HISTO- AND CYTOCHEMISTRY OF DEVELOPING LUNGS IN NORMAL AND MALNOURISHED RATS.

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"HISTO- AND CYTOCHEMISTRY OF DEVELOPING LUNGS IN NORMAL AND MALNOURISHED RATS"

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Inadequate dietary intake during pregnancy may have significant effects on the developing fetus, whose demands are high in the vital stages of cellular multiplication and differentiation that occur before birth. In the lung, a functional air-blood barrier is essential at birth, and delayed or altered lung development has been associated with pathologic conditions such as hyaline membrane disease. The purpose of this study is to compare the cellular development of the lungs of normal and malnourished rats. The detailed histology, cellular distribution of glycogen, acid phosphatase and peroxidase, and the intracellular locations of these enzymes, were studied in normal rats from day 17 of pregnancy to 3 weeks post partum. These findings were compared to results obtained when pregnant rats were deprived of food at day 13 of pregnancy, to study the effects of starvation of the mother on the lungs of the neonates.

Normal lung at day 18 of gestation shows the presence of tubular airways lined by glycogen laden, cuboidal epithelium, which is undergoing rapid hyperplasia. These cuboidal cells differentiate after day 20 into functional type I and type II cells, accompanied by a rapid decline in glycogen. Postnatally, primary saccules are separated by growing secondary crests. These form alveolar structures in which the thick septal walls eventually fuse to produce thin expanded alveoli by 14 days. The pattern of development is similar but much delayed in the progeny of starved rats; cellular hyperplasia in the fetus is slowed and glycogen is observed in the epithelial cells up to one week post partum, suggesting a slower mitotic rate for these cells. Consequently, epithelial cell

differentiation is delayed and fewer areas with a thin air-blood barrier are seen at birth. After birth, mesenchymal glycogen appears to accumulate more slowly in starved animals and the interstitium is thicker than controls, indicating that lung growth is retarded in the early postnatal phase.

Acid phosphatase activity in control lungs is observed within the tubular epithelium on day 18, followed by a steady increase in the number of reactive cells on successive days, until a maximum reactivity is recorded on day 10 postpartum. In starved animals, development of acid phosphatase is depressed; a reduced number of reactive type II cells is observed until approximately one week postpartum when acid phosphatase activity shows a rapid increase which approaches normal by two weeks. Using electron microscopy acid phosphatase activity is located in the perilamellar membranes of lamellar bodies and in multivesicular bodies of type II cells. Retarded development of acid phosphatase activity, which has been associated with surfactant synthesis and secretion, could therefore cause impaired pulmonary function. In control rats, acid phosphatase activity is also observed in alveolar macrophages around the time of birth; the number of reactive macrophages and intensity of acid phosphatase activity increases sharply after birth. In starved rats, there appears to be fewer macrophages and their acid phosphatase content is lower up to 2 weeks after birth. Since acid phosphatase is an important degradative enzyme for the role of the macrophage in pulmonary defence, retarded development of this enzyme could result in increased susceptibility of the newborn to infection.

Peroxidase activity in normal rats appears before birth and increases sharply at birth to reach maximum reactivity by day 10 post-partum. Dietary deprivation inhibits peroxidase development so that fewer positive cells are seen at parturition. The level then increases to become comparable with control lungs at 2 weeks. By electron microscopy, peroxidase is detected in the lysosomal granules of polymorphonuclear leukocytes and in type II cells. Peroxidase in type II cells is probably important in an antioxidant protective role which allows these cells to become tolerant to oxidant gases. Inhibition of peroxidase development in the perinatal period could increase the newborns' susceptibility to oxidant injury.

In conclusion, these results indicate that nutritional status has a marked effect on fetal lung growth and development by inhibiting cellular proliferation, differentiation and enzyme development. Thus malnutrition could influence surfactant synthesis, resistance to oxidant injury and susceptibility to infection in the perinatal period. There might also be long term effects on lung growth, structure, and function.

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CONTENTS

INTRODUCTION

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A. Structure and Function of Normal Lung

The respiratory system is composed of a series of conducting tubes and respiratory passages through which warmed and humidifed air is allowed to pass. The primary function of the respiratory system is gaseous exchange of oxygen from the inspired air, and carbon dioxide from the pulmonary blood, via the attenuated blood-air barrier. Secondary functions include phonation and olfaction. The conducting portion of the respiratory system includes the nasal cavity, paranasal sinuses, nasopharynx, larynx, trachea and the bronchi. The conducting portion from the nostrils to the lungs is concerned with warming, humidifying and filtering the air before it reaches the respiratory tissues. The mucosal structure reflects the various functions performed by the conducting portion of the system. Mucus is secreted by the goblet cells which entrap particulate matter and the cilia of the respiratory mucosa lining help remove the mucus and debris from the conducting system (1).

The nasal cavity is lined by squamous, ciliated, and non-ciliated respiratory and olfactory epithelium. There is a gradual transition from epidermal lining of squamous cells to predominantly ciliated lining of the nasopharynx. The trachea bifurcates into two primary bronchi lined by two distinct cell types, ciliated and non-ciliated, with the non-ciliated cells and goblet cells interdispersed among the ciliated pseudostratified columnar epithelium. The lining

cells of the bronchus appear identical to those observed in trachea. The mucosal lining of the airways gradually changes the further along one proceeds, with the cells becoming thinner and more cuboidal in shape (2).

The bronchus further subdivides into the bronchioles, respiratory bronchiole, alveolar ducts, alveolar sacs, and alveoli. The epithelium of the branching and smaller bronchi changes to simple ciliated cuboidal epithelium with numerous goblet cells at the surface. The bronchiole shows a ciliated cuboidal epithelial lining without goblet cells whereas the respiratory bronchiole, the terminal portion of the conducting system, has ciliated cuboidal epithelial cells with a number of non-ciliated cells interspersed; these are commonly called clara cells. Their function is presently not fully understood but some investigators feel that they may play a role in surfactant secretion (3). The respiratory bronchiole leads to the alveolar ducts and sacs and the bulging alveoli. The alveoli, which appear as small compartments opening into the sacs, are the smallest and most numerous subdivision of the respiratory system.

The alveolar surface is characterized by three distinct cell types: (a) the smooth attenuated type I squamous epithelium seen lining the alveolar ducts, sacs and alveoli; (b) the large cuboidal type II cells with short microvillous projections seen in alveolar corners (4); (c) the alveolar macrophage, which is free within the alveolus, though in close proximity to the epithelium. This pulmonary surface scavenger is very important in the removal of debris and

microorganisms from the lung (5,6).

It is of particular interest that the exact composition of alveolar lining was not elucidated until recently by Low using electron microscopy, though the structure has been in dispute since the middle of the 19th century. The conflict largely arose because of the inadequacies of conventional light microscopy which does not allow a precise identification of the alveolar cells and has caused considerable confusion over the exact types and nature of the pulmonary cells. The use of electron microscopy has eventually led to a much clearer and precise understanding of the morphology of the lung and a new insight into its function and origin of the various cell types (7).

Several early investigators felt the respiratory capillaries, except for supporting reticulin fibers, were naked with no visible cellular lining; later, however other investigators concluded that the alveolus may be lined with a very attenuated cell, which was not observable with the light microscope. This cell was believed to be continuous and rested on a basement membrane that was in intimate contact with the pulmonary vascular system (5,7). This situation would correspond to the blood-air barrier which intervenes between the circulation and the pulmonary air, but allows air and blood to approach each other more closely than anywhere else in the body. The attenuated epithelium would therefore facilitate the diffusion of gases and favor the transfer of oxygen to the red blood cells of the pulmonary capillaries with the release and transfer of carbon dioxide into the alveoli.

Electron microscopic studies done by Low showed that the alveolus was indeed lined by a continuous attenuated pulmonary epithelial cell (8). Later studies showed the presence of second type of pulmonary epithelial cell and it was concluded that the lining of the alveolus was comprised of two different types of epithelium. The attenuated epithelial cell which closely resembles endothelial cells as described by Low, was originally called a "membranous pneumocyte" and now is more commonly referred to as a type I alveolar epithelial cell (9). This cell type contains attenuated scanty cytoplasm with a few mitochondria, ribosomes, and cisternae of endoplasmic reticulum. These highly flattened cells are continuous with the epithelium of the terminal bronchiole and as suggested earlier, facilitate the exchange of pulmonary gases. The second type of lining cell was identified by Macklin as a large cuboidal cell with a centrally placed nucleus. The most obvious characteristic of the cell is presence of many osmophilic lamellar bodies in the cytoplasm (9, 10). Other distinguishing characteristics observed in this cell are the presence of multivesicular bodies in the cytoplasm and microvilli on the free alveolar surface (11). This cell is not considered to be phagocytic, but rather secretory as will be discussed later (12-14). These epithelial cells became known as "granular pneumocytes" and are more commonly referred now as type II alveolar epithelial cells. Besides the morphologic differences, these two types of cells can also be distinguished by their location within the alveolus. The attenuated type I cells which line most of the alveolus bulge slightly into the lumen as they overlie the capillaries, while the type II cuboidal cells are observed at the corners of alveoli and form tight junctions with the type I cells to produce an uninterrupted lining of

the alveolus (11). There are several other cell types seen in the alveolar regions, a major cell type being the endothelial cell found lining the pulmonary capillaries. The capillary wall is completely wrapped by these endothelial cells of varying size, which are contiguous with one another. The endothelium is extremely flattened with the cytoplasm containing the usual organelles, the most obvious being pinocytic vesicles, with the nucleus bulging very noticeably into the lumen of the capillary (2).

