# Postnatal Development of Alveoli

## Regulation and Evidence for a Critical Period in Rats

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## Abstract

**In many species, including humans, pulmonary alveoli are formed after birth by septal subdivision of the large gas-exchange saccules present at birth. In rats septation occurs mainly between the 4th and 14th postnatal days (Burri, P. H. 1974. *Anat. Rec.* 180:77–98), but little is known about the regulation of this process. We found that dexamethasone (0.1 μg daily) given to rats from age 4 to 13 d markedly impaired saccule septation to at least age 60 d and also diminished the extent of the increase of alveolar surface area (Sa). Underfeeding from birth to age 14 d did not diminish saccule septation but did result in diminished Sa. We conclude dexamethasone-treated rats have a critical period during which the gas-exchange saccules present at birth must be subdivided. Since Sa increased in dexamethasone-treated rats without a change in alveolar size, and, the enlargement of Sa was diminished in underfed rat pups without a deficit of saccule septation, we postulate new alveoli were formed by means other than septation of the large gas-exchange saccules present at birth. Furthermore, these various means of forming alveoli, and hence of increasing Sa, were differently regulated: dexamethasone decreased the enlargement of Sa brought about by both septation of the gas-exchange saccules present at birth and by other, as yet unidentified, means of forming alveoli; underfeeding did not diminish Sa increases produced by saccule septation but did decrease the extent of Sa enlargement due to the other means of forming alveoli.**

## Introduction

In many mammalian species, including humans, the formation of pulmonary alveoli occurs to a substantial extent after birth (see ref. 1 for a review). The events involved have been especially well-studied in the rat, where it was found that the newly born have large, thick-walled, gas-exchange structures termed, at that stage, saccules rather than alveoli (2, 3). On the fourth to fifth postnatal day the saccules begin to be subdivided into smaller compartments by the outgrowth (and elongation) of septae from their walls. The bulk of the subdivision is completed by the 14th postnatal day (2). Thinning of the alveolar wall accelerates in the third postnatal week and is accomplished by the end of that week, thereby completing the changes by which the relatively small number of large, thick-walled saccules, which compose the gas-exchange region of the newly-born, are remodeled into the more numerous, smaller, thin-walled alveoli of the mature lung. It is probable that similar postnatal changes occur in the lungs of mice (4), hamsters (Massaro, D., and N. Teich. Unpublished observations), rabbits (5), cats (5, 6), and humans (7–9), but on a different time scale.

In contrast to the detailed knowledge of these anatomical events little is known about their regulation. We undertook the present work to identify some factors that might regulate or influence architectural aspects of the lungs' postnatal development. We focused on the process of subdivision of the gas-exchange saccules and on changes in gas-exchange surface area in the early postnatal period. These parameters were assessed by measuring the surface-to-volume ratio (S/V)[1] and the mean chord length (Lm) of the gas-exchange air spaces, and lung volume. The main question asked was if subdivision could be blocked, and, if so, if the impairment was permanent.

An adrenal glucocorticosteroid (dexamethasone) and nutrition were selected as potential modulators of subdivision. An adrenal glucocorticoid was chosen for two reasons. First, formation and elongation of structures like alveolar septae are brought about by folding epithelium into ridges, a process that, in part, requires epithelial cell division. Since glucocorticosteroids inhibit cell division in several tissues (10) including the lung (11), we suspected they might inhibit the formation or elongation of alveolar septae. Our second reason for choosing a glucocorticosteroid was because the serum concentration of corticosterone, the active glucocorticoid in rats, is low in rats during the period of intense postnatal alveolar subdivision and increases as septation is ending (12). In short, from the action of glucocorticoids on DNA synthesis and from the timing of the process of saccule subdivision and the surge of serum corticosterone, we reasoned corticosterone might normally be involved in, or trigger, the process that ends septation of the large saccules that were present at birth. We chose diet as a potential modulator of postnatal lung development because lung size is closely related to body size (13), and the latter is strongly influenced in rats by diet (14).

## Methods

*Animals.* We used Sprague-Dawley albino rats originally obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and subsequently maintained in the Animal Care Facility of the University of Miami, Miami, FL. Male and female breeders were allowed food (Rodent Laboratory Chow 5001; Ralston Purina Co., St. Louis, MO) and water

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1. *Abbreviations used in this paper:* KRB, Krebs-Ringer bicarbonate; Lm, mean chord length; LV, lung volume; Ptp, transpulmonary pressure; P-V, pressure-volume; Sa, gas-exchange surface area; S/V, surface-to-volume ratio.

*Table I. Gas-exchange Alveolar Dimensions and Surface Area in Newly Born and Adult Untreated Rats*

| Age | BW | LV | S/V | Lm | Sa | $\frac{Sa}{BW}$ | $\frac{LV}{BW} \times 10^{-2}$ | $\frac{LV}{Sa} \times 10^{-4}$ |
|---|---|---|---|---|---|---|---|---|
| *d* | *g* | *ml* | $cm^{-1}$ | *μm* | $cm^2$ | $cm^2/g$ | *ml/g* | $ml/cm^2$ |
| 1 (4)* | 5.6±0.4 | 0.37±0.02 | 236±18 | 121±4 | 124±9 | 22.0±1.2 | 6.6±0.2 | 30.3±0.9 |
| 3 (3) | 8.9±1.5 | 0.57±0.2 | 259±14 | 111±2 | 207±62 | 22.3±2.9 | 6.2±0.7 | 27.8±0.5 |
| 6 (4) | 21.2±0.8 | 1.27±0.8 | 319±17 | 100±4 | 506±3 | 24.0±0.9 | 6.1±0.2 | 25.1±0.9 |
| 10 (5) | 21.2±0.4 | 1.23±0.04 | 306±7 | 91±3 | 579±29 | 27.5±1.5 | 6.2±0.2 | 22.8±0.8 |
| 14 (7) | 28.8±0.4 | 1.75±0.04 | 297±4 | 85±1 | 826±26 | 28.6±0.6 | 6.1±0.1 | 21.2±0.2 |
| Adult (2) | 117 | 7.34 | 320 | 84 | 3,495 | 30.0 | 6.3 | 21.0 |

