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PREPARATION METHODS FOR LIGHT MICROSCOPIC AND ULTRASTRUCTURAL STUDIES OF FETAL RAT BLADDER

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Abstract

Little is known about in utero urinary bladder embryogenesis and the development of the urothelium of laboratory animals. Previous scanning and transmission electron microscopy studies in rats and mice have indicated that the highly specialized superficial cells of the urothelium complete their differentiation at a very late stage of fetal development or shortly after birth. Limitations in methodology in the past have precluded extensive examination of earlier stages of bladder development. Innovations in preparatory procedures of the bladder of rat fetuses have been developed which make possible detailed scanning and transmission electron microscopic and light microscopic examination of cloaca and urinary bladder as early as day 11 of gestation.

Key Words: Urinary Bladder, Embryogenesis, Scanning electron microscopy, Laboratory animal and preparation methods.

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Introduction

Embryologically, the urinary system develops from the mesodermal ridge along the posterior wall of the abdominal cavity, and the excretory ducts of both the urinary and lower intestinal system initially enter a common cavity, the cloaca. Initially, the cloaca is continuous with the allantois (Fig. 1A). When the lumen of the allantois decreases, a thick fibrous cord called the urachus remains connected to the apex of the bladder (6).

In extensive scanning (SEM) and transmission electron microscopic (TEM) studies by Ayres et al. (1), the urinary bladder epithelium of the mouse and rat was shown to undergo rapid changes during the late fetal and early neonatal period, displaying characteristic morphologic features. A few of the dark, apparently degenerative, superficial cells appeared to slough between fetal day 18 or 19 and the day of birth. By the third postnatal week, the bladder epithelium had assumed the adult morphologic appearance as evidenced by light microscopy (LM) and TEM (1). These rapid developmental changes, along with the high mitotic activity noted by TEM, indicate the dynamic nature of the fetal and neonatal bladder epithelium of the mouse and rat (2,7).

By TEM, Firth and Hicks (3) reported that differentiation of bladder epithelium in the rat is characterized by the generation of differentiated superficial cells during the last few days of gestation. In contrast to Ayres et al. (1), they described extensive cell necrosis during the perinatal period with desquamation into the bladder lumen. The remaining undamaged basal cells proliferated and differentiated to produce the definitive characteristic superficial layer of the urothelium, which is mature by the third week after birth.

The above studies examined the bladder only during the last 4 to 5 days of the 21-day gestational period of the rat. Although the methodology for isolating the bladder was not detailed, problems with locating and identifying the bladder and fragility of the tissue were likely limitations (1-4, 7). To provide more detailed examination of bladder embryogenesis, especially to study earlier times of gestation, several LM and TEM methods were combined and modified to

trace normal differentiation of the rat urinary bladder at the earliest possible stage of development in order to more fully understand the possible susceptibility of toxic insult to the organ. Also, this study describes a dissecting technique for isolation and identification and also the preparation of the fetal bladder as early as day 11 of gestation.

Materials and Methods

Sprague-Dawley rats, 10 weeks of age, were obtained from Charles River Breeding Laboratories (Portage, Michigan) and housed in plastic polycarbonate cages with corncob bedding. Food (Agway Prolab 3000 rat chow) and deionized, distilled water were available ad libitum. The rats were housed in a room with a temperature of 22°±2° C and relative humidity of 45%. The room was lit from 23.00 to 11.00 and was dark from 11.00 to 23.00. At 11 weeks of age, males were placed individually in the cages of singly caged females at 13.00 and observed for copulation. If copulation occurred, the female was considered mated. This time was considered day 0 of gestation. The mating procedure was repeated daily until all females were mated. Males were sacrificed after mating was completed. Two pregnant females were sacrificed at 13.00 on each of days 11 through 21 of gestation. The total number of fetuses for each litter were recorded, and the number of males and females indicated when possible. Females were anesthetized by nembutal injection i.p. with fetuses removed by Caesarean section and immediately immersed in fixative. From days 16 through 21 of gestation, the fetuses were decapitated to aid in fixation of internal organs, especially the bladder.

