

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

## I. Behavior on Isopycnic Gradient Centrifugation

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### ABSTRACT

Mitochondrial biogenesis in the parenchymal cell of the mouse mammary gland appears to occur in two distinct phases: replication during cell proliferation, and maturation during cell differentiation. This study of the mitochondrial maturation phase in the mouse gland demonstrates a significant increase in organelle density on isopycnic sucrose gradient centrifugation during the transition from late pregnancy to day 8 of lactation. Differential fragility to high sucrose concentrations or changes in mitochondrial lipid composition do not satisfactorily explain the density increases.

When organelle densities were assessed by centrifugation under iso-osmotic conditions with Ficoll gradients in 0.25 M sucrose, the mitochondria from pregnant glands were observed to be more dense than those from lactating glands. The two mitochondrial populations were also found to differ in their response to changes in sucrose concentration in the Ficoll gradients. When sucrose concentration was increased, the density of both pregnant and lactating gland mitochondria increased nonlinearly, the increase being greater with the lactating gland organelles. By use of mathematical models, the differing response was interpreted as a change in the density and osmotic activity of the mitochondrial internal compartment (inner membrane plus matrix space). We have proposed that the changes reflect a large expansion of the inner mitochondrial membrane and perhaps the matrix material during the transition into lactation in the differentiating parenchymal cell.

During transition into lactation in the mammary gland, extensive growth and development of the tissue occurs in response to hormonal influences. In the mouse, the proliferation of mammary epithelial (parenchymal) cells begins early in pregnancy and is essentially complete by parturition or shortly thereafter (15, 3, 18, 13). Towards mid to

late pregnancy these cells begin differentiating to form the mature alveolar cells characteristic of the lactating gland. This phase is complete several days after the start of lactation.

The alveolar cell population which emerges upon differentiation has a greatly expanded capacity for oxidative metabolism as reflected in tissue

respiratory quotient (10). The increased oxidative metabolism which is necessary to fulfill the vast energy demands of lactation is obviously due to increased mitochondrial function. These developmental stages are therefore periods of active mitochondrial replication and expansion and provide an excellent opportunity to study the phenomenon of mitochondrial biogenesis in a mammalian system.

Observations in our laboratory (13) indicate that the major increases in mitochondrial protein and functional activities occur during the first 6–8 days of lactation which is subsequent to major cell proliferation. These increases, then, are apparently due to differentiation of individual parenchymal cells which may bring about a general maturation of mitochondria or the generation of a new highly active mitochondrial population or both.

Attempts were made to determine whether a new distinct mitochondrial population was emerging or whether the increases could be explained on the basis of a general organelle maturation. To this end, mitochondria isolated during the various stages of the pregnancy-lactation cycle were subjected to density gradient centrifugation in an attempt to resolve distinct mitochondrial populations or to assess changes in overall organelle density. The following is a summary of our findings.

## MATERIALS AND METHODS

### *Preparation of Homogenates and Mitochondria from Mammary Glands*

BALB/c strain mice were bred and females (3–5 mo old) in various stages of pregnancy and lactation obtained. During lactation, litter size was maintained at 5–7 pups.

Animals were sacrificed, glands dissected, and homogenates and mitochondria prepared as previously described (13). The isolated mitochondria were shown by respiratory control ratios, ADP:O ratios and electron microscopy to be intact, functional preparations.

### *Gradient Ultracentrifugation*

Continuous sucrose density gradients (5.0 ml, 1.0 → 1.6 M) were formed, using a Buchler gradient former (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N.J.). In the case of Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), gradients containing increasing amounts of Ficoll and uniform amounts of sucrose were prepared. The maximum and minimum concentrations of Ficoll were 20–30% and 0–10%, respectively, the amount varying with sucrose concentration.

Mitochondrial samples (2–3 mg protein) were layered on top of the continuous density gradients, and centrifugation was performed in a Beckman Model L-2 ultracentrifuge with an SW-39 swinging bucket rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). Unless otherwise stipulated, gradients were centrifuged to isopycnic equilibrium (1 h at 100,000 g, 4°C).

