Renewal of Alveolar Epithelium in the Rat Following Exposure to NO_2

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This research was undertaken to study the kinetics of Type 2 cell division and the fate of the Type 2 sister cell following exposure to NO_2. To accomplish this, male rats were exposed to NO_2. Dividing cells were labeled with ^{3}H-TdR and studied with autoradiographic technics in the light and electron microscopes for up to 72 hours after labeling. The kinetics of cell division were determined from a curve constructed from the percent of labeled mitotic figures. The fate of the Type 2 sister cells was determined by studying tissues at 24, 48 and 72 hours after labeling with ^{3}H-TdR. The results show that Type 2 cells may divide and the sister cells transform into Type 1 cells. These data support an interpretation of the mechanism for cell renewal of the alveolar epithelium in which Type 2 cells are the progenitor cells for Type 1 cells (Am J Pathol 70:175–198, 1973).

THE EPITHELIUM LINING the walls of alveoli is composed primarily of large squamous cells (Type 1 cells) which cover most of the alveolar surface. Dispersed throughout this area between the Type 1 cells are smaller cuboidal cells (Type 2 cells), which make up the remainder of the epithelial surface. Both cell types lie on a common basement membrane.[1,2] The surface of this epithelium is covered by a thin film of surface-active material (surfactant), and alveolar macrophages are found in this film on the epithelial surface.[3–5] Beneath the epithelium are capillaries and occasional interstitial cells.[6,7] The principal function of the Type 1 cells appears to be associated with gas exchange, whereas the functions of Type 2 cells have been associated with pulmonary surfactant production,[8] providing a source of macrophages,[9] and repair of the epithelium.[10]

The mechanism of cell renewal in the alveolar epithelium is not clear. Previous investigations have shown that both Types 1 and 2 cells are capable of cell division.[9,11] Type 1 cells have been classified as an ex-

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panding cell population in which cell division is associated with growth of the tissue.[9] Type 2 cells are classified as stem-type, renewing cell population.[11] In the latter type of cell population, cell division occurs, but the total number of cells does not increase because a sister cell from the division leaves the area and can transform into another type of cell.[12,13] In the pulmonary alveoli, the fate of the sister cell from division of a Type 2 cell has not been explained. From its position on the alveolar wall, we can speculate that it may be sloughed off the wall and become an alveolar macrophage, but there is no direct evidence to support this. On the contrary, recent investigations have shown that most of the alveolar macrophages are derived from hematopoietic tissues, not pulmonary tissues, suggesting that the primary role of the Type 2 sister cell is not as a source of alveolar macrophages.[14–17] Another supposition is that the Type 2 sister cell may replace damaged alveolar epithelium.[10,18] From their work on oxygen toxicity, Kapanci *et al*[10] speculated that Type 2 cells may be the reserve cells for damaged Type 1 cells.

The purpose of this study was to determine the kinetics of Type 2 cell division and the fate of the sister cell following exposure to nitrogen dioxide (NO_2). Previous work showed that, in rats continuously exposed to NO_2,[9] the number of dividing Type 2 alveolar cells increases.[19] In alveoli at the ends of terminal bronchioles, there is visible tissue damage and a large increase in the number of Type 2 cells, but in alveoli peripheral to this region the increase is only slight.[19,20] In these peripheral areas, there is generally no visible tissue damage, and the Type 2 cells act like a renewing cell population—*ie*, there is evidence of cell division but no large increase in cell numbers. In the present experiment, we studied Type 2 cell division in alveoli peripheral to the ends of the terminal bronchioles.

There are several problems involved in studying cell division in the alveoli of the lung. Since the actual amount of tissue in a section is small, compared with the size of a section, because of the alveolar spaces, large sections must be scanned to observe enough cells for a significant evaluation of the dividing cell population. This is best accomplished with the light microscope. However, identification of cells in the alveoli with the light microscope is difficult, and thus electron microscopic technics are also necessary.[11] In addition, there is no uniform organization of the cells in the tissue sections with respect to their position on the alveolar wall. The cells studied appear randomly dispersed throughout the tissue. This makes it difficult to determine the fate of sister cells after they have divided, because they may move in

any direction when dividing, and thus only a few sister cells will be seen in any one section as a pair of cells.

In the present experiments, the alveolar cells were studied with the light and electron microscopes using autoradiographic technics. Dividing cells were labeled with triated thymidine (^{3}H-TdR). Electron microscopy confirmed the identity of the cells in question and allowed specific study of their ultrastructure and morphology. This led to a set of criteria for identification of the cells with the light microscope in 1-μ plastic sections.

Materials and Methods

Experiment 1

The purpose of the first experiment was to study the ultrastructure and kinetics of Type 2 cell division following exposure to NO_2. One-month-old male Wistar rats weighing approximately 100 grams were used for this study. A total of 36 rats were exposed to 15 to 17 ppm NO_2 for 48 hours, removed from the exposure chambers and injected intraperitoneally with 500 μ Ci of ^{3}H-TdR (specific activity 6.7 ci/mmoles).[21] Animals were sacrificed, with an overdose of sodium pentobarbital, at ½, 1, 1½ and 2 hours and then hourly for a total of 12 hours after ^{3}H-TdR injection. An 18-gauge needle was inserted in the trachea, and fixative (2% glutaraldehyde in cacodylate buffer) was perfused through the airways. After the lung was filled, it was removed from the thoracic cavity and placed in a vial of the same fixative for 1 hour. The left lung was then sliced into thin (1-mm) leaflike pieces, washed in three changes of buffer and postfixed in 1% OsO_4 in veronal acetate buffer containing 7.8% sucrose. Seven additional rats served as unexposed controls. They were processed for microscopy in the same manner.

Following fixation, the leaf-like pieces were dehydrated in alcohol and infiltrated with resin (Araldite). The infiltrated slices of tissue were examined with a dissecting microscope; small squares of tissue, each containing a longitudinal section of a terminal bronchiole with its associated alveolar ducts and alveoli, were removed and embedded.[22]

Experiment 2

The purpose of this experiment was to determine the fate of Type 2 cells after they have divided. The same type of animals were used as in Experiment 1. A total of 11 rats were exposed to 15 to 17 ppm NO_2 for 48 hours, removed from the chambers and sacrificed at intervals of 1, 24, 48 and 72 hours after injection of 500 μ Ci of ^{3}H-TdR. The animals were sacrificed as in Experiment 1, and the lungs were fixed by perfusing the airways with 1% OsO_4 in veronal acetate buffer containing 7.8% sucrose. Dehydration and embedding were the same as in Experiment 1.

Autoradiography

For light autoradiography, 1-μ sections were cut using a glass knife on an ultramicrotome and mounted on glass slides subbed with a solution of 0.1% gelatin containing 0.01% chromic potassium sulfate. The mounted slides were then coated with

a 1:1 solution of emulsion (Ilford L-4) and distilled water and stored in light-tight boxes at 0 to 4 C. After 2 weeks, the slides were developed in 13.5% developing agent (Microdol X) for 8 minutes, rinsed in distilled water, fixed in 15% sodium thiosulfate for 5 minutes and washed for 10 minutes. The tissues were stained lightly with toluidine blue, rinsed in alcohol, dried and coverslip-mounted.[21]

For electron autoradiography, 10 specimen blocks from each animal studied were trimmed, and pale gold sections were cut with a diamond knife on an ultramicrotome. The sections were picked up on collodion-coated, 150-mesh nickel grids that had previously been lightly coated on the back with carbon. The specimens were double-stained with uranyl acetate and lead citrate and then covered with a light coat of carbon in a vacuum evaporator. These grids were mounted on glass slides, coated with emulsion (Ilford L-4) according to the loop technic of Caro,[23] and stored in light-tight boxes at 0 to 4 C. After 6 weeks of exposure, the samples were developed in 13.5% developing agent (Microdol X) for 3 minutes, rinsed in distilled water, fixed in 15% sodium thiosulfate for 5 minutes and rinsed in four changes of distilled water. The grids were allowed to dry for 24 hours, and then viewed on a Phillips 200 electron microscope.