* Figures in parentheses indicate the number of rats. Untreated rats raised in litters of 10 pups/dam were killed on the days listed. BW, body wt. Mean±SE are given.

ad libitum. Lighting was provided from 7 a.m. to 7 p.m. daily. Breeding was accomplished by placing one male with two females overnight for 12 h. Probable pregnancy was determined the next morning by the presence of sperm in a smear made from vaginal contents. The onset of gestation in smear-positive females was considered to be the midpoint of the 12-h cohabitation period. Pups born between 6 p.m. and 8 a.m. were considered to have been born midpoint between these times. Overfeeding and underfeeding of pups were produced by adjusting litter size to 4 or 18 pups, respectively; pups raised 10/litter were considered to be normally fed. Litters were constituted between 6 and 18 h after birth. Rats were killed by anesthetizing them with pentobarbital sodium (~60 mg/kg, i.p.) and then cutting the great vessels of the abdomen.

*Drugs.* We used dexamethasone (Organon Diagnostics, West Orange, NJ) that was diluted from 4 μg/μl to 0.1 μg/10 μl in phosphate-buffered 0.5 N NaCl. Phosphate-buffered 0.5 N NaCl served as control (diluent). All injections were subcutaneous.

*Morphometry.* Rats were anesthetized and their tracheas were cannulated through a midline cervical incision while the animal was still breathing. A bilateral pneumothorax was produced by puncturing the diaphragm from its abdominal surface. We then infused buffered 10% formalin into the trachea at a transpulmonary pressure (Ptp) of 20 $cmH_2O$. If the Ptp was steady at 20 $cmH_2O$ for 10 min, the trachea was ligated, the lungs were removed from the thoracic cavity, and fixation was continued by placing the lungs in formalin for 48 h at room temperature. Lung volume was measured by water displacement (15). The lungs were then embedded in glycol methacrylate and sections 2 μm thick were cut and stained with hematoxylin-eosin. One section was made from each pair of lungs. Corrections were made for shrinkage during fixation and in the cut sections.

Lung sections were examined at 100× with a microscope that contained five parallel lines in its eye piece. Each line had test points positioned to form a square lattice; the distance between points was 186 μm at a magnification of 100×. The total length of the test line was 3,720 μm. We placed this system over the lung section four times and at each position counted intersections with the test system in the original position and after it was rotated 90°. Thus, the final test line length was 2.98 cm. Surface density and volume density of the air space were measured and the S/V of the alveolus was calculated (16). The Lm was calculated by dividing the length of the test line by the total number of intersections (17). The Lm and the lung volume (LV) were used to calculate the surface area of the gas-exchange region of the lung (Sa) (18).

*Pressure-volume (P-V) measurements.* Lungs were degassed by ventilating rats with 100% $O_2$ for 10 min, after which the diaphragm was punctured from below and the trachea was clamped while the heart continued to beat. In experiments on 14-d-old rats, the lungs were left in the thorax, which was separated from the rest of the body. In 20- and 60-d-old rats the lungs were removed from the thorax for the P-V measurements. In all instances the preparation was immersed in 0.15 M NaCl (saline) and the lungs were inflated with saline to a Ptp of either 10, 15, or 20 $cmH_2O$ (see individual experiments) at room temperature. P-V measurements were made using a water manometer and a tight fitting syringe as the Ptp was lowered in 1-cm steps (19).

We eventually settled on a Ptp of 10 $cmH_2O$ for 14-d-old pups because higher pressures usually caused the lung to leak although we did not have that experience when a Ptp of 20 $cmH_2O$ was used to fix the lung. We cannot explain the different behavior of saline-inflated and formalin-inflated lungs. We chose a Ptp of 15 $cmH_2O$ as maximum inflation in 60-d-old rats because we had relatively few animals at that age and therefore could not tolerate too many losses due to leaks. We do not think using pressures less than 20 $cmH_2O$ in 14- and 60-d-old rats influenced our intergroup comparisons because we studied 20-d-old rats at a Ptp of 20 $cmH_2O$ and found that the P-V diagram is virtually flat above a Ptp of 10 $cmH_2O$.

*Studies on DNA synthesis.* Lungs were removed, rinsed in cold Krebs-Ringer bicarbonate (KRB) medium (20), and sliced with a McIlwain tissue slicer (Brinkmann Instruments Co., Westbury, NY) set to make

*Table II. Effect of Dexamethasone Given from Age 4 to 13 Days on Rats Killed when 14 d Old*

| Dex | BW | Lung volume | S/V | Lm | Sa | $\frac{Sa}{BW}$ | $\frac{LV}{BW} \times 10^{-2}$ | $\frac{LV}{Sa} \times 10^{-4}$ |
|---|---|---|---|---|---|---|---|---|
| $\mu g \cdot d^{-1}$ | *g* | *ml* | $cm^{-1}$ | *μm* | $cm^2$ | $cm^2/g$ | *ml/g* | $ml/cm^2$ |
| 0 (10)* | 28.7±0.4 | 1.74±0.1 | 322±7 | 81±1.2 | 858±23 | 30±0.9 | 6.0±0.2 | 20.5±0.5 |
| 0.05 (7) | 28.0±0.7 | 1.86±0.1 | NM | 91±4 | 844±39 | 31±1.1 | 6.7±0.3 | 22.1±1.1 |
| 0.1 (7) | 25.5±0.4 | 1.87±0.08 | 209±17 | 119±3 | 628±27 | 25±0.9 | 7.3±0.2 | 29.9±0.7 |
| 0.2 (4) | 24.3±0.5 | 1.73±0.04 | NM | 114±3 | 607±14 | 25±1.1 | 7.1±0.3 | 28.6±0.9 |
| 0.3 (3) | 19.2±0.7 | 1.33±0.1 | NM | 121±3 | 438±25 | 23±2.1 | 7.0±0.7 | 30.2±0.5 |
| 0.4 (4) | 20.2±0.6 | 1.61±0.1 | NM | 129±5 | 500±7 | 25±0.9 | 8.0±0.2 | 32.2±2.0 |
| 0.5 (3) | 14.2±1.5 | 1.19±0.2 | NM | 123±13 | 386±43 | 27±1.6 | 8.2±0.7 | 30.8±1.0 |

* Figures in parentheses indicate the number of rats. Rats were given diluent (phosphate-buffered 0.5 N NaCl) or the dose of dexamethasone (Dex) listed once daily from age 4 to 13 d and killed when 14 d old. BW, body weight; NM, not measured. Mean±SE are given.