The gradients were fractionated, using a Buchler tube puncturing device, and densities at 20°C determined with a Fisher refractometer (Fisher Scientific Co., Pittsburgh, Pa.). The relationship of refractive index to the density of sucrose solutions was obtained from published tables (12). For solutions containing sucrose plus Ficoll, the relationship of the refractive index to density was determined from standard solutions with known densities of sucrose plus Ficoll.

### *Enzyme Assays*

The activity of cytochrome oxidase (EC 1.9.3.1) was determined by the procedure of Cooperstein and Lazarow (6), using a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The reduction of cytochrome *c* was performed by the method of Smith (20). The samples from the gradient were frozen and slowly thawed three times to liberate latent enzyme activity, and appropriate dilutions made in 30 mM phosphate buffer (pH 7.4). A unit of enzyme activity is the quantity of enzyme that produces 1 Δ log OD/min.

Monoamine oxidase (EC 1.4.3.4) activity was measured by an adaptation of the fluorometric procedure of Krajl (14) with a modification suggested by Century and Rupp (4). An appropriately diluted enzyme sample was incubated in 2.5 ml of 0.1 M phosphate buffer (pH 7.4) for 5 min at 37°C; 0.5 ml of kynuramine dibromide (20 mg % wt/vol) was then added, and the sample was mixed. After 30 min, the reaction was terminated by the addition of 2 ml of 0.6 M perchloric acid. After centrifugation, the samples were read in a Baird-Atomic model SF-1 fluorescence spectrophotometer (Baird Atomic, Inc., Bedford, Mass.) with excitation at 315 nm and emission at 380 nm. A unit of enzyme activity is defined as that amount of enzyme that will produce 1 μg of 4-hydroxyquinolone in 1 h.

Succinate-β-hydroxybutyrate-, or glutamate-linked respiratory control ratios and ADP:O ratios were determined polarographically by an adaptation of the procedure of Estabrook (8). Modification of the procedure involved the addition of 2 mM ethylene diamine tetraacetic acid (EDTA), 7 mM MgCl<sub>2</sub>, and 4-mg bovine serum albumin to the standard reaction mixture. Oxygen consumption was monitored with a Gilson Medical Electronics (GME) oxygraph (Gilson Medical Electronics, Inc., Middleton, Wisc.) with a "Clark-type" oxygen electrode assembly (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Protein was determined by the method of Lowry et al. (16) or by absorbance at 280 nm. For the phospholipid

determinations, the samples were extracted according to the procedure of Folch et al. (9). Phospholipid phosphorus was then cleaved off and analyzed by the Bartlett procedure (1).

## RESULTS

The initial organelle density experiments involved localization of the mitochondrial band after centrifugation to isopycnic equilibrium on a linear sucrose gradient. Samples of isolated mammary mitochondria were applied to the top of the gradients and centrifuged as described in the Materials and Methods section. Fig. 1 shows the results of an experiment with mitochondria from the mammary gland of a 14-day lactating mouse. Represented are the profiles of cytochrome oxidase activity, monoamine oxidase activity, absorbance at 280 nm, and density along a sucrose gradient after centrifugation and fractionation. Both mitochondrial enzymes demonstrated a single band of activity at a mean density of approx. 1.198 g/cm<sup>3</sup>. Recovery of cytochrome oxidase activity was found to be greater than 85% of the amount applied to the gradient. Absorbance at 280 nm showed that the major protein band corresponded exactly to the bands of the enzyme activities.

Mitochondria isolated from glands at other

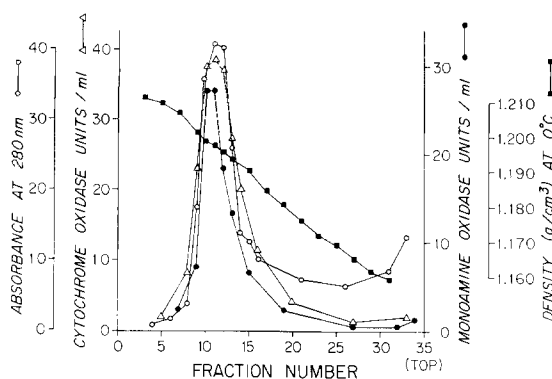


FIGURE 1 Distribution of cytochrome oxidase and monoamine oxidase activities, absorbance at 280 nm and density along a sucrose gradient. Sucrose gradient formation, ultracentrifugation, fractionation, enzyme assays, and density determinations were performed as described in Materials and Methods. Absorbance at 280 nm was determined after an appropriate dilution (1/15-1/40) of the sample with 30 mM PO<sub>4</sub> buffer (pH 7.4). The values were then expressed as absorbance of the total 150  $\mu$ l fraction. Enzyme activities were expressed as units/milliliter.