Results

Distribution of Labeled Type 2 Cells Following Exposure to NO_2

Rats exposed to NO_2 for 48 hours in Experiments 1 and 2 showed a large increase in the number of Type 2 cells labeled with ^{3}H-TdR. The criteria for classifying a cell as Type 2 with the light microscope were that it must be cuboidal, lie on the alveolar surface and contain several vacuoles (Figure 2). Electron autoradiography confirmed that large numbers of labeled Type 2 cells were present in the alveoli, and that the criteria for identifying them with the light microscope were adequate (Figure 8). Figure 1 illustrates the distribution of labeled and unlabeled Type 2 cells in a portion of the alveoli, peripheral to the opening of a terminal bronchiole, one hour after an injection of ^{3}H-TdR in an animal exposed to NO_2 for 48 hours. The distribution was the same as that previously described,[19] and there was no visible evidence of tissue damage in the peripheral areas at the light or electron microscope level. The labeling indexes (LI) obtained from sections like this were determined from counts of at least 150 Type 2 cells with the light microscope from three different sections in each animal and are presented as the percent of labeled Type 2 cells. The average LI, 1 hour after injection of ^{3}H-TdR, from 4 rats exposed to 15 to 17 ppm NO_2 for 48 hours, was 32.0 (Table 1).

The proportion of Type 2 cells in the peripheral alveoli of animals exposed to NO_2 was determined to see if the number was appreciably greater than in control animals. A minimum of 500 alveolar cells from at least three sections in each animal was counted with the light microscope. Seven experimental animals had an average of 14.1 $\pm$

Table 1—Cells in the Alveolar Epithelium Labeled with ^{3}H-TdR

Hours after injection	Type 2 cells			Type 1 cells		
	Label	Total cells	Average % label	Label	Total cells	Average % label
1	31	150		1	261	
	53	150		0	142	
	57	150		2	141	
	57	150	32.0	1	102	0.7
24	35	150		2	150	
	70	150	34.9	5	150	2.3
48	26	150		14	163	
	4	150		3	150	
	30	150	13.3	10	150	5.8
72	5	150		4	150	
	7	150	4.0	5	150	3.0

Each line corresponds to 1 animal

3.1 Type 2 cells per 100 alveolar cells; 7 unexposed controls had an average of 10.8 ± 1.2. The difference of 3.3 was significant at the 1% level.

Division of Type 2 Cells

At intervals of 1, 4, 5, 8 and 12 hours after injection of ^{3}H-TdR, 2 animals (each time) were prepared for electron autoradiography. Each grid was scanned in the electron microscope, and all labeled Type 2 cells and those in the process of mitosis were photographed. Labeled cells collected from both animals at each time interval were combined for study. Examples of cells during mitosis obtained from all animals regardless of time were combined for study.

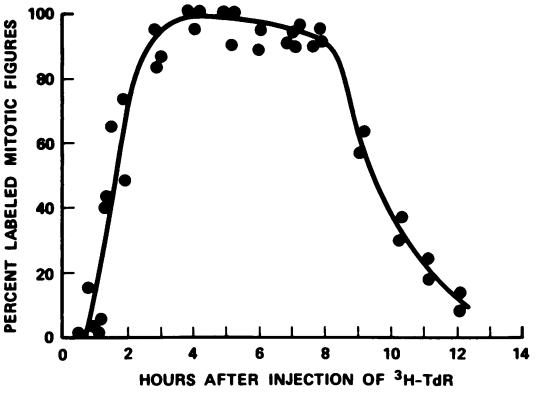
A total of 31 Type 2 cells were observed during mitosis (Figure 9). All the cells studied contained lamellar bodies, normal appearing mitochondria, microvilli on the free surface (that surface exposed to the alveoli), and were attached by at least one edge to the basement membrane. Fourteen of the 31 cells studied were partially covered on the free surface by a portion of the adjacent Type 1 cell (Figure 9). Because of this, and the fact that all cells observed were on a basement membrane, it suggests that, as the Type 2 cells divide, they move apart on the basement membrane, and one or both sister cells move under the adjacent Type 1 cell. Study of labeled Type 2 cells over the 12 hours studied supported this interpretation.

A total of 193 labeled Type 2 cells were studied in the electron microscope at 1, 4, 5, 8 and 12 hours after injection of ^{3}H-TdR. In all cases, the cells were attached by one surface to the basement membrane. If Type 2 cells do move under the adjacent Type 1 cell during cell division, then they not only should be attached to the basement membrane, but also an increasing number of labeled Type 2 cells should be covered by portions of the adjacent Type 1 cells as cell division progresses. Figures 8 and 12 are examples of cells not covered by the adjacent Type 1 cell. Figures 10 and 11 are examples of cells that are considered to be covered over more than half of their free surface by the adjacent Type 1 cell. In some cases, the Type 1 cell appears to be lifting off the underlying Type 2 cell (Figure 10). The data in Table 2 show that an increasing number of labeled Type 2 cells were covered by Type 1 cells. One hour after injection of ^{3}H-TdR, 13% of the cells were covered, whereas 45% were covered at 12 hours.

Morphologically, the labeled Type 2 cells contained lamellar bodies, normal-appearing mitochondria and cytoplasm, and microvilli on the free surfaces not covered by adjacent Type 1 cells (Figures 8–12). Because of the variable sizes and numbers of lamellar bodies observed in labeled and unlabeled cells at any of the times studied, we did not determine whether these parameters changed during cell division. At 8 and 12 hours, it appeared as if some of the labeled Type 2 cells were spreading out over the basement membrane, covering an area usually covered by Type 1 cells (Figure 12). At this time, a few examples of cells with more pronounced characteristics of both Type 2 and Type 1 cells were also observed (Figure 13).

Kinetics of Type 2 Cell Division

The kinetics of Type 2 cell division was estimated from a curve constructed from the percentage of labeled mitotic figures at ½, 1, 1½, and then at hourly intervals from 2 through 12 hours after injection of ^{3}H-TdR (Text-figure 1). Ten sections from different areas of each animal's lung were scanned in the light microscope, and the number of labeled and unlabeled mitotic figures in each was scored. Based on the electron microscopic observations of dividing Type 2 cells, the criteria for light microscopic identification were: the cell must appear to be on the alveolar surface and must contain vacuoles and a mitotic figure (Figures 2 and 3). A minimum of 20 mitotic figures were used in determining the percentage of labeled mitosis in each animal.[24]



TEXT-FIG 1—Curve constructed from the percent of labeled mitotic figures in the Type 2 cell population. Each point represents one animal.

Labeled mitotic figures first appeared 1 hour after injection of ^{3}H-TdR. The percent of labeled mitotic figures rose rapidly thereafter and approached 100% by 3 hours, where it remained until 8 hours and then decreased. The labeled mitotic figures fell below the 50% level between 9 and 10 hours.

The duration of various phases of the cell cycle was estimated from this curve.[13,24,25] The time it took labeled figures to first appear (G_2) was less than 1 hour; the time of t_2 ($G_2 + \frac{1}{2}$ M) was approximately 1.8 hours. Mitosis (M) was estimated at approximately 1.8 hours, and the duration of DNA synthesis at approximately 7.7 hours.

Fate of Type 2 Cells After Division

Because the Type 2 sister cells remain on the basement membrane after division, and some acquire characteristic of Type 1 cells, Experiment 2 was undertaken to see if the Type 2 sister cell could transform into a Type 1 cell. In this experiment, the S-phase cells were pulse labeled with ^{3}H-TdR. Tritiated thymidine is only available to the cells for about 45 minutes and is then broken down.[13] Thus, cells labeled 1 hour after injection represent those synthesizing DNA in preparation for cell division. Any labeled cell types that were not initially labeled at 1 hour and then appeared labeled at a later time would have been derived from one of the originally labeled cell types or from another cell type at a different site which then migrated into the area.[13,26]

Initial study of the surface epithelium of the alveoli from Experiment 2 showed that it was primarily composed of Type 1 and 2 cells and a few cells that had characteristics of both Type 1 and 2 cells. Light and electron autoradiography was performed in 2 animals 1 hour after injection of ^{3}H-TdR and in 2 animals 48 hours after injection. Light microscopy revealed what appeared to be labeled Type 1 cells at 48 hours (Figure 6). The autoradiographs for electron microscopy were then scanned, and all labeled epithelial cells photographed. The information from both animals at each time was combined. Electron microscopy confirmed the presence of labeled Type 1 cells at 48 hours (Figures 14–17). At 1 hour, 36 labeled Type 2 cells but no labeled Type 1 cells were observed. At 48 hours, 43 labeled Type 2 cells and 24 labeled Type 1 cells were observed. A few cells were labeled that had characteristics of both Type 1 and 2 cells similar to those shown in Figure 13. These cells were not included in the counts.