Tissue specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4° C for a minimum of 24 hrs before further processing. Specimens for light microscopy were embedded in paraffin after dehydration using an ascending series of ethanol concentrations and xylene. Sections were cut 4-5 microns thick and stained with hematoxylin and eosin. Serial sections were cut and examined by light microscopy until the cloaca was identified (Fig. 2). The location of the cloaca was identified in the remaining paraffin block tissue. This tissue was then deparaffinized in several changes of xylene and processed for routine SEM (Fig. 3) as described below. For SEM and TEM, tissues were washed with 0.1 M phosphate buffer, post fixed in 1% OsO_4 and dehydrated through an ascending series of ethanol concentrations. TEM specimens were embedded in araldite and thin sections stained with uranyl acetate and lead citrate. Specimens for SEM were immersed in Freon 113, critical point dried with Freon 13, mounted on aluminum stubs, and sputter-coated (Polaron E5100, Polaron, Inc.) with gold. TEM specimens were examined with a Philips 300. For SEM, the specimens were examined at 20 kV in an Etec Autoscan scanning electron microscope (Perkin-Elmer, Hayward, CA).

Results

Four fetuses from each of days 11, 12, and 13 of gestation were placed under a dissecting microscope with a bottom light source (Fig. 1B). After locating the cloaca, a disposable Pasteur pipette was carefully inserted completely through the fetus at the posterior end of the embryo and then carefully withdrawn; the cloaca remained in the bore of the pipette and was processed for routine transmission electron microscopy (Fig. 4).

In addition to the day 12 fetuses processed above, four additional intact fetuses were rinsed three times in 0.1 M phosphate buffer (pH 7.4) and processed for SEM without processing through paraffin (Fig. 5). After examination of the specimens by SEM, they were placed under a dissecting microscope, the allantois identified, and the tail and allantois removed by mechanical fracture revealing the neural tube and the cloaca. The remaining tissue on the SEM specimen stub was resputter-coated and re-examined using SEM (Fig. 6).

The remaining female rats from day 13, and all from days 14 through 21, were anesthetized and Caesarean section performed as described above. The fetus was placed on its back (Fig. 7A). An incision to the left side of the midline was made extending from the head to the hind leg (Fig. 7B). The incision was extended to eliminate the left half of the fetus. The liver and gastrointestinal tract were removed from the remaining exposed larger right portion of the fetus (Fig. 7C). The right kidney was identified and isolated by blunt dissection. Forcep tips were placed under the ureter and gently traced to locate the bladder. A linen string was placed around the neck of the bladder and knotted at the urethra. A needle was inserted into the bladder through the urethra and the bladder inflated with fixative. The bladder was removed and placed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C. Bladders were then divided sagitally and each half was processed for scanning electron microscopy. After critical point drying, each half was mounted on aluminum stubs, whereupon four incisions around the periphery were made to flatten the cupped bladder (Fig. 8A). Additional horizontal and longitudinal incisions were made completely across the bladder resulting in a grid that allows for scanning of one grid section at a time (Fig. 8B). This method allows for accurate mapping and quantitation of the entire bladder epithelium (5). Creation of the grid does not significantly alter the surface morphology and permits evaluation of the bladder epithelium (Fig. 8C).

Colon and stomach were also processed for TEM and LM to compare the differences between the morphology of these tissues in relationship to the cloaca or bladder.

Discussion

For SEM and TEM examination, immediate and proper fixation of tissue specimens is essential. The embryonic bladder, like other embryonic tissues, is delicate and fragile to handle. The bladder lacks a muscle wall until late in fetal life, and the tissue remains gelatinous in consistency. Immediate fixation of the entire embyro or fetus in 2% glutaraldehyde in 0.1 M phosphate buffer with refrigeration for a minimum of 24 h decreases the fragility of the tissue.

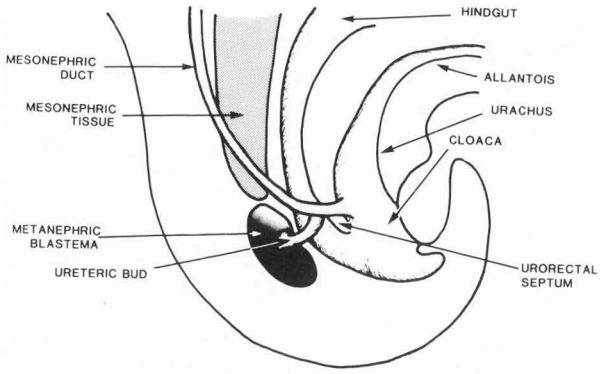


Fig. 1A Schematic drawing showing the relationship of the allantois and cloaca at the 12th day of gestation.