*Table III. Effect of Dexamethasone Given from Age 4 to 13 d on Rats Killed when 28 d Old*

| Dex | BW | LV | S/V | Lm | Sa | $\frac{Sa}{BW}$ | $\frac{LV}{BW} \times 10^{-2}$ | $\frac{LV}{Sa} \times 10^{-4}$ |
|---|---|---|---|---|---|---|---|---|
| *μg/d* | *g* | *ml* | $cm^{-1}$ | *μm* | $cm^2$ | $cm^2/g$ | *ml/g* | $ml/cm^2$ |
| 0 (12)* | 91±2 | 3.61±0.1 | 310±10 | 80±1.6 | 1,723±51 | 19.6±0.6 | 4.0±0.1 | 20.4±0.4 |
| 0.1 (3) | 83±3 | 4.20±0.3 | 192±6 | 130±2 | 1,291±95 | 15.5±0.6 | 5.1±0.2 | 32.6±0.6 |
| 0.3 (2) | 66 | 3.65 | NM | 120 | 1,218 | 18.8 | 5.6 | 30.0 |
| 0.4 (2) | 56 | 3.50 | NM | 133 | 1,068 | 19.2 | 6.4 | 33.1 |
| 0.5 (3) | 41±2 | 2.20±0.2 | NM | 110±4 | 858±118 | 20.8±1.9 | 5.3±0.3 | 25.5±1.1 |

* Figures in parentheses indicate the number of rats. Rats were given diluent or the dose of dexamethasone (Dex) listed once daily from age 4 to 13 d and killed when 28 d old. BW, body weight; NM, not measured. Mean±SE are given.

slices 1 mm thick. ~130 mg of sliced lung was placed in 5 ml of KRB medium containing 5 mM glucose, rat serum concentrations of 20 L-amino acids (21), and 15 μM thymidine. The medium had been equilibrated with 95% $O_2$–5% $CO_2$ and that gas formed the gas phase in rubber-stoppered flasks. The flasks containing lung slices were preincubated at 37°C for 1 h in a water bath shaking at 120 oscillations/min. After 1 h the medium was removed and replaced with identical medium except that the new medium contained 15 μM [$^3$H]thymidine. This concentration of thymidine was used because prior experiments showed [$^3$H]thymidine incorporation into DNA rose as the concentration of thymidine was increased until a concentration of 15 μM was reached; the rate of incorporation of [$^3$H]thymidine into DNA did not increase between thymidine concentrations of 15 and 45 μM (not shown).

*Chemical measurement and statistical analysis.* DNA was measured using calf thymus DNA as a standard (22). For each parameter measured or calculated from measurements, the values for individual animals were averaged per experimental group and the standard error of the mean was calculated. The significance of the difference between two groups was obtained using an unpaired *t* test analysis (23). Multiple group comparisons were made by analysis of variance and Duncan's multiple range test (24); Kramer's extension (25) of Duncan's test was used to identify intergroup significant differences.

## Results

*Gas-exchange region of the lung in untreated neonatal rats.* A major objective of this study was to evaluate the effect of an exogenous glucocorticosteroid on postnatal changes in the dimensions and surface area of the gas-exchange region of the lung. However, because the stress of daily injections might increase the output of endogenous corticosterone (12), thereby possibly masking the effect of the exogenous corticosteroid, we first made measurements in rat pups not injected except for the anesthetic given immediately before sacrifice. In these rats the S/V increased and the Lm decreased and both reached adult dimensions by the 14th d of age (Table I). These results can be compared with those to be provided for diluent and dexamethasone-injected rats.

*S/V, Lm, Sa, and LV: short-term and long-term effect of different doses of dexamethasone given from age 4 to 13 d.* After daily injections of dexamethasone or diluent from ages 4 to 13 d, several parameters of growth and development were measured at 14, 28, and 60 d of age (Tables II, III, and IV). The values for S/V, Lm, and LV/Sa at age 60 d were the same for males and females (see below), so the values from each sex were combined (Table IV). At each age the most striking effects of dexamethasone treatment at a dose of 0.1 μg/d were lower S/V and higher Lm. This dose also lowered body weight, Sa, and Sa relative to body weight; it increased LV relative to body weight or to Sa. Higher doses of dexamethasone did not cause further changes in Lm, Sa relative to body weight, or LV relative to Sa; the higher doses did markedly lower body weight, LV, and Sa compared with the animals that received less dexamethasone.

*Table IV. Effect of Dexamethasone Given from Age 4 to 13 d on Rats Killed when 60 d Old*

| DEX | Sex | LV | S/V | Lm | Sa | $\frac{Sa}{BW}$ | $\frac{LV}{BW} \times 10^{-2}$ | $\frac{LV}{Sa} \times 10^{-4}$ |
|---|---|---|---|---|---|---|---|---|
| *μg/d* | | *ml* | $cm^{-1}$ | *μm* | $cm^2$ | $cm^2/g$ | *ml/g* | $ml/cm^2$ |
| 0 | F (3) | 7.4±0.4 | | | 3,525±113 | 18.2±0.7 | 3.8±0.3 | |
| | | | 305±8 | 84±2 | | | | 21.1±0.3 |
| | M (3) | 9.8±0.4 | | | 4,630±91 | 15.0±0.1 | 3.2±0.1 | |
| *P** | | <0.01 | | | 0.002 | <0.01 | 0.1 | |
| 0.1 | F (5) | 9.0±0.5 | | | 2,912±129 | 15.3±0.3 | 4.7±0.2 | |
| | | | 197±0.5 | 124±3 | | | | 30.9±0.7 |
| | M (4) | 10.6±0.5 | | | 3,442±260 | 11.8±0.7 | 3.6±0.1 | |
| *P** | | <0.05 | | | <0.05 | 0.002 | 0.004 | |
| *P*‡ | | NS (F) | <0.001 | <0.001 | 0.02 (F) | 0.005 (F) | 0.04 (F) | <0.001 |
| | | NS (M) | | | 0.01 (M) | 0.01 (M) | 0.03 (M) | |

* *P* = *P* value for intersex means; ‡ *P* = *P* value for means of diluent vs. dexamethasone groups. Rats were given diluent or dexamethasone (0.1 μg) once daily from age 4 to 13 d and killed when 60 d old. BW, body weight. F, female; M, male. Mean±SE are given. Figures in parentheses indicate the number of rats. The values for S/V, Lm, and LV/Sa did not approach significant intersex differences and so the results for males and females were pooled.

