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# Progesterone Synthesis by Luteal Mitochondria *in Vitro*[1] (39326)

MICHAEL J. DIMINO AND MICHAEL D. CAMPBELL
(Introduced by P. P. Foa)

*Department of Research, Sinai Hospital of Detroit, Detroit, Michigan 48235*

It is generally accepted that luteal mitochondria contain the enzyme complex required for the conversion of cholesterol to pregnenolone (1). However, it has not been possible to determine if these mitochondria also contain the enzymes required for progesterone synthesis, or if the synthesis reported by other investigators (2–4) was due to contamination with microsomal enzymes. We have investigated this problem using highly purified luteal mitochondria prepared by isopycnic zonal centrifugation, and we have demonstrated that these organelles contain the enzymes required for progesterone synthesis.

*Materials and methods.* Mitochondria were prepared from bovine corpora lutea within 1 hr after slaughter. The tissue was passed through a cold tissue press (Harvard Apparatus Company) and then homogenized in 0.25 *M* sucrose, pH 7.4, using a chilled homogenizer (No. 22 Duall, Kontes Glass Co.) with the pestle rotating at 1000 rpm. Heavy debris and nuclei were removed by centrifuging the homogenate two times at 1000 *g* for 10 min. The supernatant was then respun at 6000 *g* for 10 min and the surface of the pellet was rinsed lightly with 0.25 *M* sucrose. The pellet was resuspended and centrifuged two more times and resuspended in 10 ml of 0.25 *M* sucrose. The mitochondria were purified further by isopycnic zonal centrifugation, as follows: the mitochondrial suspension was introduced into a Beckman Ti-14 zonal rotor containing a discontinuous–continuous sucrose gradient, as previously described (5). The rotor was spun at 25,000 rpm for approx 45 min to reach an $\omega^2 t$ value of $1.9 \times 10^{10}$, as determined by a Beckman $\omega^2 t$ integrator. At the end of the run, the rotor was unloaded and 26 fractions of 25 ml each were collected. The fractions were diluted with 15 ml of doubly distilled water and centrifuged at 7500 *g* for 15 min. The pellets were resuspended in 0.25 *M* sucrose for biochemical studies.

Cytochrome oxidase activity (a marker for the inner mitochondrial membrane), protein, and nucleic acid content were determined as previously reported (6). Mitochondrial steroidogenic activity was determined by a modification of the method of Robinson and Stevenson (7) which measures the percentage of [4-$^{14}$C]cholesterol converted to [4-$^{14}$C]pregnenolone and [4-$^{14}$C]progesterone. In our studies, 20,000 cpm of [4-$^{14}$C]cholesterol and 2 mg of mitochondrial protein were used per assay, and succinate was used as a substrate.

*Results.* Isopycnic zonal centrifugation resulted in a concentration of the mitochondria in the part of the gradient around 44% (w/w) sucrose, as indicated by the distribution of cytochrome oxidase activity (Fig. 1).

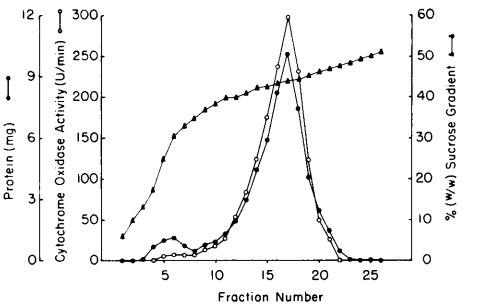


FIG. 1. Distributions of cytochrome oxidase activity and protein after isopycnic zonal centrifugation. Mitochondrial samples were prepared from four to six isolated bovine corpora lutea by differential centrifugation and purified by centrifuging them in a Beckman Ti-14 zonal rotor containing a sucrose gradient ($\omega^2 t = 1.9 \times 10^{10}$). Twenty-six fractions (25 ml each) were collected, diluted with water, centrifuged at 7500 *g* for 15 min, and the pellets resuspended in 0.25 *M* sucrose. The experiments were done on three different mitochondrial samples prepared from pooled corpora lutea, and a representative set of data are presented here.

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Protein distribution was similar to that of cytochrome oxidase activity, except for somewhat elevated values in the fractions obtained at the lighter end of the gradient. Specific cytochrome oxidase activity and specific [4-$^{14}$C]pregnenolone and [4-$^{14}$C]progesterone synthetic activities (i.e., activity per milligram of protein) of combined fractions 16, 17, and 18 were higher than those obtained with mitochondria separated by differential centrifugation alone (Table I). However, the DNA and RNA contents per milligram of protein decreased dramatically after further purification of luteal mitochondria by zonal centrifugation. The syntheses of [4-$^{14}$C]pregnenolone and [4-$^{14}$C]progesterone were linear for at least 60 min, with the [4-$^{14}$C]progesterone synthesis rate being the faster of the two (Fig. 2).

