DEVELOPMENTAL CHANGES IN MITOCHONDRIA DURING THE TRANSITION INTO LACTATION IN THE MOUSE MAMMARY GLAND

II. Membrane Marker Enzymes and Membrane Ultrastructure

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ABSTRACT

The activity of cytochrome oxidase (an inner mitochondrial membrane marker) in mouse mammary gland homogenates was found to increase five- to sixfold from late pregnancy to day 8 of lactation, while that of monoamine oxidase (an outer membrane marker) increased only about 25%. The specific activity of cytochrome oxidase in the isolated mitochondria decreased slightly over the same period while the specific activity of monoamine oxidase decreased fivefold. This reflects the fact that both cytochrome oxidase and mitochondrial protein are increasing at a much greater rate than is monoamine oxidase activity. Mixing experiments preclude the possibility that the release or removal of an inhibitor or stimulator produces the changes in enzymatic activity.

The cytochrome oxidase to monoamine oxidase ratio was followed throughout the pregnancy-lactation cycle in total mammary homogenates, isolated mammary parenchymal cells, and isolated mammary mitochondria. In each preparation the pattern was the same with little change in the ratio until late pregnancy; and then a three- to fourfold increase occurred and the values reached a maximum by day 8 of lactation. These experiments were interpreted as demonstrating that the observed enzymatic changes are reflective of alterations in the mitochondria of the mammary parenchymal cell population.

Electron micrographs of mid-pregnant and mid-lactating mammary parenchymal cells *in situ* were prepared, and distinct changes in the mitochondrial morphology noted. The most significant and obvious change is the large increase in the number of inner membrane cristae and an increase in matrix density in the lactating gland cell. Therefore, both enzymatic and morphological studies support the concept of an expansion of the mitochondrial inner membrane during presecretory differentiation in the mouse mammary parenchymal cell.

One of the most plausible explanations for the difference in the behavior of pregnant and lactating mouse mammary gland mitochondria on isopycnic gradient centrifugation is an expansion of the inner membrane during the transition from pregnancy to lactation. We have demonstrated in an earlier study (8) that several key enzyme activities associated with the mitochondrial inner membrane increase as much as fivefold during the first few days of lactation. The period of the enzyme increases corresponds exactly to the mitochondrial density increases on sucrose gradient centrifugation which were demonstrated in the preceding paper of this series (13). It appears reasonable to assume, therefore, that the behavior on sucrose gradient centrifugation and the inner membrane enzyme changes are reflecting the same phenomenon, that of a major expansion of the mitochondrial inner membrane and possibly matrix material.

To further assess this hypothesis, we compared enzyme markers for mitochondrial inner and outer membranes throughout the pregnancy-lactation cycle. In addition, the ultrastructural changes of the organelle during the transitions were studied by electron microscopy.

MATERIALS AND METHODS

Preparation of Homogenates and Mitochondria

Homogenates and mitochondria were prepared from the mammary glands of BALB/c strain mice during various stages of the pregnancy-lactation cycle as described in the preceding paper (13).

Isolation of Mammary Parenchymal Cells

The techniques for the isolation of the parenchymal cells from fat cells and connective tissue have been described previously (8).

Enzyme Assays

The method for determination of monoamine oxidase (EC 1.4.3.4) was the same as that described previously (13). The determination of cytochrome oxidase (EC 1.9.3.1) was essentially the same, except that Lubrol WX (I. C. I. America Inc., Stamford, Conn.) was used to solubilize latent enzymatic activity instead of the freezing-thawing technique.

Kynurenine-3-hydroxylase (EC 1.14.1.2) activities were determined spectrophotometrically by the method of Hayaishi (5) which measures the disappearance of NADPH at 340 nm in the presence of L-kynurenine.

Electron Microscopy

Fresh preparations of mammary tissue were fixed for 1 h in a solution containing 4% glutaraldehyde, 0.45% H_2O_2, 7.5% sucrose, and a 0.1 M cacodylate buffer (pH 7.4). Tissue preparation involved fixation with 1% osmium tetroxide in Dalton's (potassium dichromate) buffer, dehydration, embedding with Spurr low viscosity media, and staining with lead. Preparations were then examined and photographed with an RCA EMU 4R electron microscope.

Analytical Methods

Protein determination was carried out by the procedure of Lowry et al. (10) as described previously (13).