Comparisons between the age groups (Tables I–IV) show that dexamethasone (0.1 μg/d) prevented attainment of normal adult alveolar dimensions as assessed by S/V and Lm. However, note that the Sa did increase in each period in dexamethasone-treated rats even though S/V and Lm did not change. Also note Lm and S/V did not change in diluent-treated rats after the rats were 14 d old.

The LV and Sa were larger in males than females at age 60 d, whereas, when adjusted for body weight, the LV and Sa were greater in females than in males; these intersex differences were true for rats treated with diluent or dexamethasone from age 4–13 d (Table IV). The S/V, Lm, and LV/Sa at age 60 d for diluent-treated males were ($n$ = 3) 311±12 $cm^{-1}$, 85±2 μm, and 21.2±0.4 × $10^{-4}$ $ml/cm^2$; the respective values for diluent-treated females at age 60 d were ($n$ = 3) 299±9 $cm^{-1}$, 84±2 μm, and 20.9±0.5 × $10^{-4}$ $ml/cm^2$. None of the means exhibited statistically significant intersex differences. Similarly, the S/V, Lm, and LV/Sa for dexamethasone-treated male rats age 60 days were ($n$ = 4) 195±6 $cm^{-1}$, 124±4 μm, and 31±0.9 × $10^{-4}$ $ml/cm^2$; the respective values for dexamethasone-treated female rats, age 60 d ($n$ = 5), were 199±8 $cm^{-1}$, 123±5 μm, and 30.8±1.1 × $10^{-4}$ $ml/cm^2$. None of these means exhibited statistically significant intersex differences.

*Morphology in diluent-treated and dexamethasone-treated rats.* Although we have shown the effect of dexamethasone on Lm and S/V, the difference in the size of alveoli of rats given diluent compared with rats given dexamethasone (0.1 μg/d) from age 4 to 13 days can also be appreciated by viewing the histological sections of the lung at low magnification (Fig. 1 *A* and *B*). The decrease in alveolar size that occurs in rats between birth and 14 d of age is achieved by the outgrowth (and elongation) of septae from the wall of the saccules that form the gas-exchange region of the lung of the newborn (3). These buds failed to elongate in dexamethasone-treated rats (Fig. 1 *C*). We have not determined if bud formation, i.e., the eruption of septae from the wall of the gas-exchange saccule, was also impaired in dexamethasone-treated rats. Dexamethasone-treated rats have fewer alveolar attachments to the connective tissue sheath that surrounds intrapulmonary conducting airways than do diluent-treated rats (Fig. 1 *A* and *B*).

*P-V relations of lungs of diluent-treated and dexamethasone-treated rats.* We measured the P-V relations of saline-filled lungs in 14-d-old pups with the chest wall intact but the diaphragm opened. The lung recoil was not different at any Ptp between diluent-treated and dexamethasone-treated pups (Table V). P-V measurements made on lungs excised at age 20 d from pups treated with diluent or dexamethasone daily from age 4 to 13 d revealed that lung recoil at a Ptp of 3 and 4 $cmH_2O$ was diminished in lungs from dexamethasone-treated rats compared with lungs from diluent-treated rats (Table V). P-V measurements made on excised lungs from male rats age 60 d that had received diluent or dexamethasone daily from age 4 to 13 d did not reveal intergroup differences in recoil (Table V). We used the data generated in the experiments just described to calculate the change in LV between the highest and lowest Ptp. We did not find differences between diluent-treated and dexamethasone-treated rats at any age (not shown).

*Effect of dexamethasone on the DNA content of the lung and on DNA synthesis by the lung.* The DNA content of the lung of all rats was greater at 9 and 14 d than at age 3 d (Table VI). Rats given dexamethasone had less DNA/left lung at age 9 and 14 d than did those given saline. The DNA concentration in the lung was higher at age 3 than age 9 and 14 d but the concentrations of DNA at age 9 and 14 d did not exhibit statistically significant intergroup differences. The rate of DNA synthesis was greater in lung slices from 3-d-old rats than in slices from 9-d-old rats (Table VII). Dexamethasone given in vivo resulted in a decreased rate of DNA synthesis by lung slices (Table VII).

