activity in the prepartum guinea pig mammary gland and then increases as it follows the lactation profile. From an average value of 0.89 ± 0.11 total units in the prepartum gland, the activity increases about 5-fold to a peak of 2.9 ± 0.33 total units by Days 5–8 of lactation ($P < 0.01$).

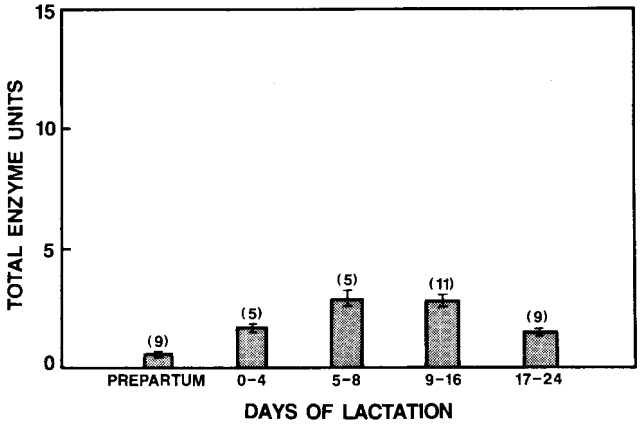

Figure 3. Total gland fructose 1,6-bisphosphatase activity throughout the pregnancy-lactation cycle. The average total gland cytosolic fructose 1,6-bisphosphatase activities (enzyme units) were determined as outlined in Materials and Methods in each of the periods of pregnancy-lactation. Each point represents the mean ± SE for the number of animals in parentheses. The periods of pregnancy-lactation are the same as those in Figure 1.

Discussion

During the transition into lactation in the guinea pig mammary gland, the cytosolic PEPCK activity increase is only twice that of general tissue expansion. The increase in mitochondrial PEPCK activity, on the other hand, is about 10 times that of tissue expansion. It is well known that during the transition into lactation in most mammals, there is a large expansion of mitochondrial function (17). This is due to an increase in the number of parenchymal cells, an increase in the number of mitochondria per cell, and to an expanded activity of each individual mitochondrion (18, 19). The increase in mitochondrial PEPCK appears to be about what would be expected for a mitochondrial enzyme during the transition into lactation in the mammary gland. The increase is actually somewhat greater than that of cytochrome *c* oxidase activity, a classical marker of the inner mitochondrial membrane, which also follows the lactation profile (Jones, D.H. and Schoelen, S.L., unpublished data). It appears reasonable to assume that mitochondrial PEPCK, along with other mitochondrial enzymes, is undergoing major enzyme induction during this period, whereas cytosolic PEPCK is only little more than keeping pace with the general tissue expansion. This would suggest that the major route of conversion of OAA to PEP in the lactating guinea pig mammary gland is through the mitochondrial enzyme and, thus, that this form plays the more important role in the lactation process.

We are assuming that the increases in PEPCK activity are a reflection of changes in the mammary epithelial (parenchymal) cell population. This appears to be justified since it is these cells which undergo the greatest increase in numbers during the transition into lactation (20). However, we cannot exclude the possibility that other cell types in the heterogeneous population may also be contributing to the change.

The preferential increase of the mitochondrial PEPCK in guinea pig mammary gland indicates that the control and function of the enzyme is different in mammary than it is in liver. Lardy and co-workers (21, 22) have shown that during starvation in guinea pig and rat, liver cytosolic PEPCK activity increases greatly while mitochondrial activity remains nearly unchanged. Many others have confirmed these results and it is now generally held that cytosolic PEPCK is the form which is induced in the liver in response to metabolic changes, the mitochondrial enzyme being largely constitutive (3). It is significant therefore that in the mammary gland it is the mitochondrial PEPCK which undergoes the greater change as it is induced, along with the other mitochondrial enzymes, by the hormones of the lactogenic process. We suggest that the guinea pig mammary gland may provide an excellent system for a study of the hormonal control of the mammalian mitochondrial PEPCK gene.

On the basis of our results, the induction of mammary gland PEPCK is different in the guinea pig than it is in the rat. It appears that in the mammary gland of the rat, cytosolic PEPCK is preferentially induced (2) whereas in the guinea pig gland it is mitochondrial PEPCK.

Although these studies do not establish the function of PEPCK in the mammary gland, we can suggest some possibilities. There are several possible functions which we believe we can reject on the basis of these and previous observations.