The ultrastructure of labeled Type 2 cells appeared similar to unlabeled Type 2 cells in the same section. We then determined how many of the labeled Type 2 cells were covered by Type 1 cells. Of the 36 Type 2 cells at 1 hour, 1 was covered by an adjacent Type 1 cell (2.8%). At 48 hours, 7 of the 43 Type 2 cells were covered by the adjacent Type 1 cells (16.3%). Most of the labeled Type 1 cells at 48 hours appeared normal, except for occasional cytoplasmic inclusions similar to lamellar bodies, microvilli or an irregular surface, and an occasional abnormal appearing nucleus (Figures 14–17). The labeled Type 1 cells were not covered by flaps of adjacent Type 1 or 2 cells.

The lung tissue of animals sacrificed at 1, 24, 48 and 72 hours were then studied with the light microscope to see if they showed an increase in labeled Type 1 cells. The criteria for classifying a Type 1 cell with the light microscope, established from observations with the electron microscope, were: a) the cell must appear to be on the surface, b) the nucleus must protrude into the alveolar space and c) there must be little cytoplasm (compared with the nucleus) and no vacuoles (Figures 6 and 7).

The numbers of labeled and unlabeled Type 1 cells were counted in at least 4 different sections from each animal with the light microscope, and the percentage of labeled cells was calculated (Table 1). The numbers of labeled and unlabeled Type 2 cells were also counted on the same slides (Table 1).

At 1 hour after injection of the isotope, there were 4 labeled cells in 646 Type 1 cells counted in 4 different animals (average LI, 0.7%). At 24 hours, there was an increase to 7 labeled cells in 300 cells from

Table 2—Number of Labeled Type 2 Cells Covered by Adjacent Type 1 Cells during Cell Division

Hours after injection of ^{3}H-TdR	Not covered	Covered
1	26	4
4	28	10
5	23	13
8	21	19
12	27	22

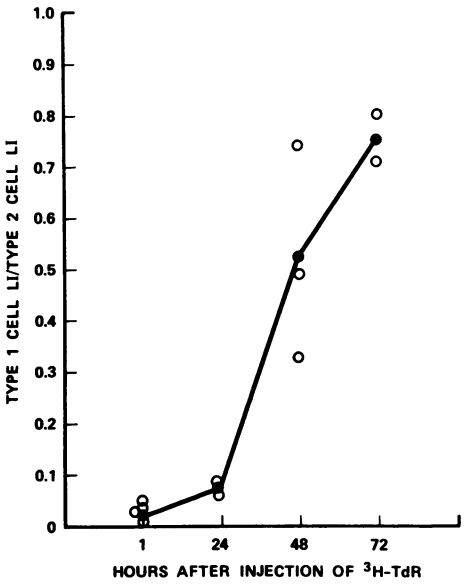
2 animals (average LI, 2.3%) and at 48 hours, to 27 labeled cells in 463 cells from 3 animals (average LI, 5.8%). At 72 hours, there were 9 labeled cells in 300 cells from 2 animals (average LI, 3.0%). Occasionally, a labeled Type 1 cell was found adjacent to a labeled Type 2 cell at 48 and 72 hours (Figure 7).

The actual number of labeled cells found in the alveoli varied considerably from animal to animal. However, if the increased number of labeled Type 1 cells is caused by transformation of a Type 2 cell, then the number of labeled Type 1 cells with respect to labeled Type 2 cells should increase with time in each animal. This was expressed by the ratio of the LI of Type 1 cells to the LI of Type 2 cells in each animal. Because of the restrictions placed on identifying Type 1 and 2 cells with the light microscope, the LI for each will be slightly in error, but the relationship of one to the other should still demonstrate this increase. This information shows that the number of labeled Type 1 cells in the tissue increases with respect to the number of labeled Type 2 cells from an average ratio of .07 at 24 hours to 0.75 at 72 hours (Textfigure 2).

The proportion of Type 1 cells in these tissues was determined from counts of 500 alveolar cells from at least 3 different sections in each animal and compared with controls to see if the increase in labeled Type 1 cells was associated with an increase in the proportion of Type 1 cells. Seven experimental animals had an average of 5.8 ± 2.6 Type 1 cells per 100 alveolar cells, and 7 controls had an average of 5.7 ± 2.3.

Discussion

The labeled Type 2 cells in the alveoli of rats exposed to NO_2 for 48 hours appear to be randomly distributed throughout the alveoli peripheral to the opening of the terminal bronchiole.[19] After exposure to NO_2, the total number of Type 2 cells was slightly but significantly greater than in control animals. Considering the large number of di-



TEXT-FIG 2—The ratio of Type 1 cell LI to the Type 2 cell LI. *Solid circles* represent the mean ratio.

viding Type 2 cells at this time (32.0%), if both sister cells remained on the alveolar wall after division, the total number of Type 2 cells in the alveoli would have increased by more than twofold. However, the measured increase was much less than this. In light of our data, the slight increase was interpreted as representing Type 2 sister cells that have not yet transformed into Type 1 cells.

The mechanism of Type 2 cell division has not been presented before. Previous investigations have described mitotic figures in Type 2 cells but not at the electron microscopic level.[9,27] The present study shows that Type 2 cells before, during and after cell division are not obviously different from nondividing Type 2 cells. They contain lamellar bodies and a normal complement of cell organelles. During actual mitosis, it did not appear necessary for the lamellar bodies to be extruded, since they were present during and after mitosis. When the two sister cells begin to separate at anaphase, they appear to move over the basement membrane and beneath the adjacent Type 1 cell. Evidence for this was presented by three different observations: a) all labeled cells observed before, during and after mitosis were attached by one surface to the basement membrane; b) about half of the cells observed during mitosis were partially covered by the adjacent Type 1 cells and c) labeled Type 2 cells covered by the adjacent Type 1 cell increased as cell division progressed (Table 2). No evidence suggested that the sister cells moved onto the alveolar surface of the Type 1 cell after division. Occasional pairs of Type 2 cells were

also observed at 8 and 12 hours, times when most of the labeled cells would have already divided, indicating that both cells could remain on the alveolar wall after division (Figure 5).

Other investigators have also reported Type 1 cells covering Type 2 cells under experimental conditions. Faulkner and Esterly [28] reported Type 2 cells covered by the adjacent Type 1 cell following treatment with Freund's adjuvant. Greenberg *et al* [29] also reported Type 2 cells covered by Type 1 cells following treatment with dilute nitric acid and suggested that the purpose of the covering cell was protective. In both of these studies, proliferation of Type 2 cells was reported, although cell division was not monitored by labeling with ^{3}H-TdR. In the present study, very few labeled Type 2 cells were covered by adjacent Type 1 cells at 1 hour, but at 12 hours (when most of the cells had divided) half of the labeled cells were covered. Later, after 48 hours, the number of labeled Type 2 cells covered by Type 1 cells had decreased to near the 1-hour levels (16.3%). Since the covering Type 1 cell did not persist, it is probable that Type 2 cells observed to be covered by adjacent Type 1 cells represent cells that had recently divided.

Evidence that Type 2 cells are able to transform into Type 1 cells came from labeling and morphologic data. Electron autoradiography showed that, at 1 hour after injection, the only labeled cells in the alveolar epithelium are Type 2 cells. Approximately 36 hours after the Type 2 cells had divided (48 hours after injection), both Type 2 and 1 cells were found labeled in the alveolar epithelium. Light microscopic autoradiography further showed that the proportion of labeled Type 1 cells increased with respect to labeled Type 2 cells in each animal, from a mean ratio of 0.07 at 24 hours to 0.75 at 72 hours. Since Type 1 cells were not labeled in large numbers initially, the increased number of labeled Type 1 cells observed at 48 and 72 hours had transformed from another cell type. The only labeled cells in the epithelium before cell division are Type 2 cells; thus, it is suggested that the labeled Type 1 cells at 48 hours were derived from Type 2 cells that had transformed into Type 1 cells. Morphologically, this interpretation is further supported by the fact that labeled Type 2 cells were found shortly after division which had characteristics of Type 1 cells; labeled Type 1 cells were found at 48 hours that had characteristics of Type 2 cells.

Two factors that could affect the number of labeled Type 1 cells observed at 24, 48 and 72 hours, and thus could affect the above interpretation, are; a) division of Type 1 cells that were initially labeled, and b) reutilization of ^{3}H-TdR from dead cells by Type 1 cells. Our

observations with the light microscope showed that some Type 1 cells have the ability to divide. Other investigators have also reported that Type 1 cells may divide, but at a very low rate.[9,11] They are classified as an expanding cell population, and the division of cells is thought to be associated with growth of the tissue.[9] In the present experiment, the number of labeled Type 1 cells observed at 48 and 72 hours (5.8% and 3.0%) far exceeds that which could be accounted for by a doubling of the Type 1 cells originally labeled at 1 hour (0.7%).