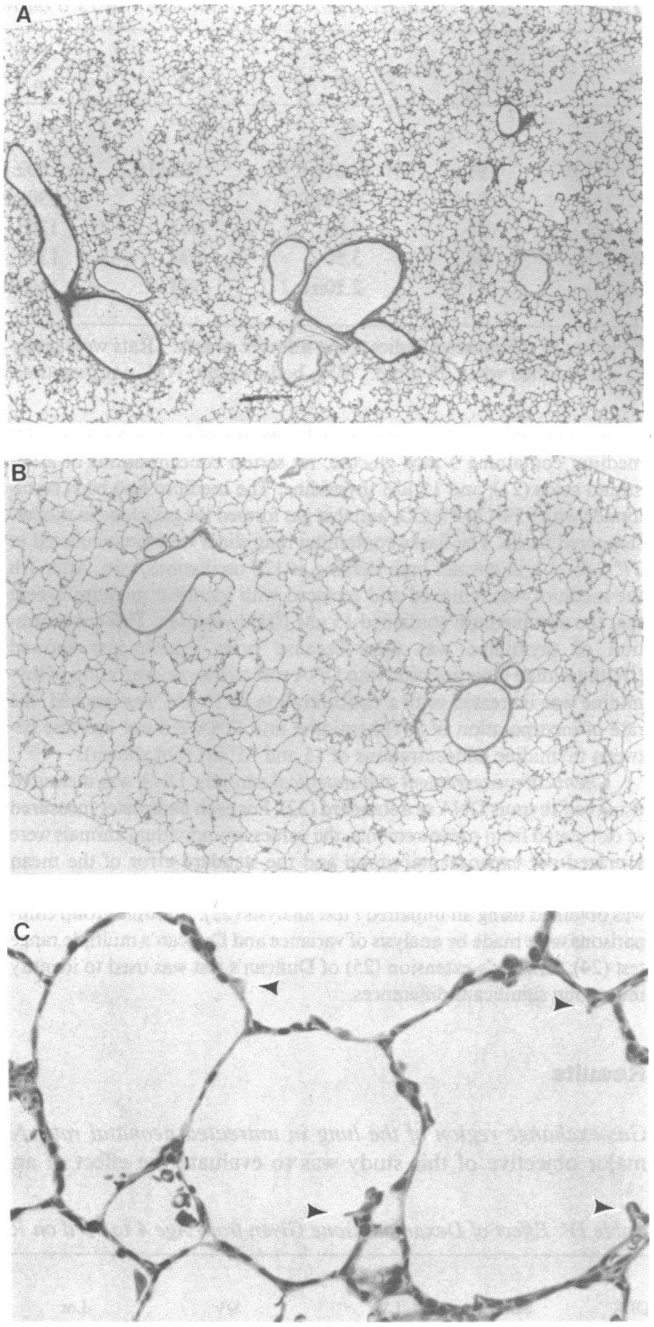


*Figure 1.* Morphology. Rats were treated with diluent (*A*) or dexamethasone (*B* and *C*) (0.1 μg/d) from age 4 to 13 d and killed at age 14 d. Their lungs were fixed at a transpulmonary pressure of 20 $cmH_2O$. Diluent-treated rats (*A*) have small alveoli and numerous alveolar attachments to conducting airways and vessels. Dexamethasone-treated pups have large alveoli (*B*), fewer alveolar attachments to conducting airways and vessels (*B*), and lack of septal elongation (*C*, arrows show short septae). Final magnification: × 21 for *A* and *B*, and × 240 for *C*.

*Table V. P-V Relations in Saline-filled Lungs*

| | | | Percent maximum lung volume | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | Dex | Ptp | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| *d* | *μg* | *cmH₂O* | | | | | | | | | | | | | | | | | | | | | |
| 14 | 0 (4) | | 23.5 | 46.7 | 64.2 | 78.3 | 88.1 | 93.8 | 96.8 | 98.1 | 99.3 | 99.7 | 100 | | | | | | | | | | |
| | | SE | 1.0 | 1.2 | 1.7 | 1.5 | 1.4 | 0.8 | 0.6 | 0.5 | 0.4 | 0.2 | | | | | | | | | | | |
| | 0.1 (5) | | 20.8 | 45.5 | 65.6 | 80.3 | 88.6 | 93.0 | 98.6 | 97.2 | 98.1 | 99.1 | 100 | | | | | | | | | | |
| | | SE | ±0.9 | ±0.8 | ±1.2 | ±1.1 | ±0.5 | ±0.3 | ±0.2 | ±0.3 | ±0.2 | ±0.2 | | | | | | | | | | | |
| | | *P* | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | | | | | | | | | | | |
| 20 | 0 (5) | | 26.7 | 36.9 | 47.2 | 58.7 | 70.4 | 80.6 | 87.6 | 91.6 | 94.1 | 95.3 | 96.9 | 97.9 | 98.6 | 99.2 | 99.5 | 99.7 | 99.8 | 99.9 | 99.9 | 99.9 | 100 |
| | | SE | 1.6 | 1.6 | 1.3 | 1.3 | 1.5 | 1.4 | 1.1 | 0.9 | 0.8 | 0.7 | 0.6 | 0.6 | 0.4 | 0.4 | 0.4 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | |
| | 0.1 (7) | | 25.8 | 37.9 | 51.2 | 66.0 | 78.6 | 86.3 | 90.3 | 92.8 | 94.7 | 96.0 | 97.1 | 98.0 | 98.7 | 99.3 | 99.6 | 99.7 | 99.8 | 99.9 | 99.9 | 99.9 | 100 |
| | | SE | 1.1 | 0.9 | 1.6 | 1.6 | 1.5 | 1.1 | 1.0 | 0.8 | 0.7 | 0.6 | 0.6 | 0.6 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | |
| | | *P* | NS | NS | NS | 0.01 | 0.01 | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | |
| 60 | 0 (8) | | 11.9 | 19.6 | 29.8 | 44.2 | 60.9 | 75.0 | 84.4 | 90.3 | 93.8 | 95.3 | 97.3 | 98.3 | 99.0 | 99.6 | 99.8 | 100 | | | | | |
| | | SE | 0.7 | 0.7 | 1.0 | 1.3 | 1.5 | 1.7 | 1.5 | 1.2 | 1.2 | 1.3 | 0.9 | 0.7 | 0.5 | 0.5 | 0.2 | | | | | | |
| | 0.1 (4) | | 10.7 | 19.2 | 30.2 | 46.4 | 64.6 | 79.4 | 88.2 | 92.7 | 95.3 | 97.0 | 97.9 | 98.9 | 99.3 | 99.6 | 99.9 | 100 | | | | | |
| | | SE | 1.1 | 1.0 | 1.2 | 3.7 | 4.3 | 1.5 | 1.0 | 0.8 | 0.7 | 0.7 | 0.5 | 0.5 | 0.3 | 0.2 | 0.1 | | | | | | |
| | | *P* | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | | | | | | |

Rats were given diluent or dexamethasone (Dex) (0.1 μg) once daily from age 4 to 13 d and killed at age 14, 20, or 60 d. Mean±SE are given. Figures in parentheses indicate the number of rats. NS, $P > 0.05$.