RESULTS

To study changes in the mitochondrial inner to outer membrane ratio of the mouse mammary gland during the pregnancy-lactation transitions, cytochrome oxidase and monoamine oxidase activities were determined throughout the cycle. Cytochrome oxidase activity has been shown to be related exclusively to the inner membrane (9, 15) and monoamine oxidase exclusively to the outer membrane and cytoplasm (15, 14). The two enzymes are thus commonly employed as mitochondrial inner membrane and outer membrane markers, respectively. In addition, these two activities can be measured in very small samples by sensitive spectrophotometric and fluorometric techniques and thus were well suited for our purposes. We have demonstrated that both enzymes travel with the major mammary mitochondrial protein band on sucrose density gradient centrifugation (13).

Cytochrome oxidase and monoamine oxidase activities were determined in the mammary gland homogenates of mice in all stages of the pregnancy-lactation cycle. Fig. 1 is a graphic representation showing profiles of the total gland activity for both enzymes throughout the cycle. The data are compiled from determinations in about 80 animals. The activity of cytochrome oxidase shows approximately a twofold increase from the virgin stage to mid-pregnancy. A further five- to sixfold activity increase begins just before parturition and is completed on or before the 8th day of lactation. During retrogression a fall to preparturition activity levels was observed. Values in late pregnancy were found to be significantly different ($P < 0.001$) than values in early, mid, or late lactation. The activity profile for the outer membrane enzyme, monoamine oxidase, however, does not follow a pattern similar to that for cytochrome

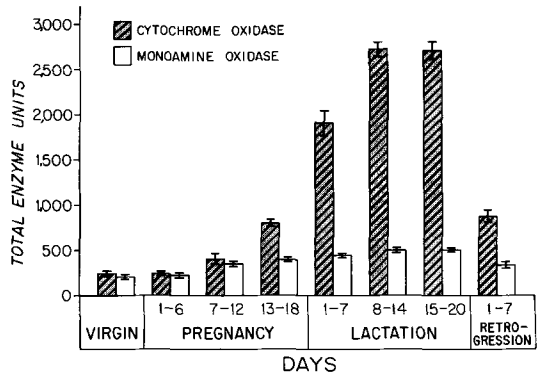

FIGURE 1 Profiles of total tissue activity of cytochrome oxidase and monoamine oxidase during the pregnancy-lactation cycle. The enzymes were determined as described in Materials and Methods and converted to activites in the total gland homogenate. The values are plotted as units per total homogenate. Values represent the mean ± standard error of the mean (SEM) for a minimum of four experiments with an average 10.

oxidase. Monoamine oxidase activity shows an approximately twofold increase from virgin to mid-pregnancy but only a 25% increase from late pregnancy to mid-lactation. Therefore, during the period of the major increase in cytochrome oxidase (late pregnancy to 8 days lactation), relatively little change was observed in the activity of monoamine oxidase.

The specific activities of cytochrome oxidase and monoamine oxidase were determined in the mitochondrial fractions and plotted against days of the pregnancy-lactation cycle. The profiles obtained are shown in Fig. 2. Mean values of cytochrome oxidase specific activity were approximately 20% lower after parturition. Since it has been established that total cytochrome oxidase activity increases greatly during early lactation, these data indicate that mitochondrial protein is expanding at an even greater rate. In the case of monoamine oxidase, a fivefold fall in specific activity is observed between late pregnancy and mid-lactation. This reflects the fact that mitochondrial protein is increasing greatly while monoamine oxidase is changing only slightly.

When large changes in tissue enzymatic activities are observed, such as was found with cytochrome oxidase, the release or removal of an inhibitor or stimulator is suspected. This possibility can be assessed by mixing experiments in which a preparation with high specific activity is added to one of low activity and the activity of the mixture determined. If the value obtained is additive of the two individual activities, this is considered evidence against the presence of an inhibitor or activator in either preparation. Cytochrome oxidase and monoamine oxidase activities were measured, as described in Materials and Methods, in the total homogenates of glands from animals in late pregnancy and mid-lactation and in mixtures of the two. The results of a representative mixing experiment with each enzyme are shown in Table I, and these indicate that, with both, the activities are clearly additive. Therefore, the changes in the

