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Tubulin Content and Assembly States in Guinea Pig Mammary Gland during Pregnancy, Lactation, and Weaning (40932)[1]

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Abstract. The microtubule (MT) protein, tubulin, was measured in mammary gland biopsies from 23 guinea pigs averaging four samples per animal from about 20 days antepartum through weaning. [^{3}H]Colchicine binding assays were carried out on supernatants from tissues homogenized sequentially in MT-stabilizing and MT-depolymerizing buffers which yielded estimates, respectively, of free (S-1) and microtubular (S-2) tubulin as well as total tubulin (S-1 + S-2). There is almost a threefold increase in total tubulin which begins about 20 days antepartum and reaches a peak by 11 to 15 days postpartum. There is an initial increase in free tubulin, also beginning about 20 days antepartum; however, about 1 week before birth, free tubulin levels off and microtubular tubulin begins to increase, reaching a peak in midlactation after a sevenfold increase from pregnancy and then declining at weaning. The results show that free tubulin is synthesized in late pregnancy and then converted to the assembled (microtubular) state, which suggests that microtubules are involved in the lactation process.

Previous studies have suggested a role for microtubules in milk secretion. These have all been based on the inhibitory effects of microtubule-altering drugs such as colchicine and vincristine (1) on *in vivo* milk flow (2), on the synthesis and secretion of casein (3), milk fat (4), and lactose (5–7), or on alveolar cell exocytosis (8) by mammary gland, either *in vivo* or *in vitro*. However, colchicine and similar drugs have other effects on cells not related to microtubules and which are sometimes toxic (9), thus compromising such indirect evidence for microtubule involvement in lactation. Here we report direct measurements of mammary gland tubulin, the primary protein of microtubules, using a [^{3}H]colchicine binding assay based on several sources (10–13) from late pregnancy through lactation. In addition, separate quantitation of free and polymerized (microtubular) tubulin was carried out on the same tissue samples. This procedure allowed study of total tubulin changes as well as apparent interconversions between free and assembled forms. The results indicate that tubulin content and assembly state are related to the lactational state of the gland.

Materials and methods. Assays of tubulin were carried out on 60- to 100-mg mammary gland biopsies (14) taken prior to, during, and after lactation, from timed pregnant guinea pigs (15) tranquilized with acetylpromazine (10 mg/kg) and locally anesthetized subcutaneously with xylocaine. From each of the 23 animals used in this study, an average of four sequential biopsies were taken over the period from about 21 days antepartum (approximately two-thirds through gestation) through weaning, alternating glands for each biopsy.

Separation of free tubulin from microtubules. In homogenates, microtubules are stabilized by buffers containing 50% glycerol and 10% dimethyl sulfoxide (DMSO) (16, 17), while disassembly is favored by increased Ca^{2+} and cold temperature (18). Tissues were minced in microtubule-stabilizing medium (MTS) containing 50% glycerol, 5% DMSO, 0.5 m*M* GTP, 0.5 m*M* $MgCl_2$, and 0.5 m*M* ethyleneglycolbis(aminoethylether) tetraacetic acid (EGTA) in 10 m*M* phosphate

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buffer, pH 6.95, at room temperature (12), with a 50:1 buffer:tissue ratio, and centrifuged for 45 min at 100,000*g* in a Beckman ultracentrifuge at room temperature. The first supernatant (S-1) was then stored on ice and the pellet was resuspended by vortexing for 1 min, in a volume equal to that of S-1, of ice-cold tubulin-depolymerizing solution (TS) containing 0.25 *M* sucrose, 0.5 m*M* $MgCl_2$, 0.5 m*M* GTP in 10 m*M* phosphate buffer, pH 6.95. The suspension was then incubated for 30 min at 4° and centrifuged for 30 min at 100,000*g*, and the second supernatant (S-2) was also stored on ice. Tubulin that was in the free or unassembled state *in vivo* remained in S-1, while tubulin in the assembled or microtubular form in the tissue was then separated and depolymerized in S-2. The second pellet (P-2) was resuspended as above and assayed in suspension to determine if any colchicine binding persisted following the depolymerization step and removal of S-2. The time interval between homogenization and incubation was the same for all samples.