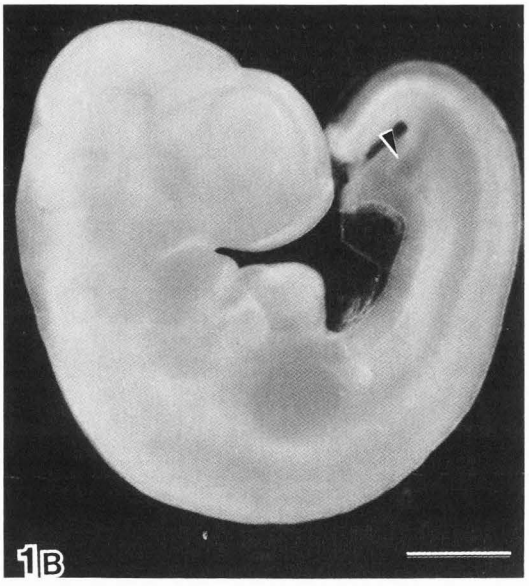
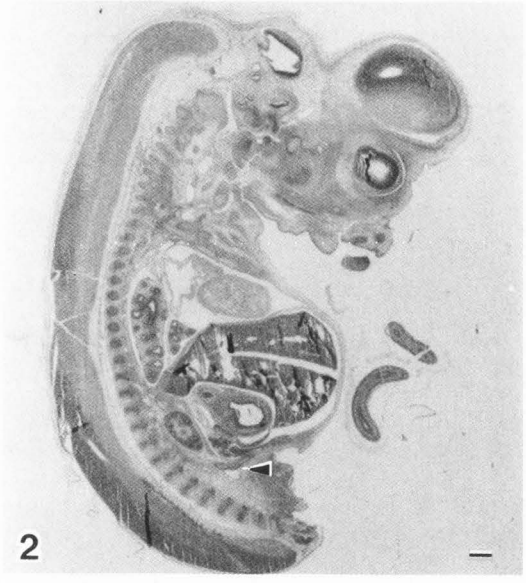
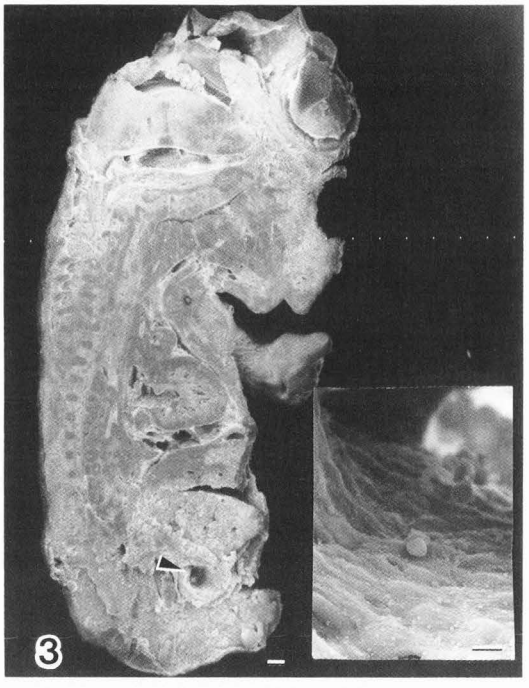


Fig. 1B Appearance of a fetus at the 12th day of gestation using a bottom light source to accentuate localization of the cloaca (arrow). Bar = 100 µm.