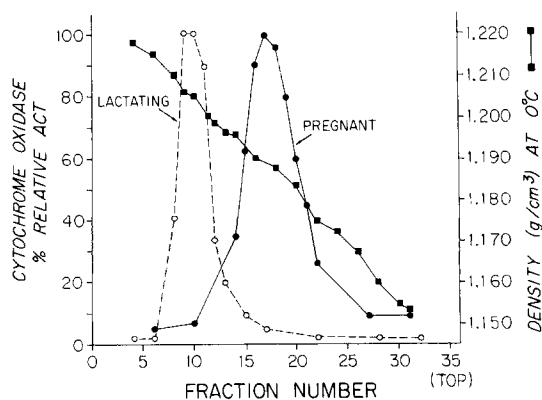
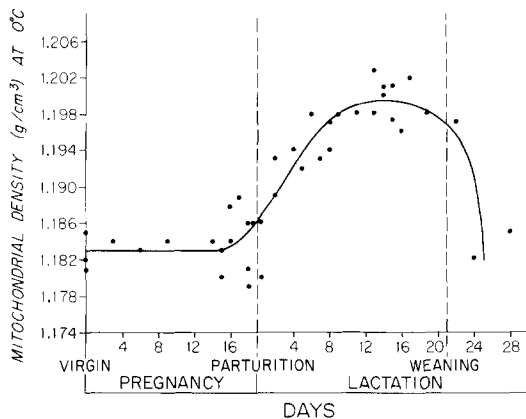


FIGURE 2 Superimposed profiles of the distribution of the cytochrome oxidase activities of pregnant and lactating gland mitochondria after sucrose gradient centrifugation. Mitochondria from 17-day pregnant and 14-day lactating glands were layered onto separate essentially identical sucrose gradients. Ultracentrifugation, fractionation, enzyme assays, and density determinations were performed as described in Materials and Methods. Cytochrome oxidase activities were expressed as percent of activity relative to the most active fraction.

stages of the pregnancy-lactation cycle also exhibited a single band during the sucrose density centrifugation, but the position of density equilibration differed during different stages of the cycle. Mitochondria from 17-day pregnant and 14-day lactating glands were subjected to centrifugation on identical sucrose gradients. When the cytochrome oxidase activities were analyzed, values converted to percentage of maximum, and the curves graphically superimposed as in Fig. 2, the density of the lactating gland mitochondria can be seen to be greater than that of the corresponding pregnant gland mitochondria. The mean values are 1.188 g/cm<sup>3</sup> and 1.203 g/cm<sup>3</sup> for the pregnant gland and lactating gland mitochondria, respectively.

To determine the profile of mitochondrial density during the entire pregnancy-lactation cycle, sucrose gradient centrifugation experiments similar to those described above were performed on mammary mitochondria obtained from mice throughout the period. The mean density of the mitochondrial band was determined at each stage, and the values were plotted against days of pregnancy and lactation. The results are shown in Fig. 3. From the virgin state to parturition, the values remained about the same with a mean density of 1.183 g/cm<sup>3</sup> (SD of  $\pm 0.003$  g/cm<sup>3</sup>). During a transition period from just before parturition to



**FIGURE 3** Buoyant density of mammary mitochondria on sucrose gradients during different stages of the lactation cycle. Sucrose gradient formation, ultracentrifugation, fractionation, and density determinations were performed as described in Materials and Methods. The mean density of the mitochondrial band was calculated and plotted against the stage of the cycle.

about day 8 of lactation, the mitochondria steadily increased in density. From day 8 to 20 of lactation, the mitochondria had a mean density of  $1.199 \text{ g/cm}^3$  (SD of  $\pm 0.002$ ). Although only a few experiments were performed with retrogressed animals, there appeared to be a return to the less dense mitochondrial form after weaning. During all the stages, only a single band of mitochondrial activity was found after centrifugation.