The possibility that ^{3}H-TdR released from dead cells is reutilized by lung tissue was shown by Baserga and Kisieleski.[30] They demonstrated an increased amount of tritium in the tissue on the fourth day after injection. However, since the increases in labeled Type 1 cells seen in the present study occurred mainly before this time, at 2 and 3 days, reutilization of labeled ^{3}H-TdR should not be a major factor in interpreting these results. In addition, reutilization shows up in autoradiographs as a slight increase in grain count. This change would not likely affect the present study because of the short exposures of the autoradiographs.[31,32]

Early transitional forms between Type 2 and 1 cells reveal a cell with characteristics of Type 2 cells, but which appears to spread out over the basement membrane instead of remaining cuboidal. Later, the Type 1 cells studied at 48 hours in the electron microscope have characteristics of Type 2 cells—namely, occasional microvilli and dense inclusions similar to modified lamellar bodies. The changes in Type 1 and 2 cells are very similar to the changes noted by Faulkner and Esterly [28] in Type 1 and 2 cells following treatment with Freund's adjuvant. They speculated that the different forms of Type 1 and 2 cells that they observed may represent a transition from one form to the other, although they did not say in which direction the transition went. Greenberg *et al* [29] also reported alterations in Type 1 and 2 cells and speculated from their study that Type 1 cells become Type 2 cells, although there was no evidence indicating the direction of the transition. Slight alterations, similar to those reported in Type 1 cells, were also reported, by Stephens *et al*,[20] during continuous exposure to NO_2. The present study indicates that the direction of the transition is from Type 2 to Type 1 cells.

Counts of the total number of Type 1 cells in the tissue showed no differences between experimental and control animals. Since there is an increase in the number of labeled Type 1 cells but no increase in the total number, it suggests that a Type 1 cell is sloughed off and replaced by a new Type 1 cell. This is supported by our finding that

labeled Type 2 cells at 48 hours were not covered by adjacent Type 1 cytoplasm, and by the example in Figure 10 which suggests that the Type 1 cell on top of the Type 2 cell is being sloughed off. However, the details of how they are sloughed off are not clear, and further research is necessary to explain this process.

The various phases of the cell cycle had not previously been measured for Type 2 cells. Other investigators have measured the duration of DNA synthesis in respiratory tissue but did not differentiate between cell types.[27,33] The results from the present study are similar to those reported for other cell populations in the rat.[34] However, since cell division was stimulated in these animals, the estimated times may be different from those in controls.[35,36]

The percentage of labeled mitotic figures rose rapidly, approaching 100% by 3 hours, and abruptly decreased between 9 and 12 hours (Text-Figure 1). This can be interpreted as showing that few dividing cells are being retained in the DNA synthesis phase of the cell cycle, and thus most of the labeled Type 2 cell population has divided by 12 hours.[13] The transition of a Type 2 cell to a Type 1 cell varies from 24 to more than 72 hours (Text-figure 2). Since few transitional forms were observed, it indicates that once the morphologic changes begin they occur rapidly.[26]

The process of cell renewal just described for the alveolar epithelium occurred following exposure to NO_2 and thus may be different in an unexposed animal. However, considerable data suggest that the process is not different, but instead represents an acceleration of the normal cell renewal process in the alveoli: first, cell renewal is basically a homeostatic mechanism for maintaining the integrity of a tissue in which there is a loss of cells.[13,37] In most epithelial tissues, the cells sloughed off represent the surface layers of that tissue. These cells are subject to chemical and physical stress, and the degree of stress placed on the tissue can affect the rate of cell renewal.[38,39] In the avleoli of the lung, the majority of the surface area is composed of Type 1 cells. Since this surface is exposed to chemical (noxious gases) and physical (particulate material) stress, it is not surprising that a mechanism for replacement or repair exists. There are many examples of changes in the rate of cell renewal in a tissue under varying conditions, and in the lung it has been shown that cell renewal rates are affected by the age, sex, strain, noxious gases and the presence or absence of mild lung infections in the animals being studied.[19,21,27,35] Since the tissue was not visibly damaged in this study and Type 2 cell hyperplasia was not seen in these peripheral alveoli, it can be implied that the life span of individual

Type 1 cells had decreased during exposure to NO_2. The increased number of dividing Type 2 cells which replace these cells is then an increase in the rate of cell renewal.

Second, the mechanics for renewal of the alveolar epithelium are essentially the same as those described for other renewing cell populations—namely, division of progenitor cells (Type 2 cells) and migration and maturation of one sister cell to a mature cell type (Type 1 cells) to replace a cell that is being sloughed off.[12,13,26] Thus, our interpretation for renewal of the alveolar epithelium is consistent with the data and existing criteria for a renewing cell population.

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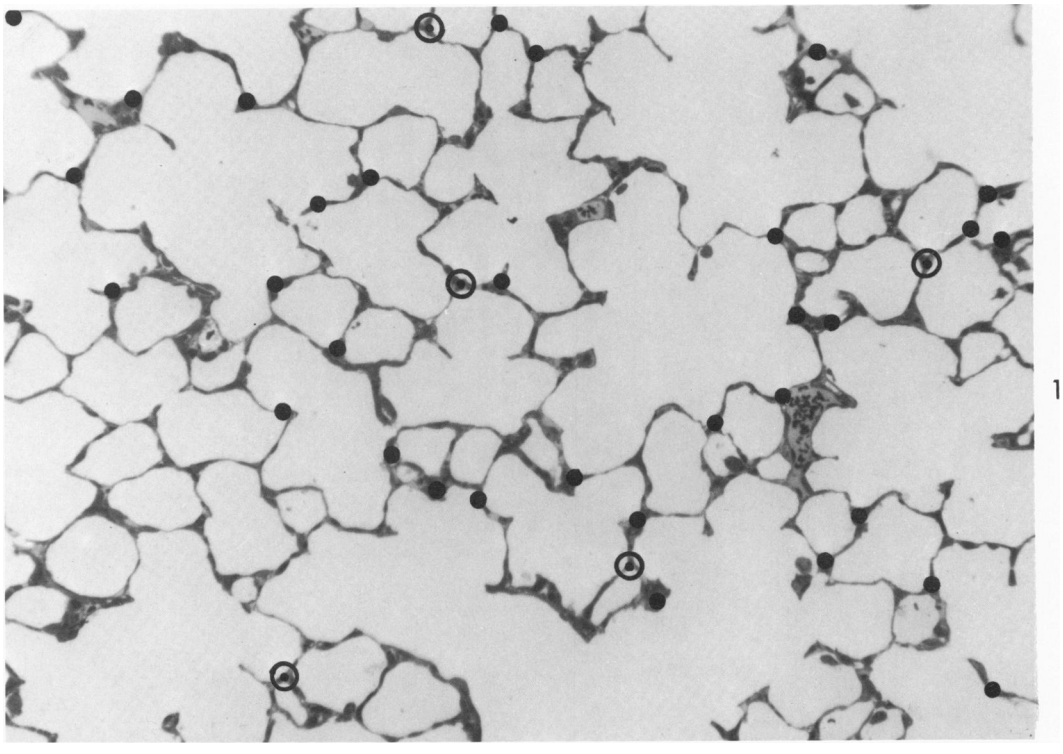
[Illustrations follow]



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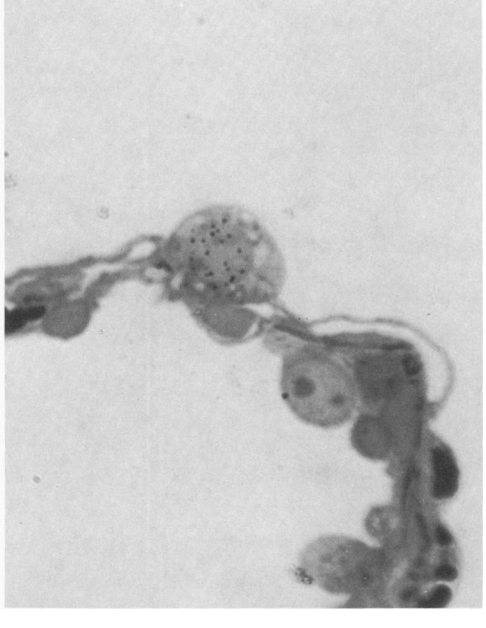