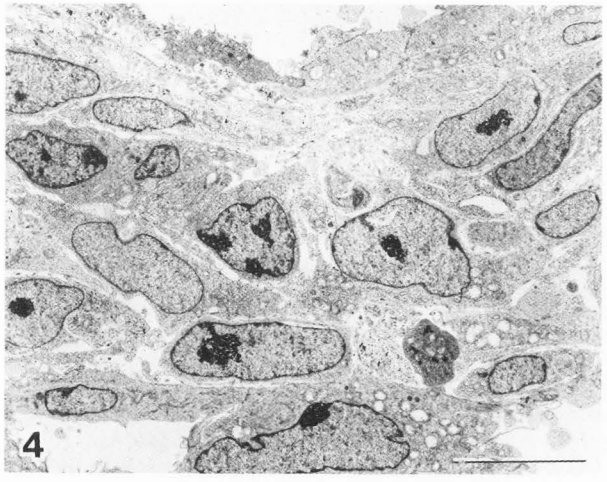
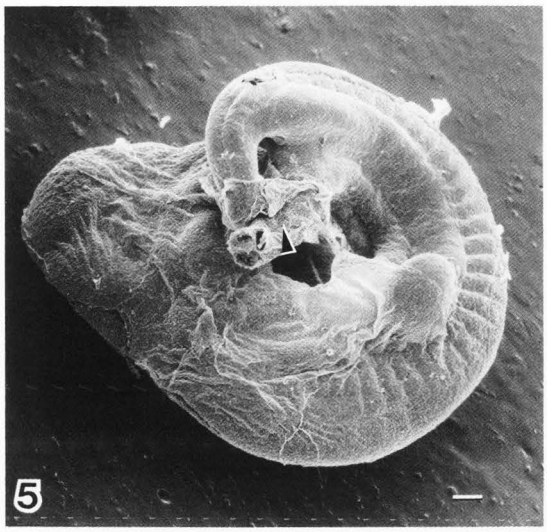
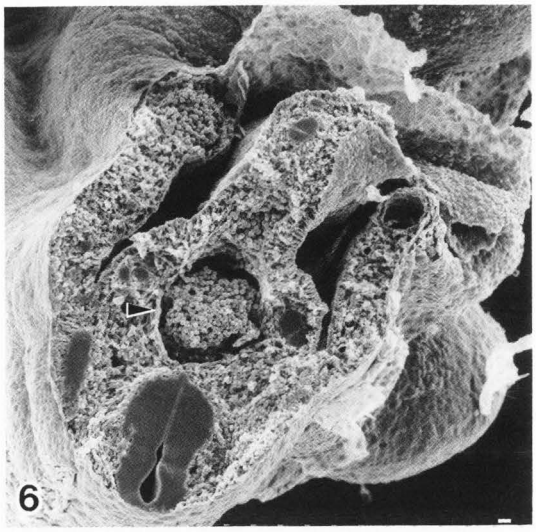


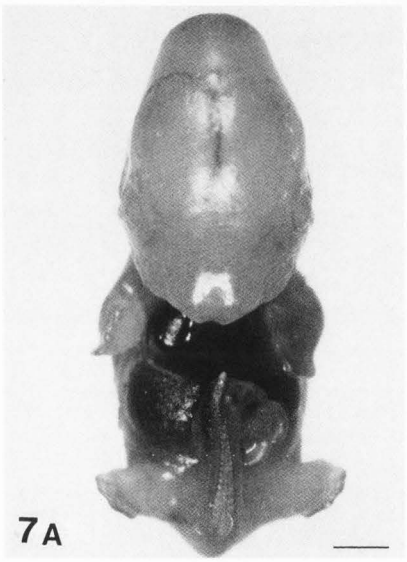
Fig. 2 Whole body section of a rat fetus on gestational day 13. The fetus measures 8.5 mm which is in accordance with the findings of Witschi (8). Arrow indicates the urinary bladder. Bar = 100 µm.

Fig. 3 The same fetus as in Figure 2 after deparaffinization and processing for SEM. Arrow again indicates the urinary bladder. Bar = 100 µm. Inset shows the well-preserved round cells covering the bladder. Bar = 10 µm.

Fig. 4 TEM micrograph from a day 13 fetus showing a section through the bladder with 2 layers of round cells. Bar = 10 µm.