To assess whether the density change might possibly be due to damage of either the pregnant or lactating gland mitochondria during sucrose gradient centrifugation, the succinate-linked respiratory control ratio was determined before and after gradient centrifugation. The mitochondrial fraction was collected from the gradients, diluted with  $0.25 \text{ M}$  sucrose ( $5 \times 10^{-3} \text{ M}$  EDTA, pH 7.4), pelleted ( $10,000 \text{ g}$ ,  $10 \text{ min}$ ), and resuspended in the sucrose-EDTA solution to a concentration of  $10\text{--}15 \text{ mg protein/ml}$ . Both this gradient-exposed mitochondrial fraction and a control mitochondrial preparation were then analyzed for respiratory control on the GME oxygraph. Two experiments were performed on mitochondria from glands in late pregnancy, and values for respiratory control ratio of 3.5 and 3.0 were obtained. The values in both cases were the same for the original preparation and the fraction off the sucrose gradient. Similarly, three experiments from glands in mid-lactation were performed, with respiratory control ra-

tios of 2.4, 2.2, and 2.1 being obtained. Again, subjecting the mitochondria to sucrose gradient centrifugation did not change the respiratory control ratios. We were limited by the amount of mitochondria obtainable from a single mouse to the one substrate. However, on the basis of these results, the mitochondrial integrity appears not to have been affected adversely by sucrose gradient centrifugation.

Another possible explanation for the change in mitochondrial density may be that it is due to a shift in the relative content of lipid to protein. To investigate this possibility, the phospholipid/protein ratio was measured in mitochondrial fractions before and after sucrose gradient centrifugation in both pregnant and lactating gland mitochondria. Since phospholipids are the predominant mitochondrial lipids, changes in the relative content of these constituents are thought to reflect alterations in overall lipid composition (11, 5). The determination of microgram phospholipid phosphorus/milligram protein on 12 different preparations gave average values of  $14.6 \pm 0.3$  and  $15.3 \pm 0.5$  for mitochondria from pregnant and lactating glands, respectively. When the unpaired data were analyzed by the Student's *t*-test, it was found that there is no significant difference ( $P > 0.3$ ) between the ratios of pregnant and lactating gland mitochondria. It appears, therefore, that during the period of mitochondrial density increase (late pregnancy to mid-lactation) no correlating change in the ratio of lipid to protein occurs.

It was next reasoned that the density differences may be the result of differing effects of high sucrose concentration on pregnant and lactating gland mitochondria due to changes in osmotic properties. To assess the organelle densities under iso-osmotic conditions, mitochondria from pregnant and lactating glands were subjected to isopycnic gradient centrifugation using Ficoll gradients with a constant amount of iso-osmotic sucrose ( $0.25 \text{ M}$ ). Mitochondria isolated from mammary glands during various stages of the pregnancy-lactation cycle were subjected to Ficoll gradient centrifugation, and the mean density was determined as described in the sucrose gradient experiments. In Fig. 4 the profiles of cytochrome oxidase activity (converted to percentage of maximum) from the pregnant and lactating gland mitochondria are graphically superimposed. The mean density values can be seen to be  $1.143 \text{ g/cm}^3$  and  $1.127 \text{ g/cm}^3$  for the pregnant gland and lactating

gland mitochondria, respectively. Each gradient exhibits only one band of mitochondrial activity.

When the Ficoll experiments were repeated, this time in the presence of hyperosmotic sucrose (1.0 M), a striking difference in the banding pattern was observed. The results, plotted as before, are shown in Fig. 5. Each mitochondrial preparation still exhibits only one band, but the lactating gland organelles are now the more dense with a mean density of 1.187 g/cm<sup>3</sup> vs. 1.171 g/cm<sup>3</sup> for those of the pregnant gland. This is similar to the pattern observed on sucrose gradients.

Table I is a tabulation of a number of determinations of mammary mitochondrial densities on Ficoll gradients in both 0.25 M and 1.0 M sucrose.