[^3H]*Colchicine binding assay* (19). One-milliliter samples of the three tissue fractions (S-1, S-2, and P-2) were incubated in triplicate with 10 μl of 5.11×10^{-4} *M* [^{3}H]colchicine (sp act, 73 μCi/mmole) at 37° for 90 min when TS was used and 150 min when MTS was used. Following incubation, 200 μl of 25 mg/ml charcoal suspension was added to each tube to remove unbound colchicine. The tubes were vortexed, allowed to stand 10 min and centrifuged at 12,000*g* for 10 min, and 200 μl of the supernatant was transferred to a counting vial with 10 ml of PCS scintillation fluid (Amersham–Searle) and counted in a Beckman liquid scintillation counter. These assay conditions were tested using homogenate dilutions over the range 10 to 100 mg of tissue/ml of the three fractions prepared in TS or MTS and gave a linear response in each case. Tubulin concentrations were calculated from [^{3}H]colchicine binding activity by assuming a 1:1 molar ratio of colchicine binding to each tubulin dimer (mol wt = 110,000) (20, 21). While much lower ratios have been reported using other buffers (22, 23), a 1:1 ratio is obtained when the results are corrected for decay in colchicine binding activity. This activity was shown to be almost completely protected by the MTS and TS buffers (12).

Results. Figure 1A shows the changes in absolute tubulin concentrations during the entire sampling period. Total tubulin (sum of S-1 and S-2) undergoes about a threefold increase from a level of 0.87 μg/mg at 20 to 16 days antepartum up to a maximum of 2.34 μg/mg in midlactation (10 to 15 days postpartum), when milk flow is greatest (24), and then declines to 1.83 μg/mg after weaning. When the values in each of the three states are grouped as shown in Table I, the average value for total tubulin in lactation is 51% higher than the average pregnancy value ($P > 0.01$). The results suggest that tubulin synthesis accelerates prior to lactation. The apparent decrease in Fig. 1A following peak lactation may be due to the reportedly increased lysosomal activity during mammary gland regression (25).

The two measured parameters, S-1 and S-2, do not parallel either the total tubulin or each other. Free tubulin (S-1) initially increases similarly to total tubulin but then, at about 1 week prior to parturition, levels off and maintains a plateau for the next 2 weeks; it then increases again for the remainder of lactation and through weaning, even while total tubulin is decreasing. Compared to total tubulin, these S-1 increases are modest. This can be seen in Table I, where the mean S-1 level increases only 23% from pregnancy to lactation and another 24% from lactation to weaning, neither of which is statistically significant. The S-2 fraction shows still a different pattern. During late pregnancy microtubular tubulin is maintained at a very low level (0.12 μg/mg) until about 1 week antepartum, when it begins to rise, reaching a midlactational peak of 0.86 μg/mg for a sevenfold increase. Following midlactation S-2 tubulin then decreases, reaching a weaned value of 0.32 μg/mg. The mean S-2 levels show a significant ($P < 0.001$), threefold increase from pregnancy to lactation and a significant decrease at weaning ($P < 0.02$) (Table I). When these data are expressed as

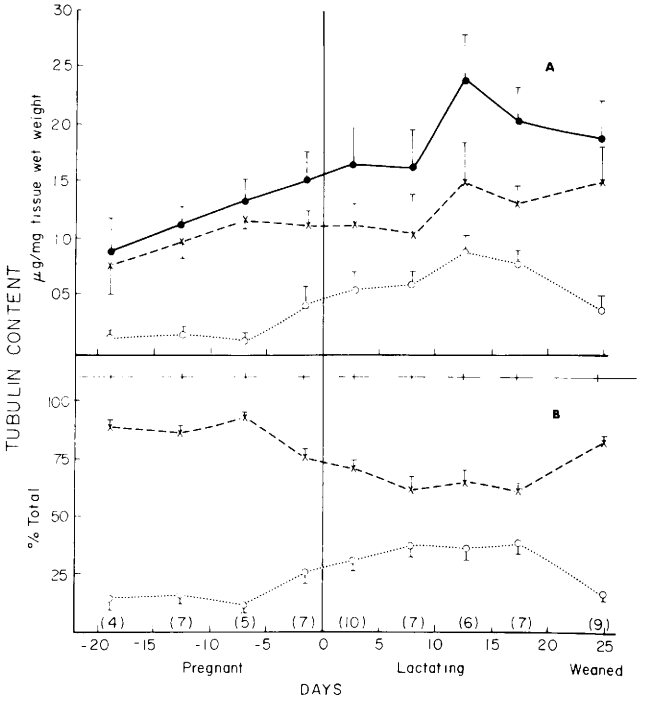

FIG. 1. Shifts in tubulin pools during pregnancy, lactation, and weaning. ×– – –×, S-1 or free tubulin; ○···○, S-2 or polymerized tubulin; ●– – –●, S-1 + S-2 or total tubulin. Each point represents a mean ± SE obtained by grouping the values within a 5-day period. The number of samples in each grouping is shown at the bottom of graph. Bars between the two graphs represent mean day ± SD. (A) Tubulin expressed as micrograms per milligram tissue wet weight. (B) Tubulin expressed as percentage of the total tubulin.