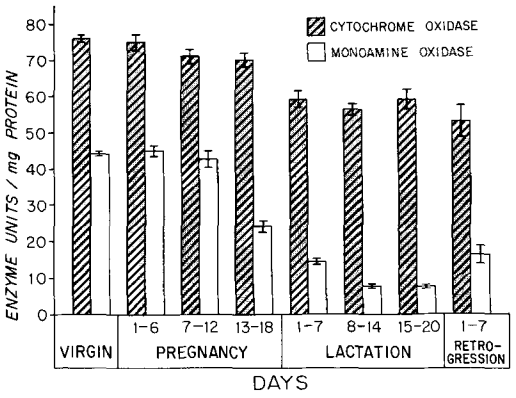

FIGURE 2 Specific activities of cytochrome oxidase and monoamine oxidase in the isolated mitochondrial fraction during the pregnancy-lactation cycle. The enzyme activities and protein values were determined as described in Materials and Methods. Values are plotted as units per milligram of mitochondrial protein and represent the mean ± SEM for a minimum of three experiments with an average of seven.

TABLE I

Additive Nature of Cytochrome Oxidase and Monoamine Oxidase Activities in Total Gland Homogenates

Preparation assayed	Cytochrome oxidase	Monoamine oxidase
	total U	*total U*
A pregnant	420	230
B lactating	1,900	580
A + B total	2,320	810
C mixture	2,250	820

Monoamine oxidase activity was determined in preparations diluted 1/1 or in a 1/1 mixture of the two preparations by techniques described in Materials and Methods. The activities are expressed as enzyme units per total homogenate.

enzyme activities appear to reflect true differences in tissue enzyme content and are not due to the effect of an inhibitor or stimulator.

In order to more clearly illustrate the change in the relative content of inner and outer mitochondrial membranes during the transition period, we determined the ratios of cytochrome oxidase to monoamine oxidase and plotted them as a function of the stage of pregnancy or lactation. These determinations were performed in total homogenates, isolated parenchymal cells, and total mitochondrial pellets from mammary glands in all stages of the cycle (Fig. 3). Using such profiles, we can determine precisely the point at which cytochrome oxidase activity increases in relation to the monoamine oxidase activity.

In the homogenate there appears to be little change in the ratio until late pregnancy, and then a three- to fourfold increase occurs reaching a peak by about day 8 of lactation. The values in mid-lactation (8–14 days) were significantly higher ($P < 0.001$) than values in late pregnancy (13–18 days). During late lactation and early regression the ratio declined. This general profile was the same for the isolated parenchymal cells, with an approximately threefold increase in the ratio occurring during the late pregnancy to early lactation period. Because of the limited amount of data in this profile, a statistical analysis was not performed. Nevertheless, the trend is clearly discernible and indicates that the total mitochondrial enzyme changes observed are due to changes in the parenchymal cell population. Finally, the profile of the enzyme ratios in the isolated mitochondria is similar to the other two. The absolute values of the ratios were slightly higher in the mitochondrial fractions. This may be due to different yields of the two enzymes in the mitochondrial fractions, the outer membrane enzyme being the more susceptible to loss. Again, the values in mid-lactation are significantly higher ($P < 0.001$) than values in late pregnancy.

In an attempt to study another marker of mitochondrial outer membrane, we measured the activity of kynurenine-3-hydroxylase in pregnant and lactating mammary gland homogenates and mitochondrial fractions. In rat liver the enzyme has been shown by several workers (2, 12) to be present and to be localized in the outer mitochondrial membrane. In our mammary tissue preparations, we found no significant activity of the enzyme. In mouse liver mitochondria, we measured levels of kynurenine-3-hydroxylase in the range of 1.3–1.7 U/mg protein. These values agree fairly well with published data on rat liver mitochondria (2, 12, 4). It appears, therefore, that this enzyme is either not present in the mouse mam-