*Table VI. Effect of Dexamethasone on the DNA Content of the Lung*

| Age | Condition | DNA/left lung | DNA wet lung |
|---|---|---|---|
| *d* | | *mg* | *mg/g* |
| 3 | None | 0.52±0.04 (6)* | 11.5±0.3 (6)‖ |
| 9 | Diluent | 1.26±0.01 (3) | 10.8±0.3 (3)¶ |
| | Dex (0.05 μg/d) | 0.96±0.01 (3)‡ | 10.1±0.1 (3) |
| | Dex (0.1 μg/d) | 0.92±0.02 (3)‡ | 10.7±0.2 (3) |
| 14 | Diluent | 1.38±0.04 (3) | 10.8±0.3 (3) |
| | Dex (0.05 μg/d) | 1.29±0.07 (3) | 10.0±0.4 (3) |
| | Dex (0.1 μg/d) | 1.14±0.02 (3)§ | 9.9±0.1 (3) |

* $P < 0.001$ vs. the mean of each group in the same column.
‡ $P < 0.001$ vs. day 9 diluent group.
§ $P < 0.01$ vs. day 14 diluent group.
‖ $P < 0.05$ vs. all other rats treated as a single group.
¶ $P > 0.05$ for intergroup means of injected rats.
Rats were killed at 3, 9, or 14 d of age. Those killed when 3 d old had not received injections except for the anesthetic immediately before sacrifice. Rats killed at 9 or 14 d had received either dexamethasone (Dex) (0.05 or 0.1 μg/d s.c.) or diluent (s.c.) from age 4 d until they were killed. Mean±SE are given. Figures in parenthesis indicate the number of pups.

The DNA content and concentration in the lung was the same at age 28 d whether rats had received dexamethasone or diluent from age 4 to 13 d (Table VIII). The increment of DNA between 14 and 28 d was greater in rats previously given dexamethasone than in rats previously treated with diluent.

*Litter size and body weight and length.* Body weight on the day of birth did not differ among pups assigned to litters of different size (Table IX). Pups maintained in litters of 18 pups weighed less and were not as long as pups raised in litters of 10 or 4 pups.

*Litter size, LV, and gas-exchange region.* LV and S/V were significantly lower and Lm of alveoli were higher at 9 d of age in pups raised 18/litter compared with pups raised 10/litter (Table X). At 14 d of age LV and Sa of pups raised 18/litter were lower than the same parameters in pups raised in litters of 10 or 4 pups, but at 14 days of age, S/V and Lm did not exhibit intergroup differences (Table X). LV and Sa, corrected for body weight, were greater in underfed than in normal or overfed pups. However, when Sa was adjusted for body length, underfed pups had a smaller surface area than normal or overfed pups.

*Table VII. Effect of In Vivo Dexamethasone on DNA Synthesis by Lung Slices*

| Age | Treatment | pmol [$^3$H]thymidine incorporated/h/mg DNA |
|---|---|---|
| *d* | | |
| 3 | None | 1,434±90 (6) |
| 9 | Saline | 817±41 (3) |
| 9 | Dexamethasone | 604±62 (3) |

Rats killed at age 3 d had not received saline or dexamethasone. Rats killed at 9 d had received saline or dexamethasone (0.1 μg/d) from age 4 to 8 d. Mean±SE are given. The figures in parentheses indicate the number of experiments. $P < 0.05$ between each group.

*Table VIII. DNA in the Lung 15 d after Stopping Dexamethasone*

| Parameter | Saline (9) | Dexamethasone (10) | *P* |
|---|---|---|---|
| Body wt (*g*) | | | |
| Day 4 | 9.2±0.6 | 8.9±0.5 | NS |
| Day 28 | 81.1±2.5 | 80.3±3.4 | NS |
| DNA (*mg/lung*) | 4.7±0.1 | 4.4±0.1 | NS |
| DNA (*mg/g lung*) | 9.3±0.2 | 8.9±0.2 | NS |
| DNA increment (*μg/d*) | 93±7 | 109±7 | <0.05 |

Rats were given dexamethasone (0.1 μg/d) or an equivolume of diluent subcutaneously from age 4 to 13 d and killed when 28 d old. DNA increment was calculated as the difference between the lung DNA content at age 14 d compared with age 28 d. Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$.

*Lung recoil and litter size.* The lungs of rats maintained in litters of 10 pups held significantly more saline at a Ptp of 10 $cmH_2O$ than lungs of rats in litters of 18 pups: 1.7±0.1 ml ($n$ = 7) for litters of 10 and 1.2±0.0 ml (4) for litters of 18 ($P < 0.01$). When corrected for lung size the recoil of lungs from large litters was not different than the recoil of lungs from pups raised 10/litter (Table XI). The specific compliance measured over the pressure range of 0 to 10 $cmH_2O$, was the same in pups from litters of 18 as in pups from litters of 10 (not shown).

*DNA and litter size.* Lungs of pups raised 18/litter weighed less and had less DNA than pups from litters of 10 but the concentration of DNA did not differ between the groups (Table XII). The rate of DNA synthesis in lung slices made from normally nourished pups killed when 9 d old was greater than in slices made from lungs of undernourished 9-d-old pups.

## Discussion

*Morphometric assessment of alveolar dimensions.* The methods available to assess alveolar size, although commonly used, seem to all have certain pitfalls (see ref. 1 for a discussion of the problem). Because of this we have chosen two, rather than one, means of assessing alveolar dimensions, since agreement between methods would increase confidence in the results. We measured S/V because the surface area of a geometric body is always $k(r^2)$ and its volume is $k^1(r^3)$, and division of one parameter by the other provides information about size (r). However, the combined parameter (S/V) depends simultaneously on shape and

*Table IX. Litter Size, Animal Weight, and Length*

| | | Litter size | | |
|---|---|---|---|---|
| Parameter | Age | 4 | 10 | 18 |
| | *d* | | | |
| Body wt (*g*) | 9 | | | |
| Initial | | | 5.7±0.1 (5) | 5.8±0.1 (3) |
| Final | | | 21.2±0.4 | 15.6±0.5 |
| Body wt (*g*) | 14 | | | |
| Initial | | 6.5±0.3 (10) | 6.0±0.2 (28) | 6.1±0.1 (58) |
| Final | | 33.1±0.5 | 28.2±0.3 | 20.2±0.5 |
| Length (*cm*) | 14 | 9.4±0.1 | 9.3±0.03 | 8.4±0.4 |

Litter size was adjusted within 18 h after birth to provide litters of 4, 10, or 18 pups. Mean±SE are given. Figures in parentheses indicate the number of pups. $P < 0.05$ for length and all final measurements between pups in litters of 10 and 18; $P > 0.05$ between pups in litters of 10 and 4.