a percentage of the total tubulin (Fig. 1B), the free tubulin starts out in the period 1 to 3 weeks antepartum as the largest tubulin pool constituting 85 to 91% of the total. Again, beginning about 1 week before parturition, the assembled tubulin fraction increases from its 9 to 15% pregnancy level to 35 to 40% of the total, which is maintained during most of the lactation period. After weaning, S-2 decreases to the antepartum levels. For comparison with other secretory tissues, assembled tubulin in liver has been reported to be 30% of the total and, in the endocrine pancreas, 35% (26).

Discussion. Analysis of the S-1 and S-2 patterns shown in Fig. 1A suggests that up

TABLE I. TUBULIN CONTENT (MEAN ± SE) DURING THE THREE BIOPSY PERIODS

	Pregnant (μg/mg)	Lactating (μg/mg)	Weaned (μg/mg)
S-1	1.00 ± 0.07	1.26 ± 0.12	1.50 ± 0.29
S-2	0.22 ± 0.04	0.65 ± 0.07[c]	0.32 ± 0.07[a]
Total (S-1 + S-2)	1.23 ± 0.11	1.85 ± 0.18[b]	1.82 ± 0.35
Pellet	0.24 ± 0.02	0.56 ± 0.05[c]	0.30 ± 0.07[b]
	N = 23	N = 30	N = 9

[a] Significantly different from preceding period at $P < 0.02$ using Student's t test for independent means.
[b] Significantly different from preceding period at $P < 0.01$.
[c] Significantly different from preceding period at $P < 0.001$.

to 5 days before parturition the increased total tubulin content is due entirely to an increase in the free or unassembled pool. After this time, while tubulin synthesis appears to continue at the same rate, the equilibrium is shifted such that free tubulin is now being assembled into microtubules. The S-1 plateau around parturition suggests that synthesis and polymerization are occurring at about the same rates; hence, the free tubulin pool becomes a steady state compartment while the assembled pool increases. At approximately midlactation there appears to be an acceleration in tubulin synthesis which is reflected in both the free and assembled states. Following this, the decrease in S-2 and the concomitant rise in S-1 suggest that the equilibrium has now shifted back to favor free tubulin (i.e., microtubules are being depolymerized) and this is occurring at the same time that total tubulin is decreasing.

Our mean lactation values of 36% polymerized tubulin and 1.85 μg/mg total in the guinea pig may be compared with values found for lactating rat mammary gland, 19% polymerized tubulin of 1.07 μg/mg total, using the same basic assay (27).

In our assays there was always some binding in the second, 100,000*g* pellet (P-2). The binding followed about the same pattern as the S-2 fraction, showing an increase during lactation (Table I), and resembled S-2 quantitatively as well. When the P-2 values are included with the tubulin data, either with S-2 or as a third compartment, the patterns described above for total tubulin, S-1, and S-2 are qualitatively unchanged and the conclusions remain the same. However, we hesitate to refer to the P-2 binding as tubulin, first, because it resisted repeated washing in TS media and, second, because of the particulate and membranous nature of the P-2 suspension compared with the 100,000*g* S-1 and S-2 supernatants. Other investigators have reported [^{3}H]colchicine binding in the 100,000*g* pellet fraction of liver (28), brain (29, 30), pancreas, spleen (31), and thyroid (32). However, analyses of this binding using a variety of techniques has yielded conflicting results (28, 32) and the question of whether this represents tubulin associated with membranes or nonspecific binding of colchicine to nontubulin membrane components is unresolved.

This study establishes the presence of a colchicine-binding component in guinea pig mammary gland which we believe to be tubulin. In the last third of pregnancy, as the mammary gland prepares for lactogenesis, the total amount of tubulin increases, presumably through increased synthesis. Then, just prior to parturition, when the gland changes from a state of growth and differentiation to one of synthesis and secretion this tubulin is assembled into microtubules. By this process the assembled form of tubulin rises sevenfold coincident with peak milk secretion. This is also a time when the alveolar epithelial cells which produce the milk constitute the greatest proportion of the mammary gland mass and cell population (33) but when the rate of mitotic division in the gland (which would also utilize microtubules) is at a minimum (34). Thus, an alternate explanation for the data might be that the rise and fall of polymerized tubulin simply reflects the increase and decrease of nondividing, differentiated, alveolar cells. Even this explanation, however, suggests a close association of nonmitotic microtubules with those cells in mammary gland whose main function is milk production. We conclude, therefore, that microtubules play a role in the lactation process and that the physiological mechanisms controlling lactation may also modulate tubulin synthesis, assembly, and disassembly.