Cells are also found between the reticulin basement membranes in the interalveolar space which communicates with the interstitium of the lung, or in vascular canals in the alveolus. These cells include fibroblasts, interstitial cells and sometimes blood cells. The other major cell type in the alveolus is the macrophage, a relatively large round cell found free in the alveolar lumen (6). They range in size from 15-30μ with a basophilic cytoplasm often containing debris and small particles, while the nucleus is variable in size and shape but is usually indented and irregular. The presence of the debris in the cytoplasm would seem to indicate that the free alveolar cell is phagocytic.

The origins, inter-relationships and functions of some of the alveolar cells have been the subject of much recent investigation. An area of confusion arose concerning the relationship and origins of the large cuboidal type II cells and the alveolar macrophage. Macklin had suggested that the two large cells were derived from a common endodermal epithelium (9), while others have suggested that the two cell types are mesodermal in origin and represent two different stages of

the same cell, and by using light microscopy methods these cells were accordingly described as vacuolated sessile alveolar cells and non-vacuolated free alveolar cells (5). However, now the most popular view is that the macrophage is derived from blood monocytes which migrate through the capillary wall to the interstitium then subsequently into the alveolar lumen to become free macrophages (6). This indicates that the macrophage is not related to the type II cell, but is rather a mesodermal cell with phagocytic abilities. The exact route the monocyte takes in order to become an alveolar macrophage is still not quite clear (7,15).

At the alveolar surface, two morphologically distinct cells, the type I squamous and the type II cuboidal, have been described. These cells represent slowly renewing populations, and a turn over time of 4-6 weeks has been suggested without distinguishing the two cell types. The results of recent investigations indicates that these two epithelial cells are not separate populations but rather that the type II cell acts as the progenitor for the type I epithelium (16).

The first evidence for this hypothesis was elucidated during experiments on alveolar injury using various gases and observing the subsequent recovery and repair. Hyperplasia of type II cells has been observed in studies using high concentration of oxygen (17) and of nitrogen dioxide (18,19). The type I cell is very susceptible to injury by exposure to toxic gases and becomes necrotic. The type II cell, however, is relatively resistant to injury and proliferates in the reparative phase. Focal hyperplasia of type II cells maintains

the integrity of the alveolus and is followed by type II cyto-differentiation into attenuated type I epithelium. This focal proliferation of the type II epithelium is a common cellular response to alveolar injury and is seen after oxygen and nitrogen dioxide exposures (16-21). It would therefore seem apparent that the type II cell is the stem cell for renewing populations of alveolar cells and acts as the reserve mechanism for replacing injured cells (22).

This repair mechanism probably reflects the normal process of cell renewal. As additional evidence, the cuboidal cells found lining the fetal rat alveolus are transformed into type I with type II thereby acting as the progenitor cells for alveolar epithelium (22). In the rat, at day 21 of gestation almost all the cuboidal cells had been transformed into recognizable type II epithelium, and with time many of these cells differentiate into attenuated type I epithelium which overlies new capillaries (23). This evidence would appear to establish an inter-relationship between the two types of alveolar epithelial cells and it is concluded that the cuboidal cell lining the fetal tubular airways is the progenitor of both types of alveolar epithelium.

Although the type II cell has this reparative function, more attention has been paid to its other possible functions. For example, whether it was phagocytic in nature or not due to the presence of hydrolytic enzymes in its inclusions (5,14), or whether the cell was actually secretory in nature and played a role in surfactant production (4,11, 24, 25). The presence of lysosomal enzymes, in particular acid phosphatase in the lamellar bodies of the type II cells, prompted the

hypothesis that type II cells are involved in phagocytosis and would be given over to degradation and digestion of ingested matter (14). However, the presence of lysosomal enzymes in certain stages of development of an inclusion is not necessarily evidence in favor of phagocytosis, since lysosomal enzymes have been identified in secretory granules (11, 26). If the type II cell does phagocytize the lipid inclusions, their phagocytic activity certainly shows an extraordinary specificity for one type of material. Presently, evidence is accumulating that strongly suggests the type II is a secretory cell producing surfactant which is extruded onto the alveolar surface (3, 11, 13, 24, 25, 27). Although it is generally agreed that the secretory material within the lamellar bodies is a phospholipid, the exact nature of lamellar bodies is still controversial. The precise intracellular site of synthesis also has not completely been established, although all the evidence favors the membrane enclosing the lamellar bodies as the actual area of production (27). The agent which is primarily responsible for the surfactant effect is the saturated phospholipid, dipalmityl lecithin which is metabolized from 1,2-dipalmityl phosphatidic acid (28). Acid phosphatase has been implicated in the metabolism of dipalmityl lecithin and has been demonstrated in lamellar bodies of type II cells (24, 25, 27, 28). In particular, acid phosphatase is seen localized in the outer membrane of lamellar bodies and in multivesicular bodies (25) which would indicate the perilamellar membrane of type II cells is the favored site of dipalmityl lecithin synthesis in the lung (27).

The relationships between lamellar bodies and multivesicular bodies has also received considerable attention, since there would appear to be a link between the two cytoplasmic inclusions found in type

II cells. Sorokin envisaged that multivesicular bodies may become incorporated or transformed into lamellar bodies with the multivesicular bodies as the primary sites of lipid synthesis in the type II cell (11). This hypothesis has received support from Meban and Goldfischer et al. based on a similar acid phosphatase enzyme content between multivesicular and lamellar bodies, indicating that they may be related functionally (14,25). Also, Kuhn found that the limiting membrane of both structures is approximately 100Å thick, which is perceptibly thicker than the 60Å-70Å membranes of mitochondria, endoplasmic reticulum and other vacuoles in the cytoplasm. A relationship between multivesicular bodies and lamellar bodies is therefore suggested by similar enzyme contents and thickness of their limiting membrane. Kuhn also observed the presence of transition structures between the two structures which would strengthen the hypothesis that the two are related (9).

B. Histochemistry and Cytochemistry

Histochemistry and cytochemistry provide valuable qualitative information on enzyme activity of the particular alveolar cells which is not readily available by quantitative fractionation, biochemical extraction and analyses. Quantitative information provides limited information on the lung, namely because the cellular elements cannot be distinguished.

Apart from the alveolar macrophage, most histochemical and cytochemical studies on the lung deal with type II alveolar cells. Type II cells exhibit a high metabolic activity with many different types of enzyme systems. The enzymes which have received the most attention are the hydrolases, and in particular the phosphatases, and the oxidases, which include dehydrogenases and peroxidases (29, 30, 31).

Acid phosphatase has been studied histo- and cytochemically in lung using a variety of techniques, the most popular being the lead capture method by Gomori which produced a visible heavy metal precipitate at the site of enzyme activity (31, 32, 33, 34). There have been many modifications made to Gomori techniques, such as Barka and Anderson's method developed for histochemistry (35, 36, 37). As indicated earlier the majority of the activity is seen in macrophages and type II cells of the lung (11, 12, 14). At the ultrastructural level acid phosphatase activity was visualized using variations of Gomori original technique (14, 25, 28, 38). Localization of acid phosphatase sites shows reaction products heavily deposited in lysosomes of

macrophages (14, 22, 39) and preferentially concentrated at the perilamellar membranes and in multivesicular bodies of type II cells. These observations have been substantiated by many investigators including Etherton, Botham, and Meban (11,12,14,25,26,28,40).