First, the role of PEPCK in the guinea pig mammary gland does not appear to be related to the release of glucose to the blood via gluconeogenesis. The entire gluconeogenic pathway is, apparently, not being induced in the mammary gland during the transition into lactation since we have shown that the activity of fructose 1,6-bisphosphatase is not changing in parallel with mitochondrial PEPCK. In addition, it has previously been determined that glucose 6-phosphatase activity is very low or is absent in the mammary gland of the rabbit and cow (23, 24). Assuming that this is true in the guinea pig as well, the direct release of glucose (formed by gluconeogenesis) into the blood from the mammary gland would not occur.

Second, mitochondrial PEPCK does not appear to have an important role in supplying glucose units for mammary glycogen synthesis. The synthesis of glycogen, although shown to occur in the mammary gland (25–28), was found to be maximal in late pregnancy and minimal during lactation (29). This does not fit our activity profile for PEPCK which follows the lactation cycle.

Third, based on studies in rat mammary gland, it appears unlikely that mitochondrial PEPCK is involved in providing glucose and/or galactose units for lactose synthesis. It has been clearly demonstrated that lactose is synthesized from free glucose and UDP-galactose (30). Since there appears to be little glucose 6-phosphatase present in the mammary gland (23, 24), it is certain that gluconeogenesis could not contribute to the glucose portion of lactose. It is doubtful that it contributes to the galactose portion either since Bartley *et al.* (31) found that when slices of lactating rat mammary gland were incubated with [2-^{14}C]acetate and [2-^{14}C]lactate, no incorporation of label into lactose occurred. This was taken to mean that no reversal of glycolysis at a level below the triose phosphates (gluconeogenesis) occurs in the lactating rat mammary gland. Lactate and acetate are believed to be important sources of lactose carbon in the intact animal only after conversion back to glucose by gluconeogenesis in the liver (32). Whether this observation would be the same in the guinea pig remains to be tested.

One possible function for PEPCK might be the generation of glycerol 3-phosphate for lipid synthesis via glycerogenesis from three-carbon intermediates. This appears to be a viable possibility since triglyceride synthesis is very important during milk production and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) is quite high in the guinea pig mammary gland during lactation as the enzyme follows the lactation profile (Jones, D.H. and Schoelen, S.L., unpublished data). The importance of glyceroneogenesis in the mammary gland is uncertain however, since it is generally held that, in this organ, glycerol 3-phosphate is synthesized almost entirely from blood glucose (1).

Carlsen *et al.* (33) have recently suggested that mitochondrial PEPCK may participate in hepatic lipogenesis. This would entail reversal of the PEPCK reaction with OAA being formed in the mitochondria from transported, extramitochondrial PEP. The mitochondrial OAA thus formed would then condense with acetate from acetyl CoA to form citrate for export to the cytoplasm. After cleavage, the acetyl CoA units would proceed on to fatty acid synthesis, the OAA being used to regenerate pyruvate for recycling back into the mitochondria to supply more acetyl CoA or OAA. This provides an attractive possibility for the function of mitochondrial PEPCK in the mammary gland in view of the large amount of lipid synthesis which occurs during lactation and the fact that the increase in mitochondrial PEPCK activity follows the lactation profile.

Another possible function, which was suggested by Lobato *et al.* (34), is that PEPCK may serve an anaplerotic role. This remains a viable possibility.

Finally, we would like to propose another function for mammary gland PEPCK which has not, as yet, been suggested. It is possible that PEPCK may be important in providing a source of PEP which is required for the synthesis of *N*-acetylneuraminic acid (sialic acid). Sialic acid, either free or polysaccharide bound, has been shown to be present in bovine and human milk (30) and also in various glycoproteins and glycolipids of the

milk fat globule membrane (35). Johnson *et al.* (36) have recently reported the isolation of a large glycoprotein called PAS-I from the fat globule membrane of guinea pig milk in which sialic acid accounts for 60% of the total sugar.

Sialic acid may serve an important membrane function in the mammary parenchymal cell, perhaps in the inner mitochondrial membrane. Söling and Kleineke (37) have demonstrated, in liver, that PEP formed from L-malate is a precursor of the sialic acid incorporated into the glycoproteins of the inner mitochondrial membrane. During the transition into lactation in the mammary gland, there is a very active mitochondrial inner membrane expansion (17) and, no doubt, a great increase in sialic acid synthesis. The enzyme PEPCK may function to supply the PEP necessary for sialic acid synthesis during the inner mitochondrial membrane expansion of the guinea pig mammary gland.

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