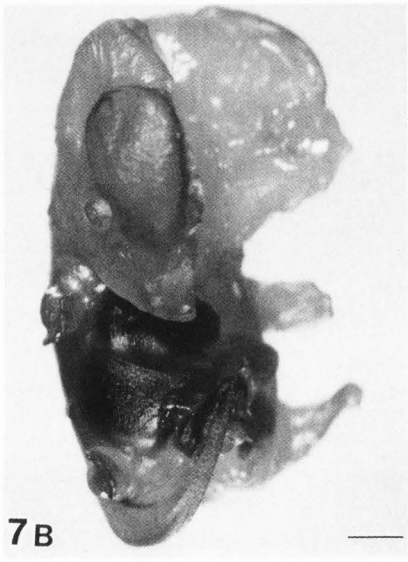


Fig. 5 SEM preparation of rat fetus from gestational day 12. Arrow indicates allantois. Bar = 100 μm.

Fig. 6 The same fetus as in Figure 5 after fracturing and removal of the distal portion of the fetus. The neural tube is located at the bottom of the figure, and the cloaca-bladder indicated by the arrow. Bar = 10 μm.

Fig. 7A Day 15 fetus after fixation measuring 10 mm. Bar = 100 μm.

Fig. 7B The same fetus as in Fig. 7A after an asymmetric incision from head to hind leg has been performed. Bar = 100 μm.

Fig. 7C The right half of the same fetus after removal of liver and intestines. The arrow indicates the urinary bladder. Bar = 100 μm.

Methods for locating the urinary bladder prior to day 15 of gestation have not been reported, and previous publications dealing with bladder morphology between day 15 and birth (1,3) are lacking in technical detail. The methods described above are reproducible and provide for the accurate isolation of the bladder or cloaca from day 11 of gestation. The cutting of a grid allows for quantitation and accurate mapping of the complete urothelium for days 15 through 21 of gestation. Kidneys can be readily identified by day 14 of gestation and occasionally on day 13, depending on the level of fetus development. By following the ureters, the urinary bladder is easy to locate. Before the time of kidney identification, the bladder appears as a cloaca and is not separated completely from the hindgut. We found that following the allantois to the urachus to the bladder-cloaca was more reliable than trying to follow the hindgut into the cloaca-bladder.

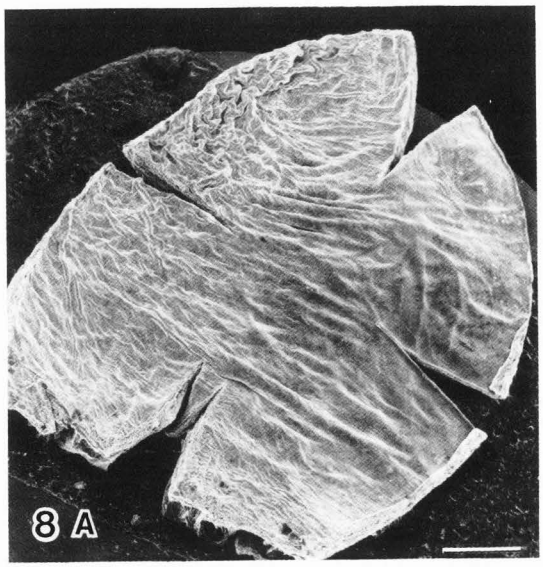


Fig. 8A Urinary bladder from a 15-day old rat prepared for SEM examination. Incisions have been made to flatten the bladder onto the specimen stub. Bar = 1000 μm.

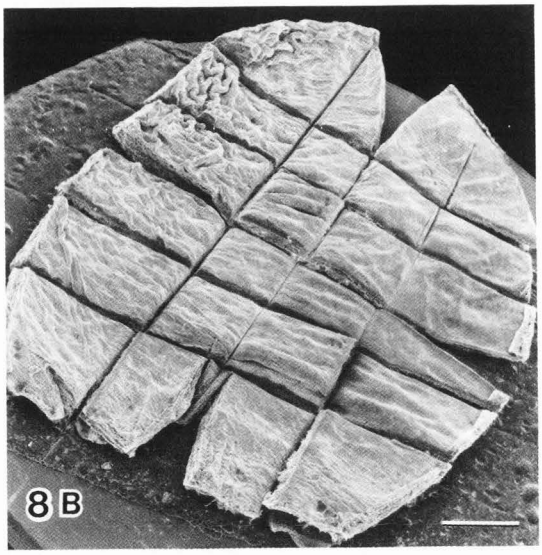


Fig. 8B The same urinary bladder as in Figure 8A after slicing the bladder into a grid-like matrix for localization of features and for quantitation. Bar = 1000 μm.