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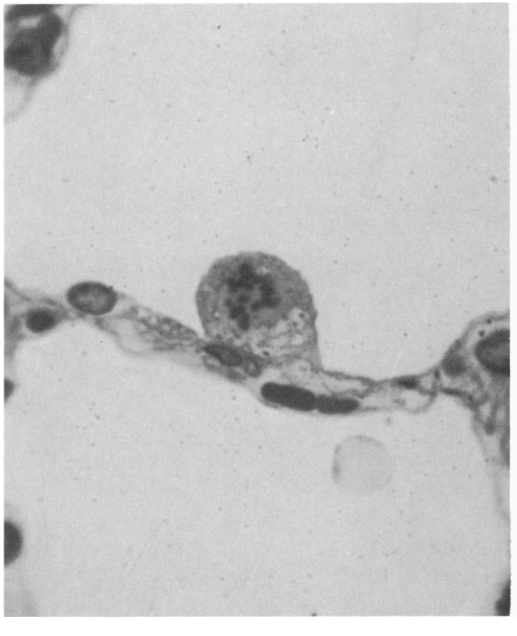

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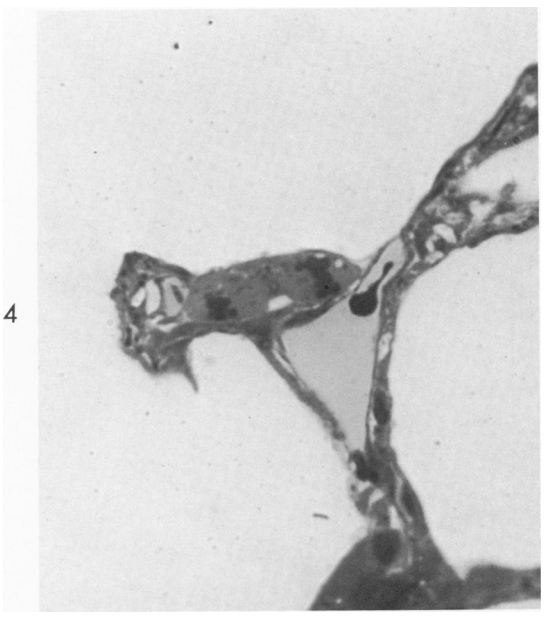
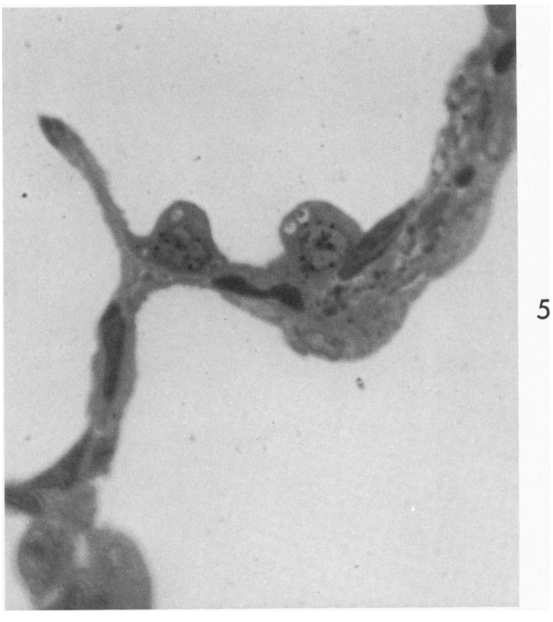
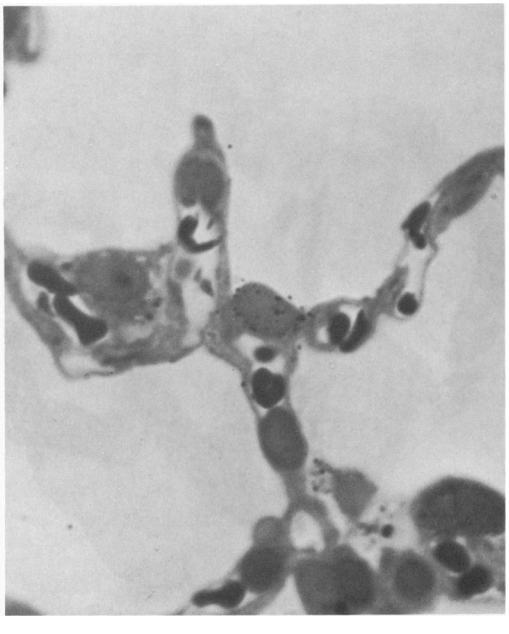
Fig 1—Pulmonary alveolar tissue after 48 hours' exposure to 15 to 17 ppm NO_2. Each point indicates the location of a Type 2 cell; the circled points indicate those which were labeled (Glutaraldehyde, osmium and toluidine blue, × 200). **Fig 2**—An example of a labeled Type 2 cell one hour after injection of ^{3}H-TdR. (Osmium and toluidine blue, × 1250). **Fig 3**—An example of a Type 2 cell during mitosis (Glutaraldehyde, osmium and toluidine blue, × 1250).







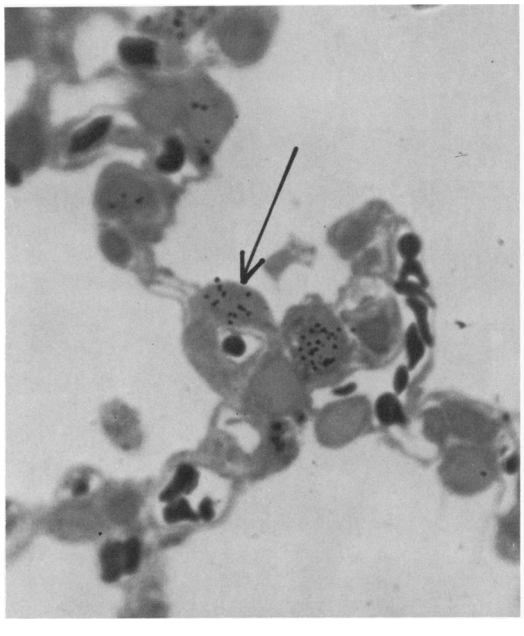


Fig 4—An example of a Type 2 cell during anaphase. Note that both cells are on the alveolar wall (Glutaraldehyde, osmium and toluidine blue, × 1250). **Fig 5**—Two labeled Type 2 cells 12 hours after injection of ^{3}H-TdR. Because most of the cells have divided by this time and because of the proximity of these cells to each other, they are considered as sister cells (Glutaraldehyde, osmium and toluidine blue, × 1250). **Fig 6**—Example of a labeled Type 1 cell 48 hours after injection of ^{3}H-TdR (Osmium and toluidine blue, × 1250). **Fig 7**—A labeled Type 1 cell (*arrow*) adjacent to a labeled Type 2 cell 48 hours after injection of ^{3}H-TdR (Osmium and toluidine blue, × 1250).