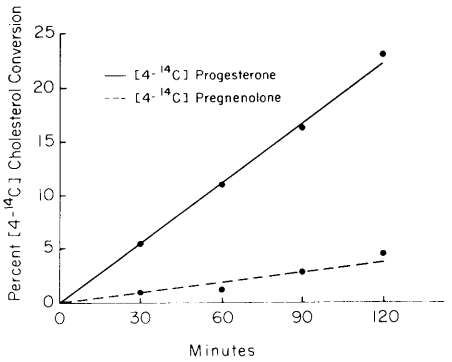


FIG. 2. The percentage of [4-$^{14}$C]cholesterol converted to [4-$^{14}$C]pregnenolone and [4-$^{14}$C]progesterone per time by luteal mitochondria purified by zonal centrifugation. The experiment was done on three different preparations of luteal mitochondria and a representative set of data is presented here.

*Discussion.* The absence of nuclear and microsomal contamination of luteal mitochondria obtained by isopycnic zonal centrifugation is suggested by their low specific nucleic acid contents, which are comparable to mitochondrial DNA and RNA values reported by other investigators (8). Furthermore, the protein distribution obtained after zonal centrifugation suggests that the higher specific cytochrome oxidase activity and [4-$^{14}$C]pregnenolone and [4-$^{14}$C]progesterone synthesis rates seen in luteal mitochondria purified by zonal centrifugation were due to the loss of nonmitochondrial protein. The fact that the mitochondria had synthesized more [4-$^{14}$C]progesterone than [4-$^{14}$C]pregnenolone supports the generally held view that cholesterol conversion to pregnenolone is the rate limiting step of ovarian steroidogenesis (1, 2).

TABLE I. SPECIFIC CYTOCHROME OXIDASE ACTIVITY, NUCLEIC ACID CONTENT, AND [4-$^{14}$C]CHOLESTEROL CONVERSION BY LUTEAL MITOCHONDRIA.

| | Differential centrifugation[a] | Differential centrifugation followed by zonal centrifugation |
|---|---|---|
| Cytochrome oxidase activity (U/mg protein)[b] | 17.5 | 23.4 |
| DNA ($\mu$g/mg protein) | 5.1 | 1.9 |
| RNA ($\mu$g/mg protein) | 16.2 | 6.5 |
| Percentage [4-$^{14}$C]cholesterol conversion to:[c] | | |
| [4-$^{14}$C]Pregnenolone | 0.9 | 1.3 |
| [4-$^{14}$C]Progesterone | 6.8 | 9.5 |

[a] Mitochondria isolated by differential centrifugation alone represent an aliquot of the sample prior to zonal centrifugation. The experiments were done on three different mitochondrial preparations prepared from four to six bovine corpora lutea, and a representative set of data are presented here.

[b] U is defined as k (sec) $\times$ $10^{-4}$.

[c] Percentage [4-$^{14}$C]cholesterol conversion values were obtained by incubating 2 mg mitochondrial samples with 20,000 cpm [4-$^{14}$C]cholesterol for 60 min in the presence of succinate.

Although the enzymes required for progesterone synthesis have been found in other subcellular fractions of luteal homogenates (9), it is likely that at least some of this activity is due to contamination from mitochondrial fragments. However, it is possible that two sites for progesterone synthesis may exist in luteal tissue. In contrast, thecal cells which primarily secrete estrogen have been reported to favor the conversion of pregnenolone to 17-OH progesterone via the $\Delta^5$-pathway (10). Thus, mitochondria of these cells may not be able to synthesize progesterone.

In conclusion, our studies strongly suggest that the enzymes required for the conversion of pregnenolone to progesterone are present in highly purified mitochondria from bovine corpora lutea.

*Summary.* Mitochondria were prepared from bovine corpora lutea by differential

centrifugation and were purified by isopycnic zonal centrifugation. A marked increase in specific cytochrome oxidase activity and a marked decrease in specific DNA and RNA content indicate that the procedure resulted in a highly purified preparation of mitochondria. These organelles had a higher rate of conversion of [4-$^{14}$C]cholesterol to [4-$^{14}$C]progesterone than did mitochondria separated only by differential centrifugation, suggesting that luteal mitochondria contain the enzyme systems required for progesterone synthesis.

1. Toren, D., Menon, K. M. J., Forchielli, E., and Dorfman, R. I., Steroids **3,** 381 (1964).
2. McIntosh, E. N., Užgiris, V. I., Alonso, C., and Salhanick, H. A., Biochemistry **10,** 2909 (1971).
3. Haksar, A., and Romanoff, E. B., BBRC **43,** 246 (1971).
4. Sulimovici, S., and Boyd, G. S., Eur. J. Biochem. **3,** 332 (1968).
5. Dimino, M. J., Kuras, R. A., McCleary, A. R., and Hoch, F. L., Arch. Biochem. Biophys. **150,** 618 (1972).
6. Dimino, M. J., and Reece, R. P., Biol. Reprod. **8,** 523 (1973).
7. Robinson, J., and Stevenson, P. M., Eur. J. Biochem. **24,** 18 (1971).
8. O'Brien, T. W., and Kalf, G. F., J. Biol. Chem. **242,** 2172 (1967).
9. Cheatum, S. G., and Warren, J. C., Biochim. Biophys. Acta **122,** 1 (1966).
10. Ryan, K. J., and Petro, Z., J. Clin. Endocrinol. Metab. **26,** 46 (1966).

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