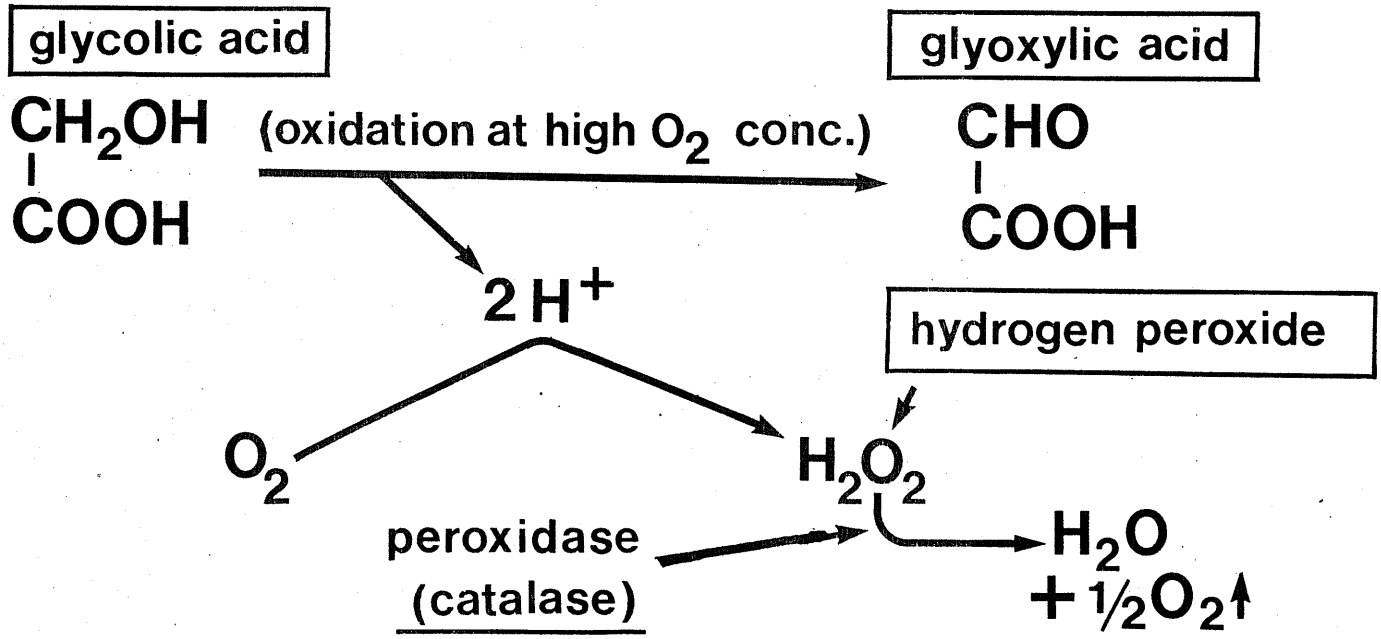
Alkaline phosphatase has also received considerable attention from investigators; Fredericsson studied the distribution of alkaline phosphatase in rat lungs and demonstrated activity in the cilia of free cells of the bronchiolar epithelium and in the septal cells (41). Petrik and Klika demonstrated significant alkaline phosphatase activity in type II cells which may indicate an active molecular transport system in lung metabolism (4,30). Kuhn observed alkaline activity at the apical plasma membrane of type II cells and in lamellar membrane as the inclusion approaches the apical plasma membrane; this was substantiated by Sorokin (39). Esterase activity in the lung has been studied by Schiff et al. (42,43); activity has been associated with lysosomal bodies in most phagocytic cells and this is also true of the alveolar macrophage. This probably indicates that esterases are concerned with intracellular digestion of phagocytized materials (34,43).

Oxidative enzymes are enzymes which are associated with the production of energy within alveolar cells. This production of energy is referred to as cellular respiration and there are many different enzymatic pathways available in cellular respiration for this production of energy (31,44,45,46). Oxidases for example require the presence of oxygen in order to catalyze a direct oxidation of their substrate (31,44). Histochemical observations on oxidative enzymes are meager, probably due

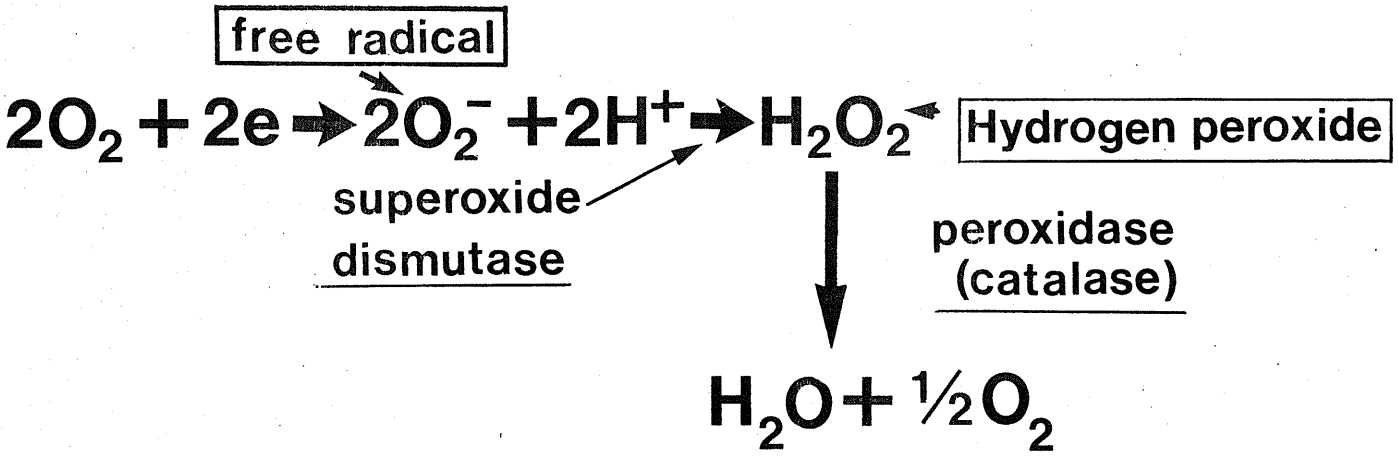
to the difficulty in producing a visible stable reaction product (47). A group of oxidative enzymes which require peroxide as a substrate are called peroxidases, one specific peroxidase being catalase, which converts hydrogen peroxide into water and oxygen (44). There have been several techniques developed which allow peroxidase activity to be visualized histochemically, the most popular method being the oxidation of benzidine to benzidine blue which can be further stabilized by the use of sodium nitroprusside (31). Peroxidase activity has been found in peroxisomes of many cell types (48-52), two of which are the type II alveolar cell and the clara cell. The ultrastructural location of peroxidase has been confirmed by several investigators using Novikoff and Goldfischer's alkaline modification (26) of Graham and Karnovsky's technique which used 3,3'-Diaminobenzidine tetrachloride (DAB) and hydrogen peroxide (53,54). This has allowed investigators to identify peroxidase activity in the lung, located in the peroxisomes of the cytoplasm of type II and clara cells (12,48,55). The function of peroxisomes is not well understood at present, although several possibilities have been suggested by de Duve and others (56,57,75). It is known that special oxidative vesicles (peroxisomes) contain peroxidase and this allows an auxillary aerobic form of oxidation to proceed when high partial pressures of oxygen are experienced by the type II cells. The type II alveolar cells of newborn mice have been shown to be more resistant to high levels of oxygen toxicity than adult mice, which would indicate that these cells may have an adaptive enzyme system which appears to act as an anti-oxidant defence mechanism against oxidative injury (59). Bonikos et al. have substantiated this finding by experimentation with oxygen toxicity in neonates. They found by exposing newborn mice to 100 percent oxygen for 7 days that

75 percent of the mice were able to survive the toxicity of the exposure. This indicates the development of an adaptive mechanism in the newborn animal which permits them to survive an exposure that is lethal to adult mice. It was also noted that type II alveolar cells showed a high rate of proliferation in the lungs during this 100 percent oxygen exposure (60,61). The type II alveolar cell proliferation after exposure to high concentration of oxygen was also observed by Adamson and Bowden (16,22). Hyperplasia of type II cells was also encountered following high levels of nitrogen dioxide exposure (18, 21). The adaptive response to high levels of toxic gases would therefore appear to involve a marked hyperplasia of type II alveolar cells, with the anti-oxidant capabilities conceivably due to the presence of adaptive enzyme system equipped to lessen the toxic effects and provide tolerance to oxidant induce pulmonary toxicity (62). Rosenbaum observed that a higher survival rate in rats, exposed to toxic concentrations of 100 percent oxygen, was obtained when this exposure was preceded by an exposure of 85 percent oxygen for 7 days. This allowed the animals to adapt and become tolerant to an otherwise toxic exposure of 100 percent oxygen. It was noticed that the free ribosomes had greathly increased in number and that the cisternae of the endoplasmic reticulum were dilated in the type II cells, thus suggesting an increase in secretion in these cells (63). Intrinsic type II cell mechanisms for mediating tolerance to high oxygen concentration may be due in part to a peroxidase or catalase enzyme present in the peroxisomes. Rosenbaum concludes that catalase may be responsible for the tolerance observed in animals exposed to 85 percent oxygen for 7 days since he noted that catalase activity remained normal in tolerant animals, but fell by 50% in animals with pulmonary lesions. Chow has also

studied the development of tolerance to oxidant pollutants and he observed that peroxidase provided a protective mechanism against oxidant injury by breaking down hydroperoxides to less toxic forms (65). It is known that glycolic acid, a major substrate, can be oxidized to glyoxylic acid utilizing oxygen, when it is in high concentrations to yield hydrogen peroxide. Peroxide must then be decomposed by peroxidase or catalase in the peroxisomes of type II cells in order to prevent toxic affects on the cells (44,58).