*Table X. Litter Size and Lungs Gas-exchange Region*

| Parameter | Age | Litter size 4 | 10 | 18 |
|---|---|---|---|---|
| | *d* | | | |
| | 9 | | | |
| Lung volume (*ml*) | | | 1.23±0.04 (5) | 1.14±0.06 (3)* |
| S/V ($cm^{-1}$) | | | 306±7 | 263±15* |
| LM ($\mu m$) | | | 91.3±3 | 96.7±2.3 |
| SA ($cm^2$) | | | 579±29 | 473±29* |
| Sa/BW ($cm^2/g$) | | | 27.5±1.5 | 30.2±1.0 |
| | 14 | | | |
| Lung volume (*ml*) | | 1.83±0.05 (4) | 1.75±0.04 (10) | 1.34±0.09 (10)‡ |
| S/V ($cm^{-1}$) | | 302±9 | 297±4 | 301±9 |
| Lm ($\mu m$) | | 85.3±0.2 | 84.6±0.6 | 83.4±1.7 |
| Sa ($cm^2$) | | 858±13 | 828±26 | 634±42‡ |
| Sa/BW ($cm^2/g$) | | 25.4±0.5 | 28.6±0.6§ | 34.5±1.0‡ |
| Sa/body length ($cm^2/cm$) | | 91.6±1.5 | 89.1±2.8§ | 75.5±5.0‡ |

* $P < 0.05$ vs. 10 pups/litter; ‡ $P < 0.05$ vs. 4 and 10 pups/litter; § $P < 0.05$ vs. 4 pups/litter. Mean±SE are given. Figures in parentheses indicate the number of pups.

size and can be used for intergroup comparisons of size only if the shape of the bodies being compared is the same. We think we can use S/V for such comparisons because we did not discern by microscopy differences of alveolar shape between diluent- and dexamethasone-treated rats. In addition, the results of our measurements of S/V agree with those of the other estimator of alveolar dimensions used (Lm). These considerations led us to conclude, from the results of measurements of S/V and Lm, in conjunction with the microscopic appearance of the lung, that alveoli in sections from dexamethasone-treated rats are larger than alveoli in sections from diluent-treated or from uninjected rats.

Even if we accept that measurements of S/V and Lm reflect alveolar size, the sensitivity of these parameters to detect differences, or changes, of alveolar size has been questioned (26). Hence, use of these measurements to make inferences about the mode by which Sa increases—formation of new alveoli, distention of pre-existing alveoli, or increased length or complexity of already formed alveolar septae—is a separate, and more difficult to resolve, issue. However, because we did detect a substantial increase of S/V (~35%) and a fall of Lm (~40%) between birth and age 14 d, it is clear, as reported by others (2), that subdivision of saccules present at birth contributed to the increase of surface area (six- to sevenfold) that occurred over this period in untreated and in diluent treated pups. Since the same methods did not detect changes of Lm or S/V in dexamethasone-treated pups we think it is equally reasonable to conclude that septal subdivision did not take place (or occurred to a minimal degree) in dexamethasone-treated rats; thus, septal subdivision of saccules present at birth must have contributed much less, if anything, to the fivefold increase of Sa that took place in rats treated with 0.1 $\mu$g of dexamethasone per day over the first 2 wk of age. Similarly, our failure to detect changes of S/V and Lm between age 14 and 60 d in rats previously treated with dexamethasone argues against septal subdivision, of the large sacs present at age 14 d, contributing much, if anything, to the 4.5-fold (in females) and 5.5-fold (in males) increase of Sa that took place between age 14 and 60 d. Identical reasoning, with respect to further alveolar partitioning, pertains to the increase of Sa in diluent-treated rats between age 14 and 60 d (a fourfold increase of Sa in females and a 5.4–fold increase in males), and to the differences in Sa between male and female rats (Table IV).

Sa could be increased without the formation of new alveoli if alveolar walls become more complex, i.e., more folds and wrinkles, and lung volume increased by virtue of this increased complexity. We cannot exclude this possibility but are unaware of any direct evidence to support the notion that the measurement of Lm is too insensitive to detect changes in alveolar dimensions responsible for such large increases of Sa.

These considerations lead us to favor the notion that some mechanism, or mechanisms, other than (*a*) subdivision of the large saccules present at birth, (*b*) over-expansion of alveoli, or (*c*) increased complexity of already formed alveolar walls, is re-

*Table XI. Litter Size and P-V Relations in Saline-filled Lungs*

| Ptp | Percent maximum LV 10 pups/litter (7) | 18 pups/litter (4) | *P* |
|---|---|---|---|
| $cmH_2O$ | | | |
| 0 | 24.2±0.8 | 21.8±1.7 | NS |
| 1 | 46.8±0.8 | 45.9±0.8 | NS |
| 2 | 64.8±1.0 | 62.8±0.8 | NS |
| 3 | 79.1±1.0 | 77.1±0.7 | NS |
| 4 | 88.6±1.0 | 87.4±0.7 | NS |
| 5 | 93.9±0.5 | 92.4±0.3 | NS |
| 6 | 96.9±0.3 | 95.7±0.3 | NS |
| 7 | 98.2±0.3 | 97.5±0.4 | NS |
| 8 | 99.4±0.3 | 98.8±0.3 | NS |
| 9 | 98.8±0.2 | 99.4±0.2 | NS |
| 10 | 100 | 100 | |

Rats were assigned to litters of 10 or 18 pups/dam within 18 h after birth and killed at age 14 d. The P-V measurements were made on saline-filled lungs that were in the thorax with the diaphragm opened; the thorax was immersed in saline. Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$.