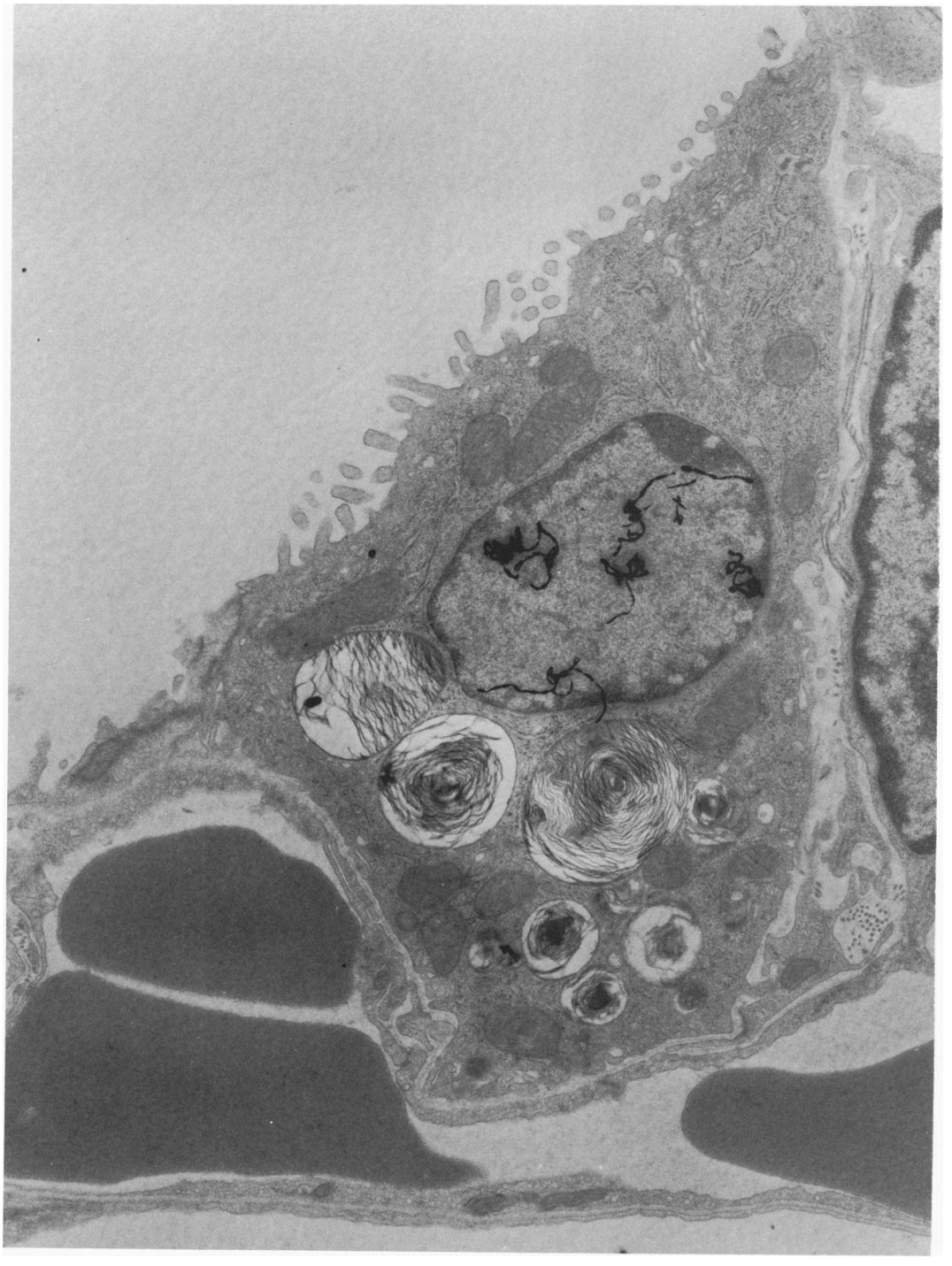


Fig 8—Electron micrograph of a labeled Type 2 cell 1 hour after injection of ^{3}H-TdR (× 13,000).

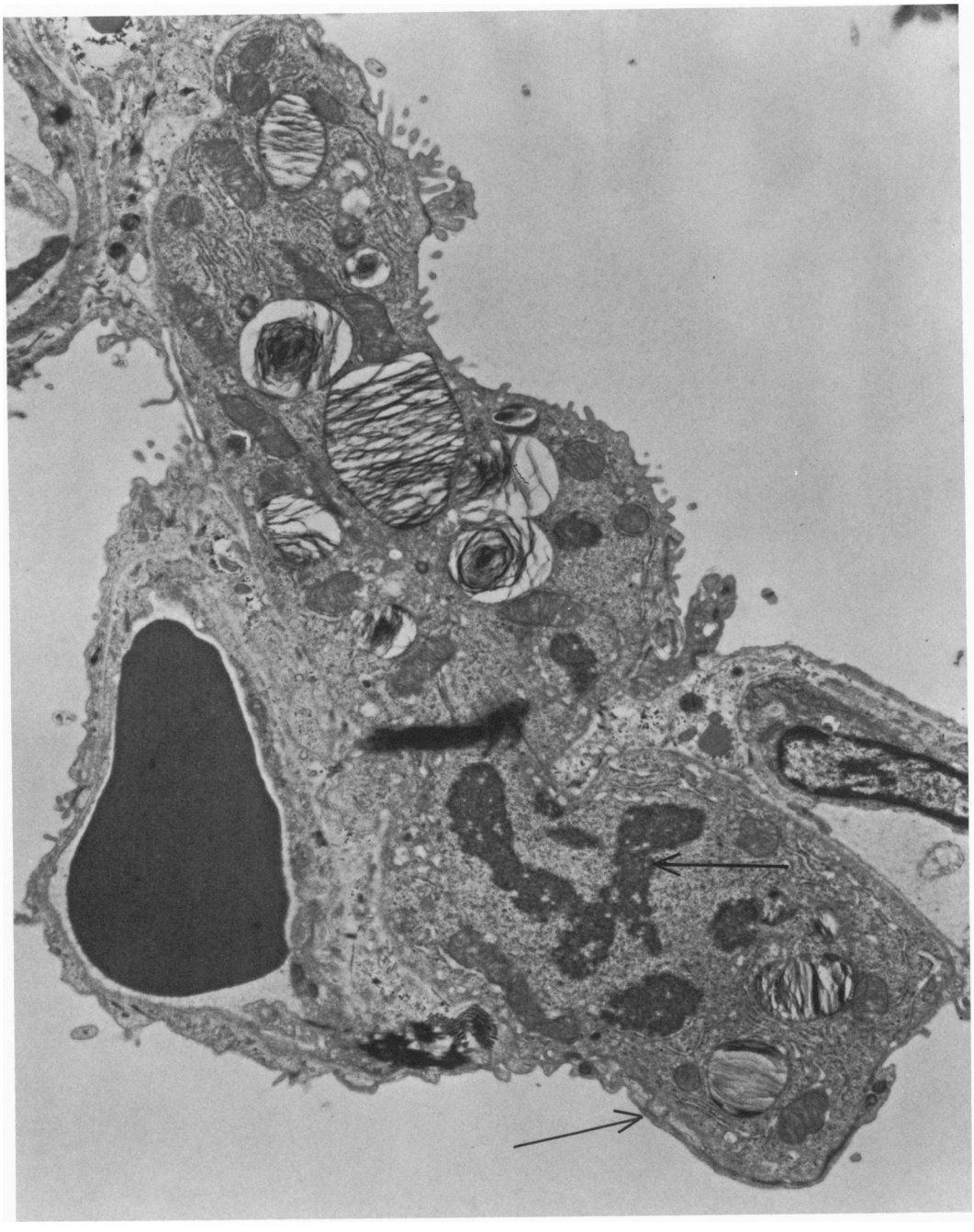
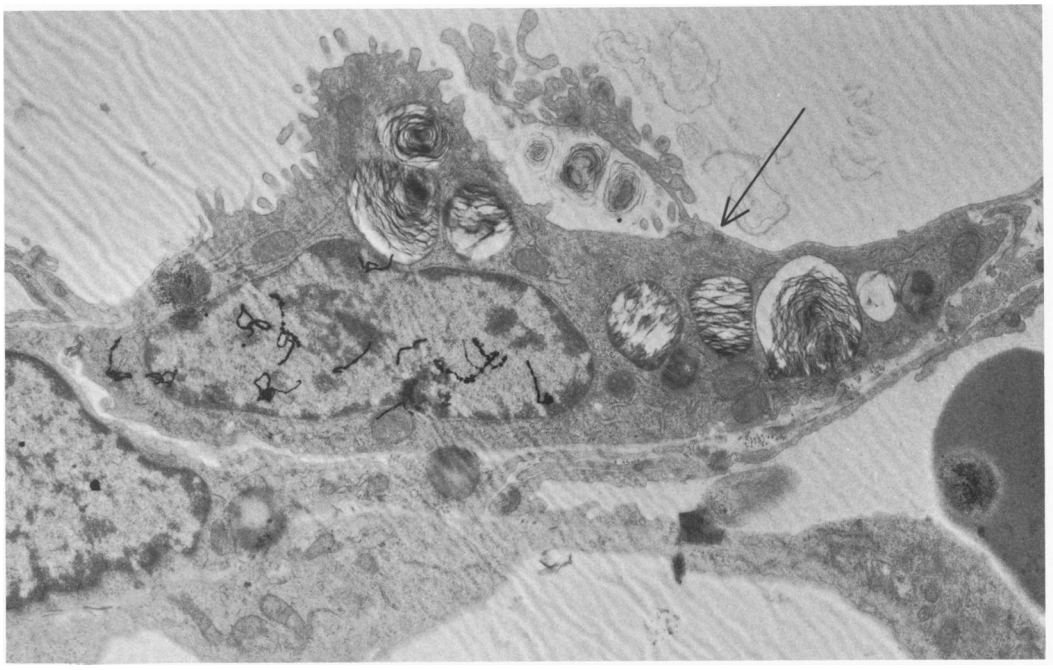


Fig 9—Electron micrograph of a Type 2 cell during mitosis. *Arrows* illustrate covering by the adjacent Type 1 cell and the condensed chromosomes (× 9000).