Another enzyme which has also received considerable attention in conjunction with the defence or protection against pulmonary oxygen toxicity is superoxide dismutase (SOD) which dismutes the free radical oxygen to less toxic forms. It was observed that the development of oxygen tolerance paralleled the increase in superoxide dismutase activity. Similarly SOD activity decreased as the animals progressively lost their tolerance to oxygen over a period of 30 days. These observations support the theory that superoxide dismutase and a peroxidase (catalase) may provide an in vivo defence against the oxygen toxicity (64,66).



Lactic acid dehydrogenase is an oxidative enzyme which will oxidize substrates in the complete absence of free oxygen (anaerobically) (31). Dehydrogenases (LDH) can be demonstrated by employing tetrazolium salts which will yield a highly colored insoluble formazons (67). This enzyme has been studied by Sherwin et al. in connection with type II cell hyperplasia after an exposure to nitrogen dioxide. He noted that accompanying the marked hyperplasia of type II was a sharp increase in lactic acid dehydrogenase activity (18,20), suggesting that LDH could also play a role in lessening the toxicity of high concentrations of gaseous pollutants.

C. Development of the Lung

The lung begins growth and development as an epithelial groove in anterior aspect at caudal end of the foregut which eventually forms a small pouch. Later, the foregut, caudal to the pouch, begins to elongate and separates into the future stomach, allowing for differentiation into a primary lung bud and digestive system. The foregut, anterior to the now formed primary lung bud, forms the primative esophagus and trachea. The lung bud and the esophagus-trachea divide by fusion of the lateral ridges or folds which begins at the primary lung bud and extends toward the developing larynx. As the trachea and the esophagus are separating into individual structures the lung bud or pouch begins to be divided into left and right secondary buds, which segment into lobes with growth at this point being extremely rapid as those outgrowths of endodermal tissue and mesodermal tissue project into the pleuroperitoneal cavity. The lung buds continue their rapid fetal growth as a bronchial pattern of lobes and fissures begins to emerge out of the undifferentiated embryonic tissue (68).

The intrauterine and perinatal growth and development of the rat lung has been divided into three recognizable stages.

The first period is termed the glandular period of development and is the most extensive period "in utero" for the rat, occupying more than 3/4 of its total intrauterine life. In the glandular phase of development, the lung consists of a loose mass of mesenchymal connective tissue with branching airways called tubules, lined by columnar

or cuboidal epithelial cells, ramifying through the mesenchyme. Blood vessels are not in direct contact with the epithelial lined tubules at this time but are widely distributed in the mesenchyma (67). The complete cuboidal epithelial lining of the tubules transforms into functional type II cells, marking the transition from the glandular to the canalicular phase of development (70). The first appearance of surfactant in the fetal lung marks this change which usually occurs at approximately day 20 of gestation in the rat (71). In the transition period, enzyme systems first become detectable. Esterases have been identified at day 20 of gestation (13,43) coincident with the first appearance of acid phosphatase in lamellar bodies and surface activity (13,29,71,72). Oxidative enzyme systems, such as peroxidase, have also been identified at approximately day 20 gestation, as noted by Schneeberger (46,73).

Physiological changes within the fetal lung marking the change from glandular to canalicular are characterized by several morphological changes which are rather sudden and easily recognized. Blood capillaries are in contact with the cuboidal epithelium and appear to bulge into the airways. At this point attenuated epithelium is observed, as cytodifferentiation occurs to give a primitive air-blood barrier (23,69,70). The rat is born in this phase of lung development; it has no alveoli but rather a lung built up of primitive air sacs (74). The blood-air barrier thereby becomes functionally developed in the critical perinatal period. The primitive air sacs have very thick septa and contain two capillary networks, each one facing a sac (75). This pattern remains practically unchanged for the

first 5 days of life. The development of lung from the canalicular to the alveolar stage involves a change in degree rather than kind and takes place postnatally. This indicates the relative immaturity of the lung at parturition.

Postnatally, rat lung development is characterized by several distinct changes in the lung architecture. The transition from canalicular to alveolar stage is marked by the presence of secondary crests which begin to grow and subdivide the primary terminal air sacs into alveoli, whose partitions carry with them the capillaries. During this process the septa become thinner and the interstitium is reduced to a single capillary network between adjacent alveoli (74). Burri has described postnatal development of the lung as having three recognizable parts. Firstly, a period of lung expansion in which the lung grows or enlarges by expansion of the existing air saccules. This period lasts up to approximately day 4 in the rat; it is followed by a period of rapid tissue proliferation which lasts until about two weeks after birth (76). In this period there is a vigorous increase in tissue mass which is followed by an increased capillarization. This process of septation, which produces actual alveoli, greatly increases the actual surface area of the lung (74). The third period is referred to as "equilibrated growth" phase, which begins some time after 2 weeks and continues until the lung reaches maturity. During this phase, the newly formed alveoli are slowly enlarged with some alveolarization still taking place at a reduced rate, with growth confined largely to alveolar expansion (74-78).

D. Malnutrition and Development

The problem of fetal malnutrition and subsequent retardation of growth and development has become increasingly prominent in recent years. It now appears that there are inter-relationships between intra-uterine malnutrition with pre-and post-natal structural and functional changes. It is recognized that malnutrition in the fetus affects all the body organs as well as the total body weight and size. From information available it appears that malnutrition studies have been concentrated on the brain, however, malnutrition studies do indicate that other areas such as liver, lung, kidney, thymus, skeleton, and endocrine organs are also affected (78). These changes, therefore, must be considered important for the individual's ability to function properly in his own physiological dimension.

Previous studies on development in fetal malnutrition have shown that the growth of cells is retarded. Malnutrition has its greatest influence on cellular growth during the proliferative stages which is characteristic of fetal growth, hence prenatal growth is most vulnerable to malnutrition (79). Malnutrition may damage the cells of any organ, including the brain if it occurs during a vulnerable period of growth, that is, during a period of most active cell replication. The general effects of starvation are obvious with a reduction in body weight, often as high as 40 percent. The lungs, adrenals, kidney, all appear to be reduced in direct proportion to the total body weight with the brain showing least reduction in size and the liver the most (80). These reductions are due in part to the retarded

cellular growth reflected in a reduced rate of DNA synthesis, altered RNA metabolism and a reduced rate of new protein synthesis. This retardation in growth may lead to reduced or impaired cell activities with decreased cellular function (81).

During the past few years evidence has accumulated that there is a relationship between early malnutrition and subsequent retarded lung development, which could lead to reduced lung function. There appears to be a parallel effect in the reductions of body weight and lung weight in malnutrition (81). If animals are subjected to prenatal and postnatal malnutrition a profound decrease of 40-50 percent can be demonstrated in body and lung weights (81). It is also possible that during early development, enzyme systems necessary for proper physiological functioning have not evolved to the extent that is necessary for the lung cells to carry out normal activities (83).

The critical division and differentiation of pulmonary epithelium may be altered with consequent changes in the ability of newborn lungs to function adequately. Faridy has studied the effects of maternal malnutrition on fetal lung growth and development of lung surfactant system. He demonstrated that malnourished fetuses had 35 percent less lung phospholipids and decreased surface activity in conjunction with reduced lung sizes (84). Gross et al. studied the inhibition of enzymes in relation to pulmonary fatty acid and phospholipid synthesis after acute nutritional deprivation. It was found enzymes necessary for pulmonary surfactant production are reduced in activity ranging from 36-55 percent. This would appear to establish

the premise that lung growth and function are greatly altered by dietary deprivation (85).

Retarded cellular growth experienced in fetal malnutrition, particularly in the proliferative phase may be due to several reasons. Although the exact nature of how a reduction in caloric intake affects the developing lung is not entirely clear, it is obvious that in malnutrition there is a reduction in available nutrients necessary for normal energy production and a decrease in availability of material for DNA and protein synthesis. Low energy levels are likely due to reduced nutrient supply and decreased protein synthesis, as protein synthesis is dependent upon the availability of amino acid precursors. The synthesis of nucleic acids requires general proteins as well as specific enzyme proteins which are altered during malnutrition with reduction in both general and specific synthesis (83). This would explain the observed changes in total weight, protein content, DNA content and RNA content which are reduced up to 25 percent of the control values in severe prenatal nutrition restrictions (80,81).