*Table XII. Effect of Litter Size on DNA in the Lung*

| Parameter | Age | Litter size 10 | Litter size 18 | P |
|---|---|---|---|---|
| | *d* | | | |
| | 9 | | | |
| Lung wt (*g*) | | 0.280±0.009 (5) | 0.096±0.005 (5) | <0.05 |
| DNA/lung (*mg*) | | 3.3±0.2 | 2.8±0.01 | <0.05 |
| BW | | 20.8±0.3 | | |
| DNA (*mg/g lung*) | | 12.1±0.5 | 12.5±0.7 | NS |
| [$^3$H]thym. incorp. ($pmol \cdot mg\ DNA^{-1} \cdot h^{-1}$) | | 2,830±194 | 1,523±356 | <0.05 |
| | 14 | | | |
| Lung wt (*g*) | | 0.300±0.003 (3) | 0.244±0.019 (5) | <0.05 |
| DNA/lung (*mg*) | | 3.3±0.1 | 2.9±0.09 | <0.05 |
| DNA (*mg/g lung*) | | 10.8±0.04 | 11.2±0.2 | NS |

Litter size was adjusted within 18 h of birth to provide litters containing 10 or 18 pups. Mean±SE are given. Figures in parentheses indicate the number of pups. Thym. incorp., thymidine incorporation.

sponsible for much of the increase of alveolar surface area in dexamethasone-treated rats, and, after 14 d of age, in diluent-treated rats. The formation of additional alveoli, either by *de novo* formation of new structures or by retrograde alveolarization of bronchioles, could contribute to an increase of Sa without altering S/V or Lm.

*Critical period.* As just noted, we think alveolar size diminished between birth and age 14 d in rats not treated with dexamethasone but did not diminish from birth to age 60 d in dexamethasone-treated rats. Since the recoil of the liquid-filled lungs from diluent-treated and dexamethasone-treated rats was the same at high transpulmonary pressures, the persistence of a small S/V and a large Lm in the latter does not reflect over-expansion due to diminished lung recoil during fixation. Rather, as supported by the microscopic appearance of the lung, the persistence of large alveoli seems to be due to failure of septae to arise from saccule walls, or, if septae did form they did not elongate to a normal extent. This failure would leave the large saccules, normally present in the newborn, intact rather than subdivided into the smaller alveoli of the mature lung. Persistence into adulthood of alveoli with dimensions of the gas-exchange saccules present at birth demonstrates that, under the conditions of our study, i.e., treatment with dexamethasone, there is a critical period in which subdivision of the original saccules must take place.

*Determinants of alveolar dimensions.* We interpret the results of our measurements of S/V and Lm to mean that alveoli formed in dexamethasone-treated rats, both during and after dexamethasone treatment, are the same or nearly the same size as alveoli present before dexamethasone treatment. Similarly, alveoli formed in diluent-treated rats after age 14 d are the same size as alveoli present at age 14 d, i.e., after saccule subdivision has been complete. Put somewhat differently, if our interpretation of the data is correct, dexamethasone-treated rats continue to form large alveoli after dexamethasone treatment is stopped, whereas diluent-treated rats continue to make small alveoli after age 14 d.

These observations have led us to wonder if there is a template or "size-setter", for alveolar dimensions, which, in rats, would be normally determined between age 4 to 13 d, and, which may be altered by dexamethasone treatment during that time. Such a template could be envisioned in a system where the gas-exchange region of the lung is considered to be a series of branching tubes.[2] In this system the Sa could be increased up to about age 14 d in diluent-treated rats by septation of saccules present at birth and through elongation of the branching tubes by the *de novo* formation of alveoli. In contrast, septation in dexamethasone-treated rats does not occur, so increases of surface area would occur only by formation of alveoli by a process not involving septation of the original saccules. In both diluent-treated and dexamethasone-treated rats the new alveoli would be added to an already formed tube of a certain dimension that could act as the putative template.

Irrespective of the appropriateness of the proposed model, the addition of alveoli after age 14 d in rats previously treated with dexamethasone suggests there are at least two controllers for the formation of alveoli. One controller, which is responsible for alveoli produced by subdivision of the primary saccules present in the newlyborn rat, is permanently blocked by early treatment with dexamethasone. The other controller, which is responsible for formation of new alveoli by a mechanism other than septation of the original saccules, is partially but not permanently impaired by early treatment with dexamethasone, as evidenced by the increase of Sa during and after treatment with dexamethasone.

In this regard it is important to point out that food deprivation from birth to age 14 d, produced by increasing the litter size from 10 to 18 pups, did not diminish the extent to which S/V rises and Lm falls compared with pups raised to age 14 d as 10/litter. However, Sa is diminished in food-deprived pups. Since both groups (10/litter and 18/litter) were the same weight before adjusting litter size, and since both groups had the same S/V and Lm at age 14 d, the difference in Sa must be due to the effect of food deprivation on the controller responsible for alveolus formation by a method other than septation of the saccules present at birth.

*Mechanism of dexamethasone effect.* We have not elucidated the mechanism by which dexamethasone impairs elongation of

2. We considered the lungs' gas-exchange region as a series of branching tubes at the suggestion of Dr. Joan Gil.

septae. Dexamethasone inhibits DNA synthesis in the lung as indicated by a lowered rate of incorporation of [$^3$H]thymidine into DNA and the smaller amount of DNA in lungs from dexamethasone treated rats. This inhibition of DNA synthesis may blunt septal elongation because the formation of ridges (septae) and their elongation in epithelia is partly accomplished by division of epithelial cells. Epithelial cell replication would throw the epithelial sheet into a ridge whose height would be modulated by the rate of epithelial cell proliferation. However, if inhibition of DNA synthesis by dexamethasone does play a role in impairing elongation of septae, inhibition in a specific cell type, or in a few cell types, must be a key element, because food deprivation causes as great an overall impairment of DNA accumulation in the lung without preventing the rise of S/V and the normal fall of Lm.

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