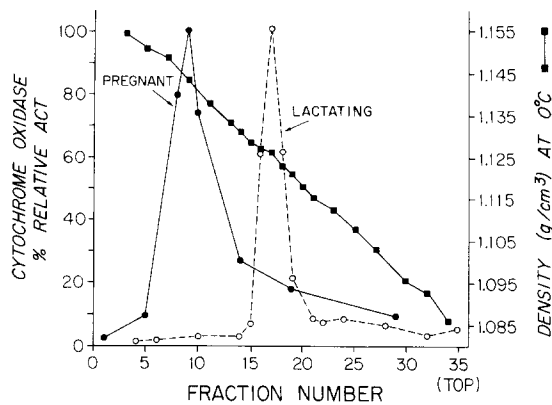


FIGURE 4 Superimposed profiles of the distribution of the cytochrome oxidase activities of pregnant and lactating gland mitochondria after centrifugation in Ficoll gradients containing iso-osmotic (0.25 M) sucrose. Mitochondria from 17-day pregnant and 14-day lactating glands were layered onto separate, essentially identical Ficoll gradients. The ultracentrifugation procedure and representation of results are similar to those in Fig. 2.

The experiments cover the span from mid-pregnancy to late lactation. In the Ficoll gradients with 0.25 M sucrose, the decrease in mitochondrial density from pregnancy to late lactation was shown by statistical analysis to be significant ( $P < 0.005$ ). In the Ficoll gradients containing 1.0 M sucrose, the increase in mitochondrial density over the same period was also significant ( $P < 0.01$ ). In both types of Ficoll gradients, a transitional density was found for mammary mitochondria in the early lactating gland. From the results shown in the table, it appears obvious that high sucrose does affect, very significantly, the banding patterns of the mitochondria on gradient centrifugation. In addition, the effect observed is greater for the

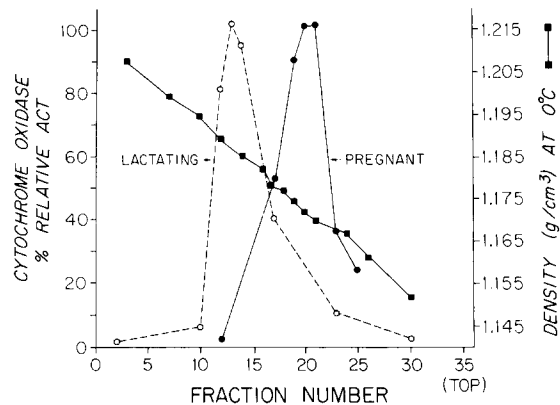


FIGURE 5 Superimposed profiles of the distribution of the cytochrome oxidase activities of pregnant and lactating gland mitochondria after centrifugation in Ficoll gradients containing hyperosmotic (1.0 M) sucrose. Mitochondria from 16-day pregnant and 17-day lactating glands were layered onto separate, essentially identical Ficoll gradients. Ultracentrifugation procedure and representation of results are similar to those in Fig. 2.

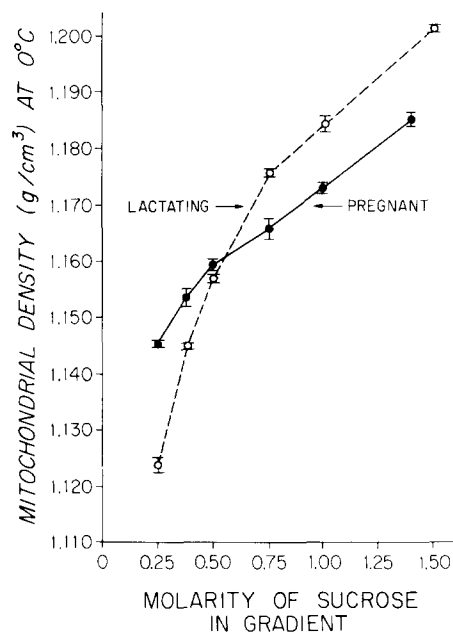
TABLE I  
Mitochondrial Density on Ficoll Gradients During Different Stages of the Lactation Cycle

Stage of lactation cycle	Mitochondrial density on Ficoll gradients containing a constant sucrose concentration	
	1.0 M sucrose	0.25 M sucrose
Pregnancy (9-18 days)	1.172 ± 0.001 (4)	1.146 ± 0.002 (4)
Early lactation (1-10 days)	1.176 ± 0.001 (6)	1.133 ± 0.002 (5)
Late lactation (11-20 days)	1.182 ± 0.001 (8)	1.125 ± 0.003 (10)

Mitochondria were isolated from mammary glands during the designated stages of lactation, Ficoll gradient centrifugation carried out in 1.0 M or 0.25 M sucrose, and densities determined as described in Materials and Methods section. The mitochondrial band density (g/cm<sup>3</sup>) is represented as mean ± SEM with the number of experimental determinations in parenthesis.

lactating gland mitochondria than it is for pregnant gland mitochondria.