Reduced or impaired enzyme synthesis may be anticipated because of the reduced protein synthesis observed in dietary deprivation studies. It appears that each enzyme system in developing fetal lung follows its own specific pattern of ontogenesis and this may be of significance when considering the effects of malnutrition on the derangements of biochemical patterns in the developing lung. Acute nutritional deprivation has been shown to significantly reduce enzyme activity levels which are associated with the production of surfactant

(84,85,86). e.g. Acid phosphatase which has been closely linked with surfactant production and is seen in association with lamellar bodies in type II epithelium (25). It is feasible that reduced enzyme activity is a consequence of delayed cellular differentiation and so would result in delayed production of surfactant. This relationship and the energy requirements as judged by glycogen utilization has not been fully investigated (87).

The development of peroxidase in malnourished animals is also unknown. Type II cells which are more resistant to oxidant injury than type I cells (22) and this factor may be related to levels of adaptive oxidative enzymes found in this cell type. The effect of malnutrition on the peroxidase content of epithelial cells is not known.

PRESENT STUDY

The purpose of this investigation is to correlate the morphology of pulmonary development and cellular differentiation in the perinatal period with the cellular distribution of glycogen and enzymatic activities. The effects of maternal starvation on these parameters is also studied in the developing fetal and neonatal lungs.

There are many reports on changes in lung morphology and biochemistry before and after birth in several species (46,64,70,84,88), but good correlative studies into the timing of differentiation and enzyme development are scarce. Specifically, this study is designed to examine the morphology of the developing rat lung using light microscopy.

Histochemical methods are also utilized to furnish information on the glycogen content of lung cells and map important metabolic and adaptive pathways present in alveolar cells. In particular, two enzymes are chosen to represent major metabolic pathways: (a) acid phosphatase to provide information concerning the hydrolytic enzyme system considered important in surfactant secretion, and (b) peroxidase to provide information on an oxidative enzyme system may be implicated in a "protective" role for type II alveolar cells.

Electron cytochemistry is carried out to allow definite cellular identification and to give precise localization of enzymatic activity in the alveolar cells. These examinations, using light and electron microscopy, will identify a pattern of normal morphological and biochemical development.

The second part of this study is designed to elucidate any differences between normal lung development and development after severe dietary deprivation is imposed upon the pregnant rats. The maternal rats are starved from day 13 of gestation until two weeks after birth to focus on the problem of fetal and postnatal malnutrition and its effects on lung development as indicated by any morphologic and enzymatic changes.

MATERIALS AND METHODS

A. Animals

Forty-eight timed pregnant rats (Charles River Albino) received on the 13th day of gestation, were utilized for the experiments. These rats were subsequently divided into two groups. Group one, the control group, consisted of 16 rats, while the second group, the test group, consisted of 32 rats. The control group was allowed laboratory animal chow and water "ad libitum", while the test group was subjected to a diet of total solid deprivation but were allowed 2 percent glucose in water "ad libitum".

The control and the experimental animals were sacrificed daily during the gestation period. Fetal rats were harvested by Caesarean section and thoracotomy was carried out with the use of a magnifying lens to aid in the removal of the fetal lung tissue. Two pregnant rats from each group were sacrificed from day 17 to day 22 of gestation, while five neonates were sacrificed by decapitation on postnatal days 1,2,3,5,7,10,14,21, and 28 in the control and similarly to day 14 in the test group.

The lungs removed from the fetal and neonatal rats were treated in several different ways in order to prepare them for subsequent examinations. Lung tissue to be examined with light microscopy for morphology, glycogen and electron cytochemistry was prepared by placing excised lungs in 3 percent buffered glutaraldehyde fixative pH 7.4 at 4^{o}C. for 24 hours. If the size of the animal permitted it, the lungs were gently inflated "in situ" with 3 percent glutaraldehyde

via the trachea and then suspended in the fixative for 24 hours. The lungs after fixation were placed in millionigs buffer pH 7.4 for 4-24 hours at 4°C. Lung tissues to be examined for histochemistry were immediately frozen on a carbon dioxide freezing microtome and maintained at -20°C. If the size of the lungs permitted it, the lungs were inflated with cryoquik and the tissue was then sectioned on an International cryostat at 6μ in an expanded state and placed on glass microscope slides, briefly dried and maintained at -20°C. until further treatment in histochemical solutions (89). Sections prepared in this fashion were utilized for acid phosphatase and peroxidase enzyme studies.

B. Light Microscopy

Methacrylate sections:

Glycol methacrylate sections were utilized in the examination of lung tissue for cellular morphology and glycogen content. The lung tissues, after they were fixed and buffered at 4°C., were removed from the buffer and cut by hand with a razor blade to produce thin tissue blocks measuring 5 x 10 x 20 mm. These blocks were post fixed in 10 percent phosphate buffered formalin for a further 24 hours at 4°C (47). After fixation was complete the tissue blocks were transferred to ascending concentrations of ethyl alcohol commencing with 50 percent, 80 percent, 90 percent and three changes of absolute alcohol for complete dehydration. The tissue blocks were mixed in a 1:1 mixture of absolute alcohol and solution A of the glycol methacrylate embedding kit for 1 hour, then transferred to solution A for 5 hours and finally placed in a mixture of 42 parts solution A with 1 part solution B on a shaker with ice cubes for 1-2 hours until infiltration was complete. The tissues were then embedded in plastic moulds using the 42:1 mixture of solutions A and B, and allowed to polymerize at 27°C. overnight. After polymerization was completed the blocks were removed from the moulds and prepared for sectioning. Sections were cut with a dry glass knife on J.B4 Porter-Blumm rotary microtome at thicknesses of 3/4μ and 1 1/2μ. The sections were transferred to a water bath at 35°C. and picked up on glass slides and dried thoroughly on a hot plate (The J.B4 plastic embedding kit consists of solution A (glycol methacrylate, butoxyethanol and benzoyl peroxide) and solution B (polyethylene glycol and dimethylaniline)(91).

i). Morphology: Sections to be used in studying the developing lung morphology were stained using Gomori silver methanamine technique (33). The tissue sections were oxidized in 0.5 percent Periodic acid for 10 minutes and rinsed thoroughly in distilled water and then incubated in silver methanamine solution at 60°C. until the sections turn brown. (The medium consisted of 3 percent methanamine-5.0 ml., 5 percent silver nitrate-2.5 ml., and 9 percent borax-5.5 ml.) (31). The slides were then rinsed in distilled water, toned briefly in 0.2 percent gold chloride, rinsed in distilled water, fixed with 5 percent sodium Thiosulfate and rinsed in distilled water again, before dehydrating in ascending grades of alcohol, clearing in xylol and mounting with Permount.

ii). Glycogen: Lung tissues examined for glycogen content utilized 1 1/2μ sections which were pre-soaked 30 minutes in distilled water prior to exposure with histochemical Schiff's reagent. The technique utilized was the Periodic Acid Schiff's method for carbohydrates (31). The sections were immersed in 0.5 percent periodic acid for 5 minutes and washed in running tap water, then rinsed in distilled water and incubated in Schiff's reagent at 23°C. for 20 minutes. The sections were then washed in running tap water for 30 minutes, dehydrated in alcohols, cleared in xylol and mounted with Permount. Glycogen appeared a magenta to pink color in the lung sections.

iii). Acid phosphatase: Tissue sections cut on the cryostat at 6μ were fixed for 5 minutes in 3 percent glutaraldehyde and then rinsed in distilled water prior to incubation in Barka and Andersons

modification of Gomori's medium for acid phosphatase for 60 minutes at 37°C. (31,33,35). (The medium consists of 10 ml. of Tris Maleate buffer, pH 5.0 and 10 ml. of distilled water with 20 ml. of 1.25 percent glycerol phosphate and 20 ml. of 0.2 percent lead nitrate which was preheated to 37°C. before use.) After incubation the tissues were rinsed briefly in distilled water and then placed in 2 percent ammonium sulfide for 2 minutes. The tissues were then rinsed briefly in distilled water and counter stained for 1 minute in 0.5 percent Eosin before dehydrating in ascending grades of ethyl alcohol, clearing in xylol, and mounting in Permount. The final reaction color product indicating acid phosphatase activity was black and the background was stained a light pink color to provide contrast.

iv). Peroxidase: Tissue sections cut at 6μ on the cryostat to be examined for peroxidase activity were very quickly rinsed for 2-3 minutes in 10 percent neutral Buffered formalin, rinsed in distilled water, then incubated in Diamino-Benzidine (DAB) solution for 1 minute at 4°C., transferred to nitroprusside solution A for 10 seconds, then to nitroprusside solution B at 23°C, for one hour (52,53). The sections were rinsed in distilled water, counterstained in 0.5 percent Eosin for 1 minute before dehydrating, clearing and mounting in Permount. (Solution A consists of 4.5 gm. of nitroprusside dissolved in 35 ml. of absolute alcohol and diluted with 15 ml. of distilled water. Solution B consists of 15 ml. of solution A to which 35 ml. of absolute alcohol and 2.5 ml. of 0.2M acetate buffer are added (31). The final reaction product is a bright blue color indicating peroxidase activity with a light pink background stain for contrast.