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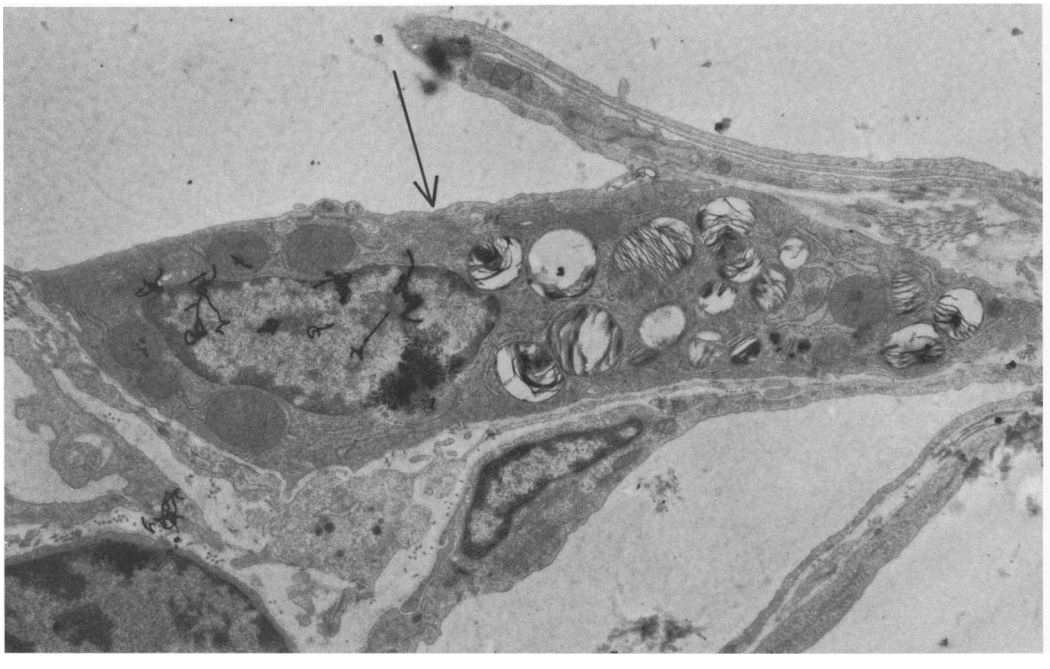

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Fig 10—Type 2 cell 8 hours after injection of ^{3}H-TdR. Note the covering Type 1 cell that appears to be peeling away (*arrow*) (× 8600). **Fig 11**—Type 2 cell 8 hours after injection of ^{3}H-TdR. The free surface is covered by a Type 1 cell (*arrow*) (× 9500).

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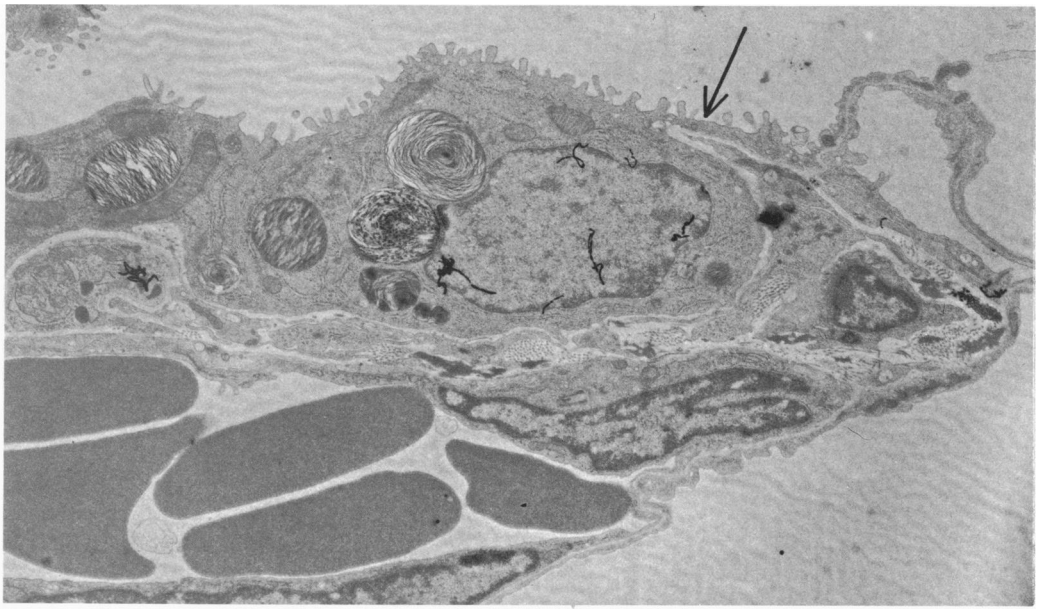

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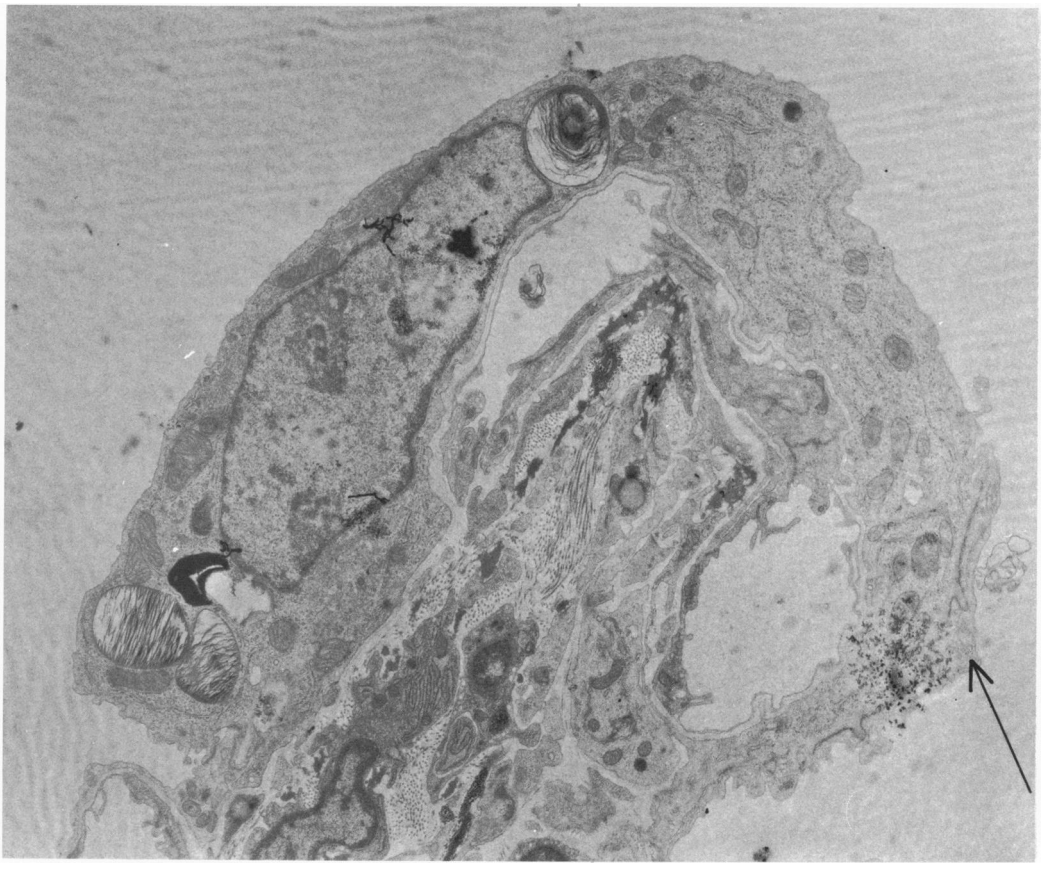


Fig 12—Type 2 cell 8 hours after injection of ^{3}H-TdR. The cell appears to be spreading out and one process is covering an area usually occupied by a Type 1 cell (*arrow*) (× 7700).
Fig 13—A type 2 cell with characteristics of a Type 1 cell 12 hours after injection of ^{3}H-TdR. The *arrow* points out the overlying portion of the adjacent Type 1 cell (× 8200).