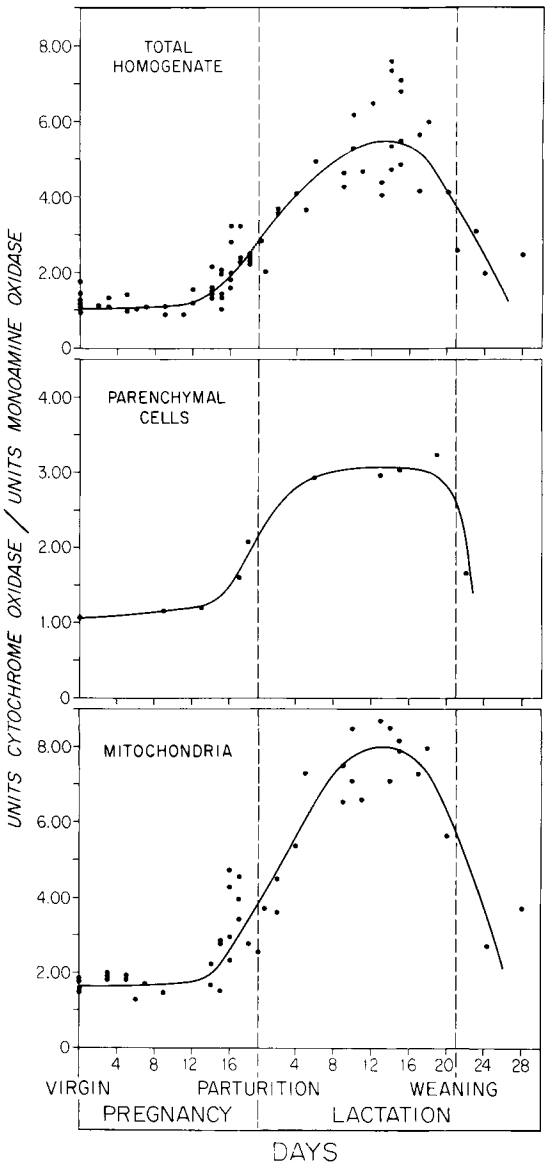

FIGURE 3 Ratio of cytochrome oxidase activity to monoamine oxidase activity in mammary gland homogenates, parenchymal cells, and mitochondrial fractions during the pregnancy-lactation cycle. The various preparations were obtained and enzyme activities determined as described in Materials and Methods. The ratios of the two enzyme activities were plotted in relation to the stage of the lactation cycle.

mary gland or was below the level of detection by our techniques. We were thus unable to use it as a marker for the outer mitochondrial membrane.

As a final criterion of the mitochondrial membrane changes, ultrastructural studies were carried out with electron microscopy. Undifferentiated parenchymal cells from the mammary gland of a 12-day pregnant mouse and fully differentiated parenchymal cells from the gland of a 12-day lactating mouse were examined. After sacrificing the animals by neck fracture, a piece of the inguino-abdominal glands was immediately removed and fixed as described in Materials and Methods. Several sections from different blocks were observed under low power, and the glandular parenchymal cells were identified and photographed. This was necessary to assure that the mitochondria under study were clearly from the parenchymal cell population. Higher power (× 30,000 to 40,000) magnification was then used to examine the cellular ultrastructure, with special emphasis on the mitochondria.

Fig. 4 *a* is a representative electron micrograph of a portion of a mammary gland parenchymal cell from a 12-day pregnant mouse. The mitochondria (in cross section) appear to be generally round or ovoid in shape with relatively few cristae (inner membrane invaginations) present. In the micrograph of a 12-day lactating gland parenchymal cell (Fig. 4 *b*), the mitochondria show a much more extensive inner membrane development. A marked increase in the number of cristae is noted, resulting in a very dense appearing internal structure. There also appears to be an increase in the density of the organelle matrix material. The mitochondria (in cross section) are now predominantly rodlike with only a limited number of round or ovoid forms present. A comparison of organelle sizes during the different stages was not attempted because of the differences in shape and because of a lack of three-dimensional information.

DISCUSSION

Our observations which demonstrate about a 10-fold increase in total mammary cytochrome oxidase activity during the entire pregnancy-lactation transition correlate well with other studies of changes in mitochondrial inner membrane enzymes in mammary glands of the mouse (8), the rat (1, 3), and the guinea pig (11). All of the studies suggest that these enzymes increase as part of an overall expansion of oxidative metabolism in response to the large energy demands of the lactation period. Accompanying the increase in mitochondrial inner membrane activities there is an equivalent increase in total mitochondrial protein (8). It is therefore concluded that the observed changes in enzyme activities are due to an overall mitochondrial development and are not just selective increases of the individual enzymes.

In this communication, we have presented evidence which indicates that the mitochondrial development from late pregnancy to mid-lactation involves an expansion of the inner membrane, with the outer membrane increasing only slightly. Part of the evidence supporting this contention is the relatively small increase in monoamine oxidase during the period when cytochrome oxidase is increasing five- to sixfold. The profiles of specific activities of the two enzymes showed a slight (20%) drop in cytochrome oxidase and a four- to fivefold drop in monoamine oxidase. This reflects the fact that mitochondrial protein and cytochrome oxidase are increasing at a much greater rate than monoamine oxidase.