C. Electron Microscopy

Cytochemistry: Acid Phosphatase

Tissues to be examined for electron cytochemistry were fixed in 3 percent glutaraldehyde buffered at pH 7.4 millionig's buffer containing 10 percent dimethylsulphoxide and 7.5 percent sucrose for 30 minutes at 4^{o}C. (47,90). The lung was then cut into small blocks by hand using a razor blade and fixation for optimum preservation was continued for 1-3 hours at 4^{o}C. in the above mentioned fixative. The tissues were then rinsed in millionig's buffer for 30 minutes and 40μ sections were cut on an international cryostat and floated on 0.05 M acetate buffer containing 7.5 percent sucrose for 30 minutes.

The tissue slices were then incubated at 37^{o}C. for 60 minutes in a freshly prepared medium modified by Etherton and Botham (28). (The incubation medium contains 40 ml. of 0.05 M acetate buffer with 7.5 percent sucrose mixed with 40 mgm. lead acetate and 16 mgm. of α-napthyl phosphate having a pH of 5.0). After incubation the tissues were transferred to 0.05 M acetate buffer and 7.5 percent sucrose mixture for 30 minutes at 4^{o}C. (40).

The tissues were diced into 1 mm. cubes using a sharp razor and post fixed in cold millionig's buffered oxmic acid for 1 hour, on a shaker surrounded with ice cubes. The tissues were then washed twice in distilled water with dehydration in 2 changes of 70 percent alcohol for 30 minutes, followed by 2 changes in 95 percent alcohol for

30 minutes and finally 2 changes of 100 percent alcohol for 15 minutes to complete dehydration. Impregnation was started by placing the specimens in a 1:1 mixture of Spurr plastic and absolute alcohol for 30 minutes and finally placing the tissue in 100 percent Spurr plastic for 30 minutes and then changing to a new solution of Spurr for 2 hours to complete impregnation. The tissues were then embedded in small gelatin capsules with Spurr and allowed to dry at 65°C. overnight. (The Spurr plastic mixture consists of vinyl cyclohexene dioxide, diglycidyl ether of propyleneglycol, monenyl-succinic anhydride and Dimethylamino-ethanol).

After polymerization the gelatin capsule was trimmed off at the end to form a pyramid and then sections (pale gold to silver) were cut on a Sorvall Porter-Blumm ultramicrotome using glass knives. The thin sections were picked up on copper 200 mesh grids, blotted and dried. The grids were then examined unstained on a Phillips 201 Electron microscope.

Cytochemistry: Peroxidase

The ultrastructural identification of peroxidase activity in the developing rat lung was done using Graham and Karnovsky's medium (26,49,53). The lungs were immediately placed in 10 percent formalin and 5 percent sucrose after excision, and, if the lungs were large enough to permit, fixative was perfused via the trachea, and fixation was allowed to proceed for 30 minutes at 4°C. (72).

After washing in buffer, frozen sections 40μ in thickness were cut on an international cryostat and briefly rinsed in buffer.

After rinsing, the sections were incubated for 60 minutes at 37°C. in a medium containing 10 mg. of 3,3'-diamino benzidine tetrachloride (DAB) (Sigma Chemical Company) in 10 ml. of Tris Hcl, buffer pH 7.6, containing 0.2 ml. 0.01 percent H_2O_2 (54, 93).

The sections were washed in 3 changes of distilled water and post fixed in 1 percent cold millionig's buffered osmic acid and 5 percent sucrose for 1 hour on an ice cube shaker. They were dehydrated in graded alcohols and embedded in Spurr plastic as previously described. Thin sections were prepared and viewed unstained on a Phillips 201 electron microscope as described earlier.

D. Cell Counts

Acid phosphatase:

Frozen sections cut at 6μ were examined with the light microscope sequentially from day 17 of gestation to birth and at regular intervals after parturition to four weeks postnatally in control animals and similarly in experimentally starved neonates up to two weeks post partum. A cell count was done noting the number of positive cells out of 1,500 alveolar cells utilizing at least three different animals, at each time recorded. The precise identification of the cell types in fetal and early neonatal rat lung was difficult and limited by the quality of the frozen section and the expertise of the observer. Positive cells were designated as epithelial and non-epithelial; cell identity was confirmed by electron microscope cytochemistry.

Peroxidase:

Frozen sections cut at 6μ were examined under the light microscope at similar time intervals. The positive identity of cell types was impossible in fetal and neonatal lungs and only possible 10 days after birth. Thus, it was necessary to approximate the amount of peroxidase activity seen at the various times studied. Positive cells were counted in 15 random high power fields (x400) on 3 different animals at each time recorded. The total number of positive cells was plotted against time to give relative amount of peroxidase activity in control and started animals at various fetal and postnatal ages.

RESULTS

Lung growth and development was studied sequentially from day 17 of gestation to four weeks post partum by morphologic and histochemical techniques in the control group of rats, which received solid nutrients and water ad libitum. Litters were produced after 22 days of gestation with the average weight of 6-7 grams per neonate with an average number of 12 neonates per litter. There was no mortality observed in either the maternal or newborn rats.

The test group of pregnant rats were deprived of all solid nutrients from day 13 of gestation until parturition. However, the maternal rats were allowed 2 percent glucose in water during this period, and after birth the mother rats were given a very small daily portion of laboratory animal chow in an attempt to minimize cannabilism and the high mortality rates in the maternal rats. The mortality rate reached as high as 50 percent of the total number of adult rats particularly between day 21 of gestation and 3 days post partum. The dietary deprivation also caused a significant number of stillbirths which was between 20-30 percent of the total number of neonates; after live birth the mortality rate reached as high as 30 percent of the total number of neonates up to 3 days post partum. The cause of death in stillborns and neonates was not determined. The lungs of stillborn and dead newborns were removed and examined with light microscopy and appeared similar to survivors sacrificed at similar times. It would appear that the deaths were not due to lung disease such as hyaline membrane disease but were likely related to other causes of

severe malnutrition. The cannibalistic tendencies of the maternal rats was also much greater in the starvation studies than in the control studies and this contributed to a greatly reduced number of neonates for this portion of the study. At parturition the progeny of the starved rats were much smaller than normal; the average weight of the neonates was 4-5 gm. indicating approximately a 30 percent reduction from the controls. The size of the lungs was also noticeably less than control lungs. This difference in size can be seen in Figure 1...where the lung from a starved neonate is considerably smaller than the lung from a control animal both at 10 days after birth.

A. Morphology of the Developing Lung--Control

FETAL At day 17 of gestation, the lungs are very glandular in appearance. Lying in the mesenchyme are large tubular structures with relatively wide lumens which are lined by a high cuboidal, one layered epithelium. Mitotic figures are seen frequently in the cuboidal epithelium, indicating rapid cellular division (fig. 2). The mesenchyme located between the widely spaced tubules occupies the majority of the space in the fetal lung. Mesenchymal cells are not tightly packed and the lung has the appearance of islets of tubular tissue supported in the loosely arranged mesenchyme. The cuboidal epithelial cells of the tubules are more tightly arranged and are distended by a pale cytoplasmic material which has been shown to be glycogen, as demonstrated later using the P.A.S. stain.

Lung appearance does not noticeably change on day 18; there appears to be a slightly increased number of tubular structures present with a few solid epithelial buddings which do not have open lumens. Blood vessels can be detected lying in the poorly differentiated mesenchyme but do not appear to be in contact with the cuboidal epithelium at this time. Similarly on day 19 of gestation the fetal rat lung still has an immature glandular appearance. The number of glandular tubules has increased significantly and these are still lined by pale staining cuboidal epithelium.

At day 20 of gestation, the morphology of the developing lung begins to undergo change (fig. 3). The tubules are closer together and

the mesenchyme is more closely packed, causing the blood vessels in the mesenchyme to come into close contact with the tubular epithelium. The lumens of the tightly packed tubules are widened giving a more expanded, less glandular appearance to the lung. The cells lining the tubules are still cuboidal in shape with a pale staining cytoplasm, however, the mitotic figures observed in earlier fetal days are not as prominent.