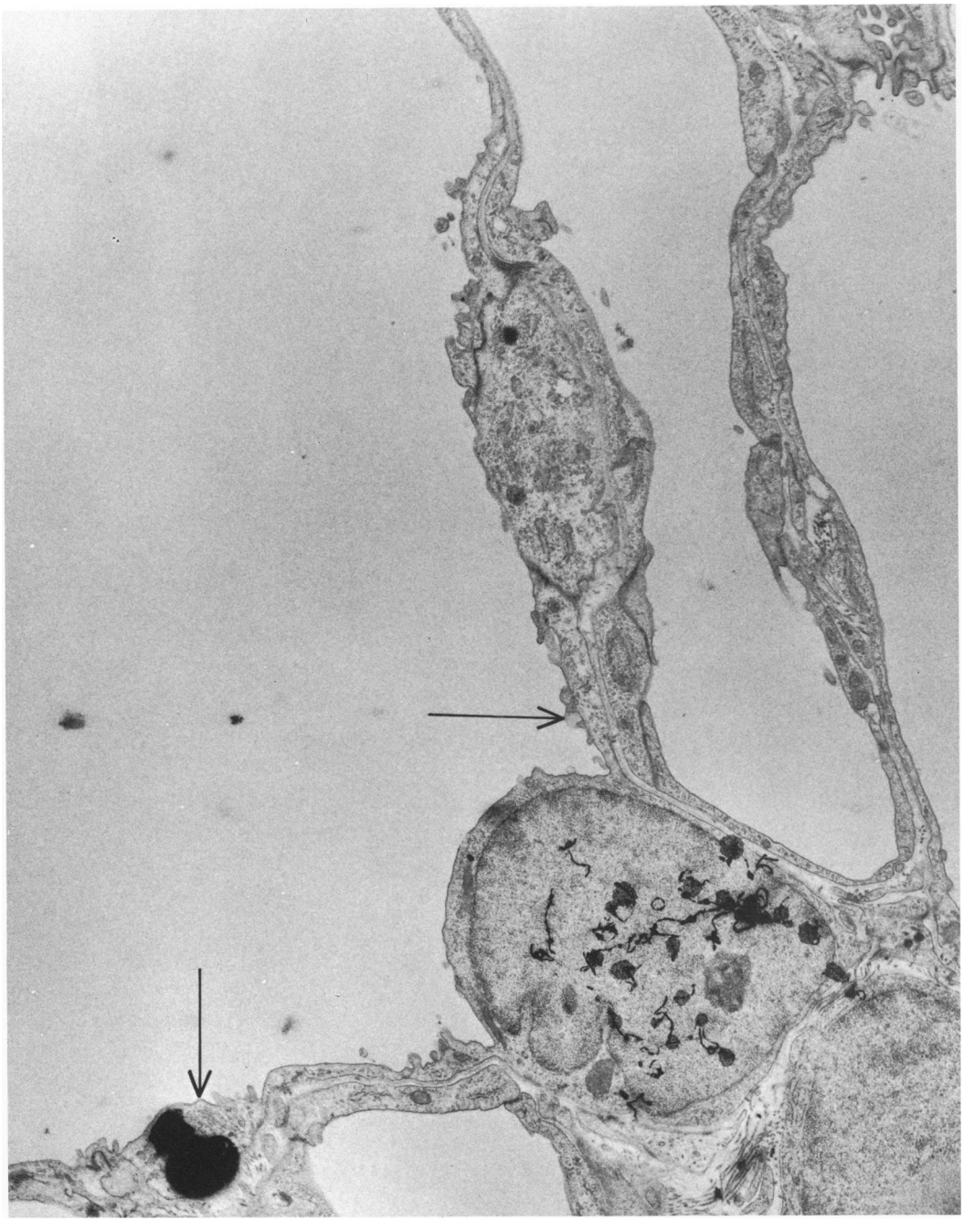
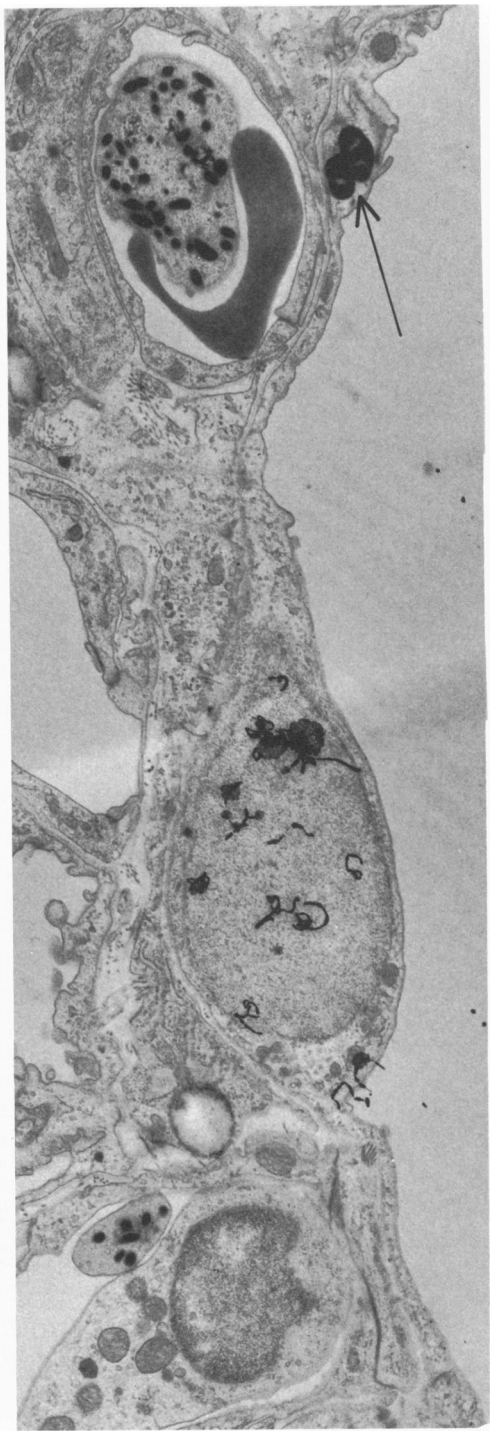


Fig 14—A labeled Type 1 cell 48 hours after injection of ^{3}H-TdR. Note the dense inclusion and irregular surface (*arrows*) (× 11,600).



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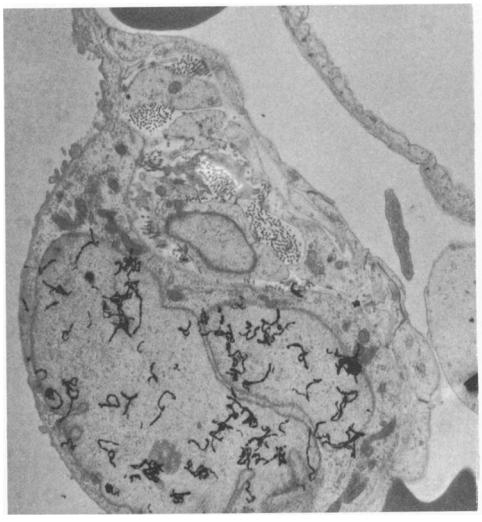

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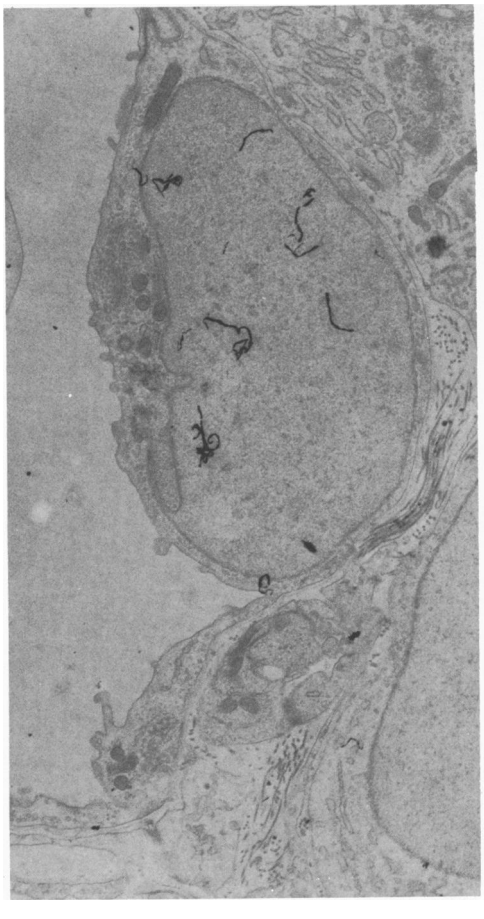

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Fig 15—A labeled Type 1 cell 48 hours after injection of ^{3}H-TdR. Note the dense inclusion at the edge of the cell (*arrow*) (× 11,200). **Fig 16**—A labeled Type 1 cell with an irregular nucleus 48 hours after injection of ^{3}H-TdR (× 6000). **Fig 17**—A labeled Type 1 cell with an irregular surface 48 hours after injection with ^{3}H-TdR (× 11,200).