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1. Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., and Creswell, K. M., Fed. Proc. 33, 158 (1974).
2. Patton, S., FEBS Lett. 48, 85 (1974).
3. Ollivier-Bousquet, M., and Denamur, M. R., C.R. Acad. Sci. Paris 276, 2183 (1973).
4. Patton, S., Stemberger, B. H., and Knudson, C. M., Biochim. Biophys. Acta 499, 404 (1977).
5. Loizzi, R. F., dePont, J. J. H. H. M., and Bonting, S. L., Biochim. Biophys. Acta 393, 20 (1975).
6. Guerin, M. A., and Loizzi, R. F., Amer. J. Physiol.: Cell Physiol. 3, C177 (1978).

7. Sasaki, M., and Keenan, T. W., Exp. Cell Res. **111**, 413 (1978).
8. Knudson, C. M., Stemberger, B. H., and Patton, S., Cell Tissue Res. **195**, 169 (1978).
9. Mizel, S. B., and Wilson, L., Biochemistry **11**, 2573 (1972).
10. DiPasquale, A. M., McGuire, J. M., Moellmann, G., and Wasserman, S. J., J. Cell Biol. **71**, 735 (1976).
11. Pipeleers, D. G., Pipeleers-Marichal, M. A., and Kipnis, D. M., Science **191**, 88 (1976).
12. Pipeleers, D. G., Pipeleers-Marichal, M. A., Sherline, P., and Kipnis, D. M., J. Cell Biol. **74**, 341 (1977).
13. Sherline, P., and Mundy, G., J. Cell Biol. **74**, 371 (1977).
14. Loizzi, R. F., and Guerin, M. A., Lab. Animal Sci. **29**, 221 (1979).
15. Elvidge, H., J. Inst. Animal Tech. **23**, 111 (1972).
16. Filner, P., and Behnke, O., J. Cell Biol. **59**, 99a (1973).
17. Rubin, R. W., and Weiss, G., J. Cell Biol. **64**, 42 (1975).
18. Weisenberg, R., Science **177**, 1104 (1972).
19. Sherline, P., Bodwin, C. K., and Kipnis, D. M., Anal. Biochem. **62**, 400 (1974).
20. Weisenberg, R. C., Borisy, G. G., and Taylor, E. W., Biochemistry **7**, 4466 (1968).
21. Bryan, J., and Wilson, L., Proc. Nat. Acad. Sci. USA **68**, 1762 (1971).
22. Wilson, L., Ann. N.Y. Acad. Sci. **253**, 771 (1975).
23. Battacharyya, B., and Wolff, J., Proc. Nat. Acad. Sci. USA **71**, 2627 (1974).
24. Mepham, T. B., and Beck, N. F. G., Comp. Biochem. Physiol. **A45**, 273 (1973).
25. Helminen, H. J., and Ericsson, J. L. E., J. Ultrastruct. Res. **25**. 214 (1968).
26. Pipeleers, D. G., Pipeleers-Marichal, M. A., and Kipnis, D. M., J. Cell. Biol. **74**, 351 (1977).
27. Nickerson, S. C., and Keenan, T. W., Cell Tissue Res. **202**, 303 (1979).
28. Stadler, J., and Franke, W. W., J. Cell Biol. **60**, 297 (1974).
29. Wilson, L., Biochemistry **9**, 4999 (1970).
30. Lagundo, J. R., Lyons, C., and Wickremasinghe, G., Biochem. J. **122**, 56 (1971).
31. Gillespie, E., J. Cell Biol. **50**, 544 (1971).
32. Bhattacharyya, B., and Wolff, J., J. Biol. Chem. **250**, 7639 (1975).
33. Pitelka, D. R., Kerkof, P. R., Gagne, H. T., Smith, S., and Abraham, S., Exp. Cell Res. **57**, 43 (1969).
34. Nagasawa, H., and Vorherr, H., Amer. J. Obstet. Gynecol. **127**, 590 (1977).

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