At a later fetal stage, day 21 of gestation, a series of "airways" or terminal air sacs are observed, causing the lung to appear more mature (fig. 4). The lumens of the terminal air sacs are distended considerably bringing the cuboidal epithelium of the air sacs into closer contact with blood vessels of the shrinking mesenchyme. The lung now is entering a phase of cellular differentiation, with some of the previous cuboidal epithelial cells starting to flatten out or attenuate as the blood vessels expand beneath them. This marks the beginning of cytodifferentiation of the terminal airways from cuboidal epithelium into functional type I and type II pulmonary alveolar cells, however, the majority of epithelial cells still appear cuboidal in shape with a distended pale staining cytoplasm. The unequal flattening of the previously uniformly cuboidal epithelial cells provides the first means of distinguishing the potential type I and type II alveolar cells. The development of these cell types are essential for normal functioning lung and are necessary for the production of the critical blood-air barrier in the perinatal period.

One day prior to birth, on day 22 of gestation, the lungs develop a more mature look, with numerous widely distended terminal air sacs (fig. 5). The cytodifferentiation of the epithelial cells noted earlier is progressing rapidly. In some parts of these terminal airways, a thin blood-air barrier is thereby functionally developed by day 22 prenatally, however, alveoli as such are not yet developed even though respiration could be maintained. The airways are still referred to as terminal air sacs at this time and have a relatively thick interstitial septum which is an obvious distinguishing characteristic of prenatal lung.

POSTNATAL The lung at birth is still an immature organ in as much as it contains only terminal air sacs and has not yet developed recognizable alveoli. This necessitates that the lung must undergo major postnatal developmental changes before becoming a fully functional unit. These maturational changes include the thinning of the interstitium, expansion of the air spaces, formation of secondary alveolar septa, actual alveolar formation and remodelling of the capillary network. These changes take place simultaneously as the lung develops.

At day one after birth the lung is essentially composed of smooth walled terminal saccules with relatively thick walled interstitial septa (fig. 6). The interstitial wall shows little or no branching or folding and the septum appears to be composed of a double capillary network. This likely fuses in later postnatal life to form a single capillary network and so accounts for the septal wall becoming much thinner. The

terminal saccules are lined by differentiated alveolar cells of both types. Morphologically a very similar appearance is observed in the succeeding 3 days following birth.

At day 5 postnatally, the air spaces are more expanded and seem to be more irregular in shape than observed earlier. The septa are irregular in thickness; some areas are quite thin while other areas are considerably thicker. One week after birth the lung develops some interesting changes on the route to maturation. The smooth walled primary septa show thickenings or buds, which are called secondary crests (fig. 7). These secondary crests or septa grow and divide the primary air saccules into alveolar sacs and eventually give rise to alveoli. The primary septa are still thick in most regions and obviously still composed of a double capillary network. By day 10 the septation process has shown considerable progress. The secondary septa exhibit substantial growth and subdivision to produce structures which could be considered alveoli. The double capillary network system is still in prominence in the septal walls resulting in a relatively thickened appearance (fig. 8). Similarly the morphology of the lung 2 weeks after birth has not changed to any great extent and appears much like the 10 day postnatal lung.

Three weeks after birth the lung appears much more mature with the most obvious change being the thinning of the primary and secondary septal walls resulting in a thinner air blood barrier (fig. 9).

The primary air saccules have almost all been replaced by alveolar ducts, alveolar sacs and alveoli to produce much more mature lung. There was little morphologic difference between rats examined subsequently and normal adult lung.

Figures 1 - 9. Methacrylate Sections Stained with Silver Methanamine.

Figure 1. Control lung (upper) and starved (lower) at 10 days postpartum showing size differences, x10.

Figure 2. Day 17 prenatal control, air tubules (T) lined by cuboidal epithelium with several mitotic figures (M), x400.

Figure 3. Day 20 prenatal control, lung has more alveolar tubules (T) with wider lumens, x400.

Figure 4. Day 21 prenatal control, terminal air sacs (TAS) are distended causing blood vessels (arrows) to come into closer contact with cuboidal epithelium, x400.

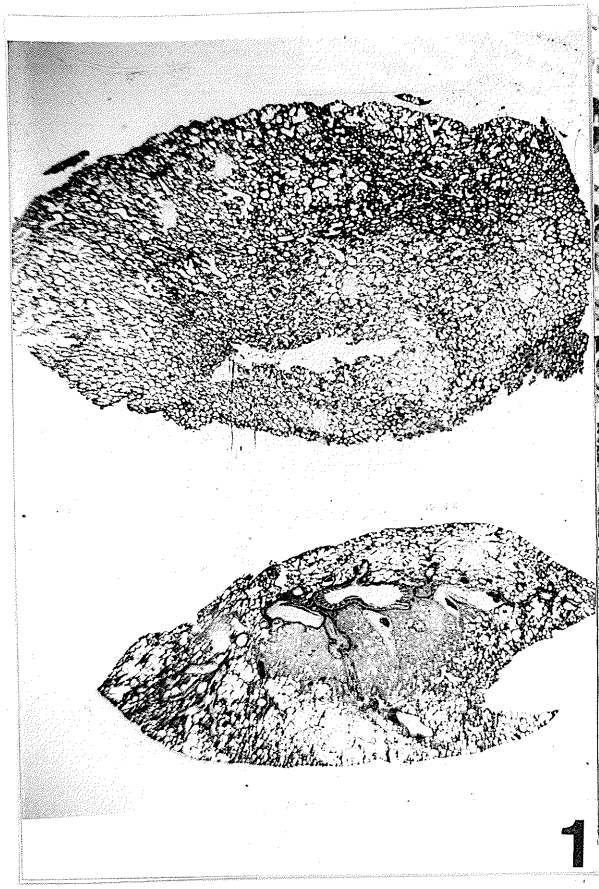
Figure 5. Day 22 prenatal control, the lung has widely distended terminal air saccules, x400.

Figure 6. Day 1 postnatal control, smooth walled saccules (S) with thick primary septa (PS), x400.

Figure 7. Day 5 postnatal control, small secondary crests (SC) can be seen in the walls of saccules (S), x350.

Figure 8. Day 10 postnatal control, the secondary crests (SC) show considerable growth, x400.

Figure 9. Day 21 postnatal control, a mature appearance is now seen, alveoli (A) have thin septal walls, x400.



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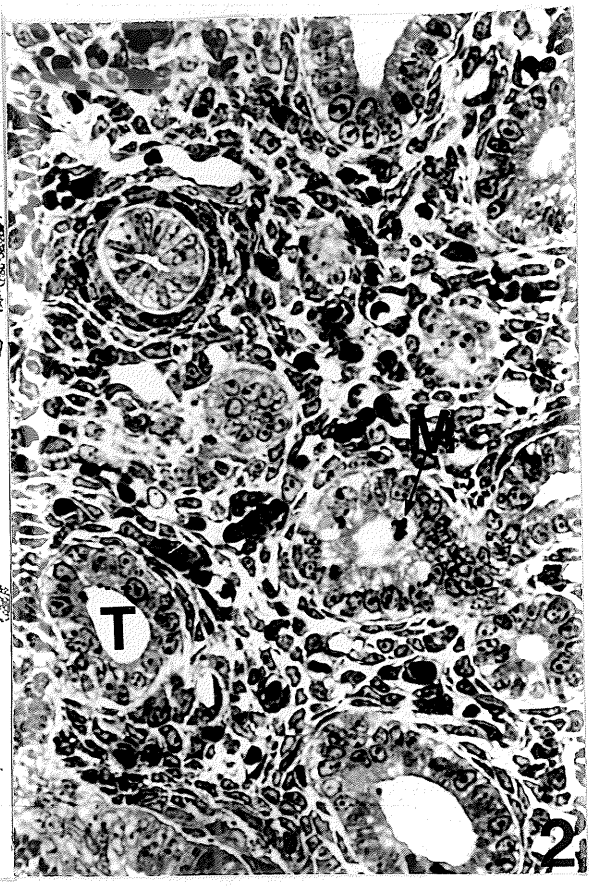

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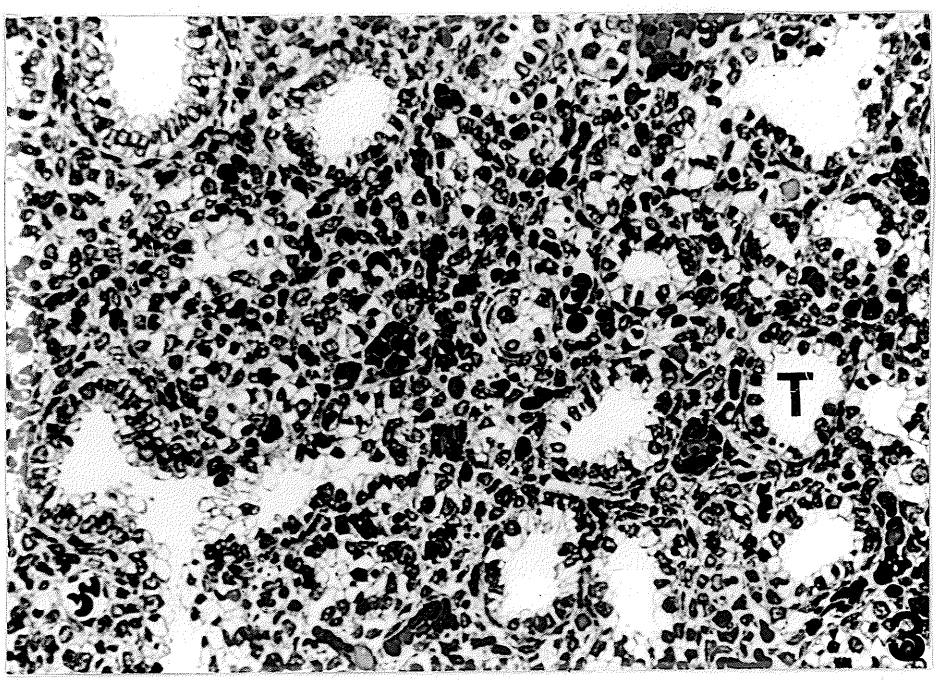