The increase in the ratio of cytochrome oxidase to monoamine oxidase was found to be essentially identical in the total gland homogenate, the isolated mammary parenchymal cells, and the isolated mitochondrial fraction. These experiments clearly delineate that the period of inner mitochondrial membrane expansion is from late pregnancy to about day 8 of lactation, and, also, that the enzymatic changes observed in the total gland homogenates truly reflect changes in the mitochondria of the parenchymal cell population.

Additional evidence supporting the contention that the inner mitochondrial membrane expands during the transition is provided by electron micrographs of the organelles *in situ*. Mitochondrial structure was shown to be significantly changed as a result of the pregnancy-lactation transition.

Our electron microscope findings which demonstrate a general change to more rodlike organelles with a greatly expanded inner membrane are in general agreement with observations in the mouse mammary gland by other workers. In early light microscopy studies, Weatherford (16) found that during functionally quiescent states the mammary mitochondria were small and round in shape. On the other hand, mitochondria from actively secreting cells during lactation were large and filamentous. In later electron microscope investigations, Wellings et al. (17) observed that, compared to the prelactating state, the lactating gland mitochondria have more conspicuous cristae and more

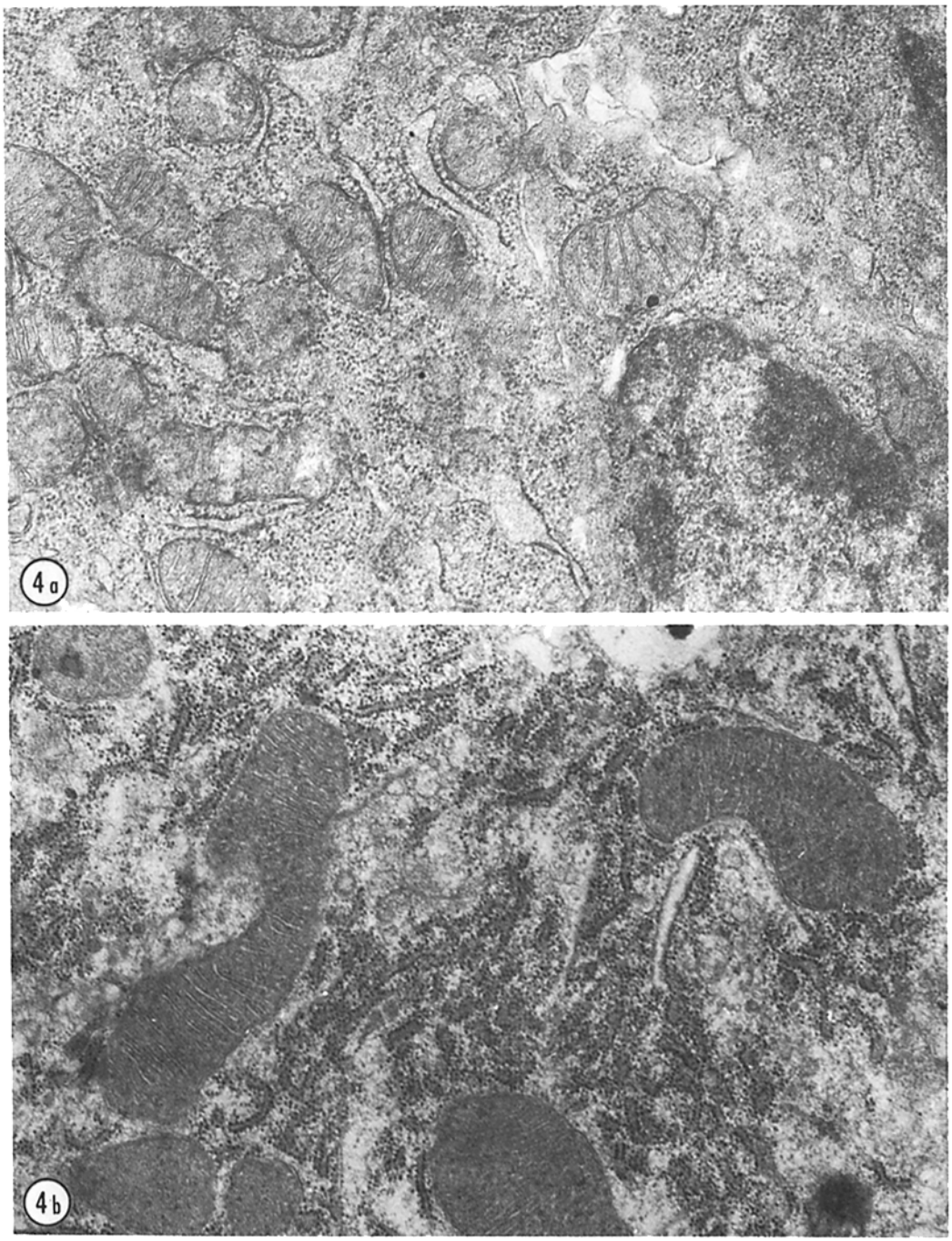

FIGURE 4 Electron micrographs showing mitochondria from mammary gland parenchymal cell sections of mice at two stages of the pregnancy-lactation cycle. Tissue preparation, sectioning, and microscopy were performed as described in Materials and Methods. Magnification of both micrographs is × 40,000. (*a*) 12-day pregnant mouse and (*b*) 12-day lactating mouse.

dense internal matrices. Hollman (6) observed a significant increase in inner membrane cristae during the lactating state.

The period of the enzymatic and morphological changes reported in this communication corresponds closely to that of the organelle density increases on sucrose gradient centrifugation demonstrated in the preceding paper (13). Therefore, three lines of evidence (ultracentrifugation, enzymatic, and morphological) appear to substantiate the concept of an expansion of the mitochondrial inner membrane during the transition into lactation in the mouse mammary gland.

Several workers have demonstrated (16, 17, 6, 7) that the numbers of mitochondria per mammary parenchymal cell increase throughout pregnancy and into early lactation. It has also been well established that the parenchymal cell population increases greatly from early pregnancy until about parturition (8, 6). It is obvious therefore that total mitochondrial numbers in the mammary tissue are increasing greatly during this period of major cell proliferation. Along with this increase in the mitochondrial population we have shown that there are significant increases in the oxidative capacity of individual organelles due to the expansion of the inner membrane. This expansion, however, does not parallel the period of major cellular and mitochondrial replication but is more directly related to the cell changes which occur just before secretory function. This appears to be a type of organelle maturation or differentiation which is part of epithelial cell differentiation.