To further assess the response of mitochondrial density to sucrose concentration, the densities of late pregnant and mid-lactating mammary gland mitochondria were determined by centrifugation in Ficoll gradients containing 0.25 M, 0.38 M, 0.50 M, 0.75 M, and 1.00 M sucrose. The mean density of the mitochondrial population for each gradient was determined and plotted as a function of the concentration of sucrose in the gradient (Fig. 6). Also plotted in the figure are the densities of the pregnant and lactating gland mitochondria on sucrose gradients (last points to the right). From these results the following observations can be made. First, the density of pregnant and lactating



**FIGURE 6** Mean density of late pregnant and mid-lactating gland mitochondria as a function of the molarity of sucrose in the Ficoll gradients. Mitochondria from late pregnant and mid-lactating glands were centrifuged in Ficoll gradients containing 0.25 M, 0.38 M, 0.50 M, 0.75 M, and 1.0 M sucrose as described in Materials and Methods. After fractionation, the mean densities of the mitochondrial bands were calculated and plotted against the concentration of sucrose in the gradients. The density value for each sucrose concentration represents the mean and range for two experiments performed with mitochondria from either pregnant (16 and 17 days) or lactating (14 and 15 days) glands. The last value to the right in both plots represents the molarity of sucrose at which the mitochondria equilibrated in sucrose gradients.

gland mitochondria responded differently to the increasing sucrose concentration. In sucrose concentration up to 0.5 M, pregnant gland mitochondria are more dense than those of the lactating gland. Lactating gland mitochondria show a sharper rise in density, and at sucrose concentrations greater than 0.5 M they become more dense than pregnant gland organelles.

It was of interest to determine whether mitochondria, which had been previously subjected to sucrose gradients, would behave normally on iso-osmotic Ficoll gradients. Therefore, pregnant (15 and 17 days) and lactating (13 and 16 days) gland mitochondria were centrifuged on sucrose gradients under the isopycnic centrifugation conditions used in previous experiments. The mitochondria were collected from the gradient and resuspended in 0.25 M sucrose ( $5 \times 10^{-3}$  M EDTA, pH 7.4) by the same method used in respiratory control studies. These gradient-exposed mitochondria and samples of the original mitochondrial preparation were then subjected to isopycnic centrifugation in iso-osmotic Ficoll gradients. In all cases the mitochondria that had been subjected to sucrose gradients equilibrated the same as those which had not. Therefore, centrifugation on sucrose gradients had no effect on the subsequent equilibration of mitochondria on iso-osmotic Ficoll gradients. This is further evidence that sucrose gradients do not irreversibly damage the organelles.

## DISCUSSION

In this investigation which employed both sucrose and Ficoll gradients, only a single mitochondrial band was observed in any one gland preparation on equilibrium centrifugation. In both types of gradients the mitochondrial band of the mid-lactating gland was quite sharp, which appears to reflect a rather homogeneous population of mitochondria. On the other hand, during late pregnancy to early lactation the band was more diffuse, and this indicates that more mitochondrial heterogeneity exists during this transitional period.

In the sucrose gradient studies, the mitochondrial density increased during the transition from late pregnancy to mid-lactation. This reflects a distinct change in physical properties at a time of drastically increasing energy demands within the tissue. Various explanations were considered in an attempt to clarify the physical basis for the observed changes in mitochondrial density and to

relate these to alterations in membrane structure or composition. Evidence was presented which indicates that neither differential damage nor alterations in lipid composition can explain the observed density changes.