The proposed methods are applicable to studying the development of other organs. They may be used alone or in combination for LM, TEM and SEM.

Acknowledgments

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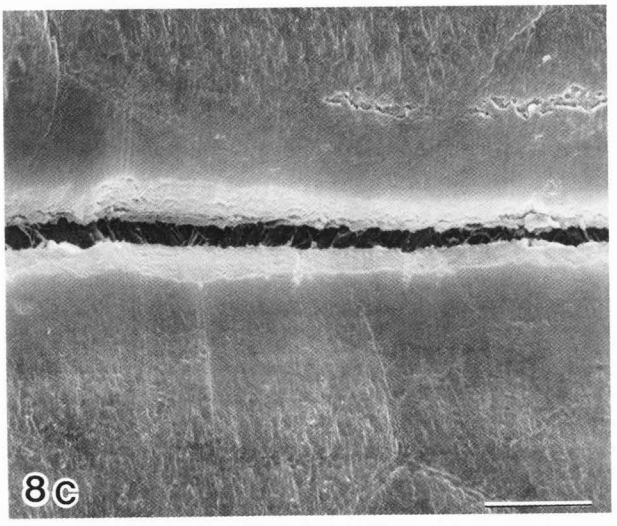

Fig. 8C Close-up of the bladder showing a cutting line. Note that the morphology of the urothelial cells is well preserved and allows complete evaluation of surface morphology. Bar = 10 μm.

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Discussion with Reviewers

Reviewer II: Why is it necessary to "flatten" the bladder by cutting it prior to SEM observation? SEM should allow one to observe curved, as well as flat, regions.
Authors: For strictly qualitative evaluation, it is not necessary to flatten the bladder by cutting it prior to SEM observation. However, when observing a curved specimen, one usually has to tilt and rotate it, and, at times, change magnification in order to rotate the specimen that is obscured by the curve. Flattening the bladder reduces the need for these manipulations and makes quantitative procedures easier to perform and more reproducible.

Reviewer II: Does cutting of the grid disrupt the surface of the bladder in significant areas around the cut? What alternatives are available? Can a grid be electronically positioned over the image or an overlay placed on the screen and the sample appropriately positioned?
Authors: The cutting of the grid and also laying the specimen flat allows for complete quantitation of the entire bladder urothelium. Each section of the grid can be completely observed with the SEM thereby decreasing the chances of missing cells. Obviously, cutting of the grid does disrupt the surface of the bladder, but not in significant areas beyond the cells that are actually cut (Fig. 8C). A grid can be electronically positioned over the image or an overlay placed on the screen and the sample appropriately positioned. However, the purpose of cutting the grid is to perform quantitative evaluations reproducibly. An electronic grid cannot be positioned on a specimen permanently, causing some differences if the specimen is manipulated during the procedure, if the magnification is changed, or if the specimen is removed and replaced in the microscope chamber.

Reviewer III: Have the methods described in this study been used for studies of fetal rat and mouse bladders? If so, do your investigations indicate any substantial differences in the bladder embryogenesis in rats relative to man, or does the rat serve as a useful model for mammalian and human development?
Authors: We are concluding a study of fetal rat bladder embryogenesis, and our preliminary findings confirm and extend the light microscopic observations of Witschi (8) involving rats and of Gyllensten's investigations of bladder embryogenesis of 42 human embryos (L. Gyllensten, Contributions to the embryology of the urinary bladder. Acta Anatom., 7, 305-333, 1949). Based on these findings, it seems likely that the rat is a useful model for human bladder development.

Reviewer III: Why have you anesthetized your animals? The removal of the fetuses should be fast enough when using cervical dislocation. In addition, this method gives less side-effects from hypoxia and hypothermia than the use of nembutal. It has been found by others (Reitan and Tvera, Cell Tissue Kinet., 18, 631-639, 1985) that nembutal has considerable effects on the bladder epithelium a short time after injection. Please comment.
Authors: The effects of nembutal on cell proliferation as reported in Reitan and Tvera were several hours after injection of the drug. In our studies, the rats are killed within a few minutes after nembutal injection. In our experience, this has not had an effect on the morphology or cell kinetic parameters that we are measuring.

Reviewer III: K. Feren and J. B. Reitan