The mechanism by which the internal structure of the mitochondrion is altered has not been fully elucidated by these studies. Two general schemes are plausible. First, there may be the production of a new population of mitochondria during the transition into lactation. This new population would possess a different shape and a more complex internal structure than the organelle which predominates during the virgin and early pregnancy stages. On the basis of this mechanism, one would expect to find at least two different mitochondrial populations coexisting during the transition period. One population would possess the properties characteristic of the nonlactating gland organelle, i.e., less dense on sucrose gradient, simpler internal structure, low ratio of cytochrome oxidase to monoamine oxidase. The new population would be more dense on sucrose gradient, have a more complex internal structure, and show a high ratio of cytochrome oxidase to monoamine oxidase. The second possibility is that the entire mitochondrial population matures during differentiation in the pregnancy-lactation transition. This maturation would involve an expansion of the inner membrane.

With either mechanism, one would expect to find an increase in the ratio of cytochrome oxidase to monoamine oxidase activities around the time of parturition. The statistical averaging effect of the enzyme measurements would preclude a distinction between the two theories on the basis of enzymatic data. In the case of the ultracentrifugation studies (13), it seems that, if different populations of mitochondria coexist during the transition period, it should be possible to resolve at least two distinct bands on sucrose density gradient ultracentrifugation. We observed, however, only one band in any stage of development, and this single band increased in density during the transition period. In addition, there was no evidence on electron microscopy of any significant morphological heterogeneity in any given stage of gland development. It appears, therefore, that our findings tend to support the concept of overall organelle maturation during cellular differentiation rather than development of a new, more active mitochondrial population.

Regardless of the exact mechanism, these studies have shown that major changes in the physical, enzymatic, and structural composition of the mitochondrial population take place within the mammary parenchymal cells during the changing cell physiology. Therefore, the genetic system of the cell appears to have the ability to produce an organelle tailored to meet specific metabolic demands.

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