The experiments employing Ficoll gradients demonstrated an interesting relationship between mitochondrial density and sucrose concentration. Ficoll is a high molecular weight synthetic polymer of sucrose which can be used to form density gradients of very low osmotic pressure. In Ficoll gradients with iso-osmotic sucrose present, the pregnant gland mitochondria banded at a greater density than those from the lactating gland. Therefore, the pregnant gland organelles appear to be intrinsically more dense under iso-osmotic conditions.

When the concentration of sucrose in the Ficoll gradients was increased, the density of both pregnant and lactating gland mitochondria increased nonlinearly with the sucrose concentration, the increase being greater with the lactating gland organelles. This nonlinear response of organelle density was similar to that found by Beaufay and Berthet (2) for liver mitochondria centrifuged in glycogen gradients containing increasing sucrose concentrations. By the use of a mathematical model, those authors were able to describe this behavior in terms of a particle containing both an osmotically active compartment (matrix space) and a sucrose-accessible region (intermembrane space). As the sucrose concentration within the gradient increased, the osmotically active compartment dehydrated. The sucrose space equilibrated with the higher sucrose concentration in the gradient medium, and as a result of these two factors the density of the organelle increased. We attempted to see if the differential density responses of our two organelle populations to sucrose could be explained in terms of a difference in the osmotic and sucrose-accessible spaces. The model used was originally formulated by de Duve et al. (7). The density of the mitochondria is related to the composition of the density gradient medium by the following relationship

$$\rho_p = \rho_w \frac{\rho_d \alpha + (\rho_d + \rho_m \beta) m}{\rho_d \alpha + \rho_w (1 + \beta) m},$$

where  $\rho_p$  is the density of the mitochondria,  $\rho_d$  is the mass of the particle entirely deprived of solvent,  $\rho_m$  is the density of the sucrose in the gradient medium,  $\rho_w$  is the density of the solvent

(pure water),  $m$  is the molality of the sucrose solution,  $\alpha$  is the amount of the osmotically active substances inside the particle, and  $\beta$  is the fraction of the particle that is freely accessible to sucrose. Using the experimentally determined values  $\rho_p$ ,  $\rho_m$ ,  $\rho_w$  and  $m$ , we calculated the most probable values of  $\alpha$ ,  $\beta$ , and  $\rho_d$ . The parameters were estimated by minimizing the sum of squared deviations, using the simplex algorithm of Nelder and Mead (17). For pregnant gland mitochondria, the values of  $\alpha$ ,  $\beta$ , and  $\rho_d$  were 0.018, 0.248, and 1.186, respectively. For lactating gland mitochondria,  $\alpha$ ,  $\beta$ , and  $\rho_d$  were 0.132, 0.207, and 1.220, respectively. A plot of the equations for pregnant and lactating gland mitochondria, using the best fit values of  $\alpha$ ,  $\beta$ , and  $\rho_d$ , fits closely the experimental data, and the root mean square deviation for either pregnancy or lactation was less than  $1.5 \times 10^{-6}$ .

Based on the above model, the difference in the density response to sucrose for pregnant and lactating gland mitochondria was due to a difference in the size and content of the osmotic and sucrose-accessible spaces, and to a difference in the mass of the total organelle. The value of  $\alpha$ , which represents the relative amount of osmotically active substances inside the organelle, was sevenfold greater for the lactating gland mitochondria. The relative values for  $\rho_d$  also indicated that the density of the mitochondria (solid components) was greater during lactation than pregnancy. The similar values for  $\beta$  (20% higher during pregnancy) represented only a small change in the relative size of the sucrose space.

These changes in the osmotic activity and density could be explained in several possible ways. The most plausible explanation appears to be an expansion of the inner membrane and an accompanying increase in the amount and the organization of the matrix material. This would explain the increase in osmotically active substances and the greater effect of sucrose on the lactating gland mitochondria. Since sucrose crosses the outer but not the inner mitochondrial membrane, the dehydration effect would be primarily on the inner membrane. The presence of increased amounts of inner mitochondrial membrane and matrix material would cause a greater increase in organelle density upon osmotic dehydration.

In the second paper in this series (19), further data supporting the inner membrane-matrix expansion is presented. Since the mitochondrial structure changes take place in response to the

greater energy demands of lactation, it seems reasonable that the inner membrane and matrix material (site of Krebs cycle, electron transport, and oxidative phosphorylation) would increase during the transition into lactation.

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