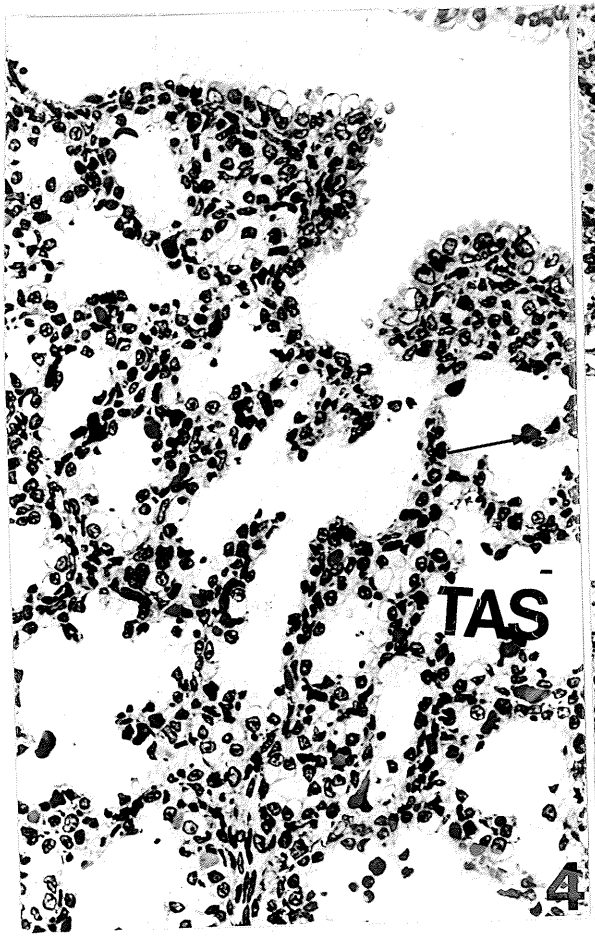
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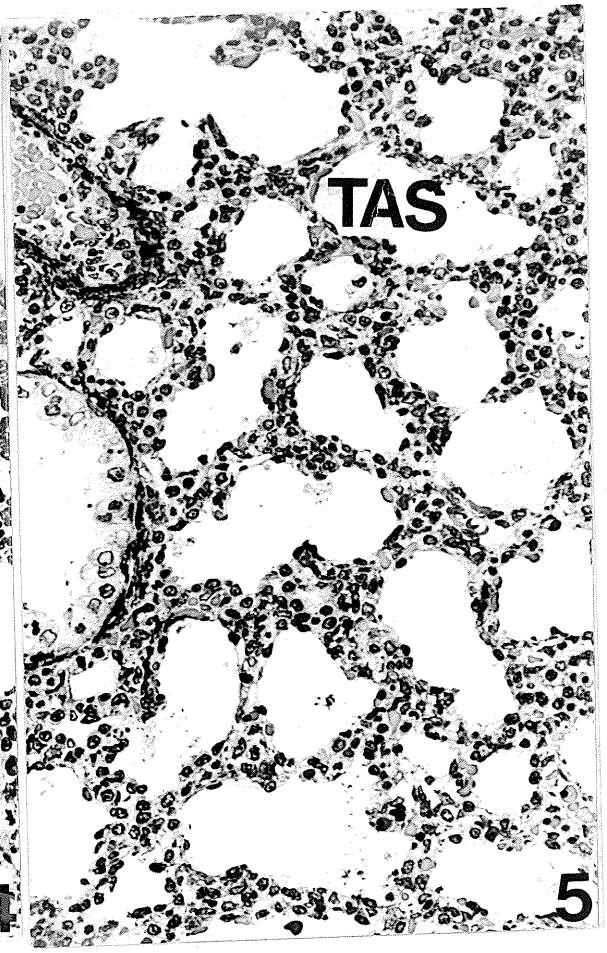
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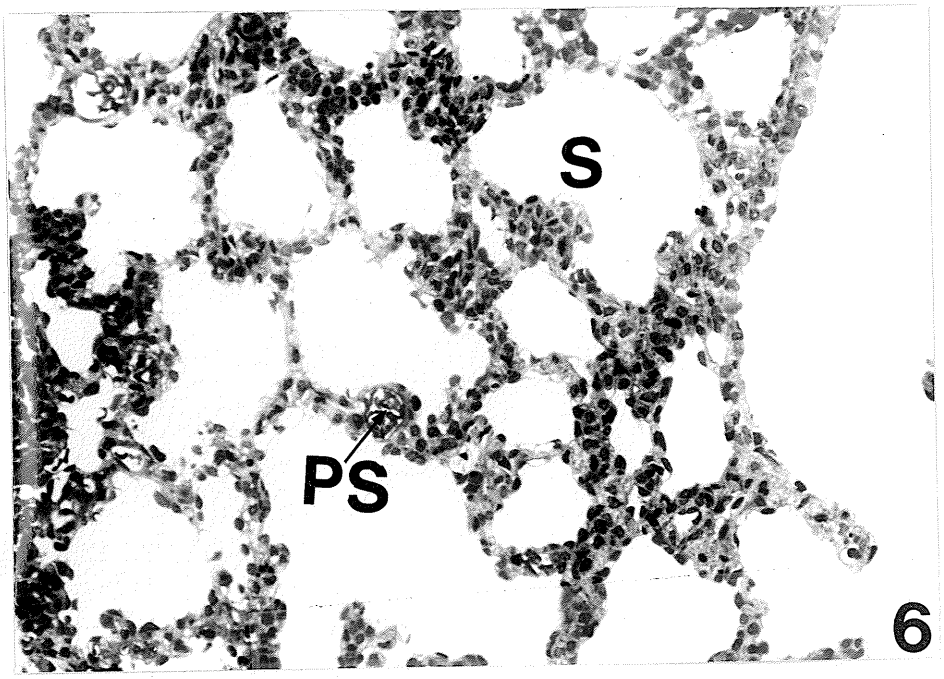
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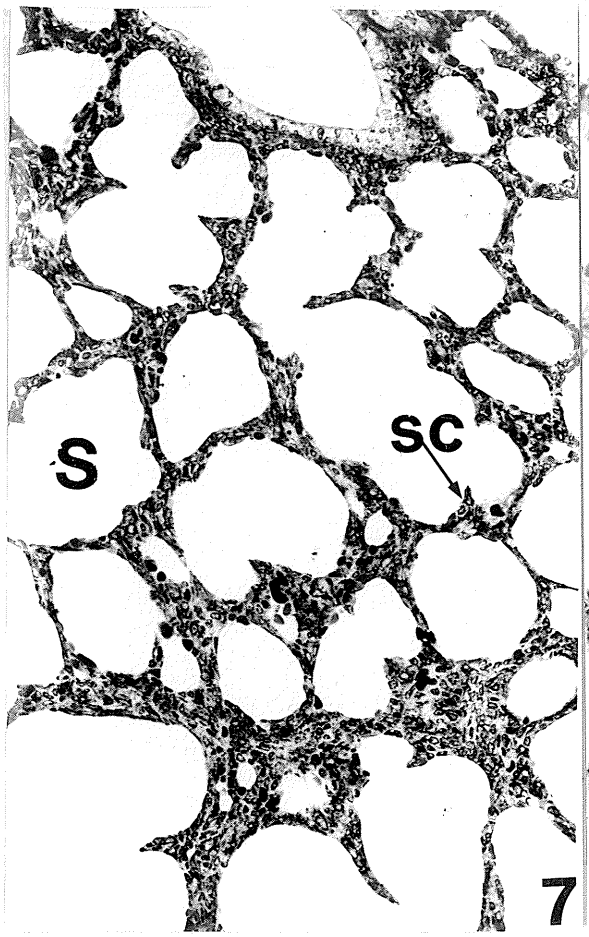
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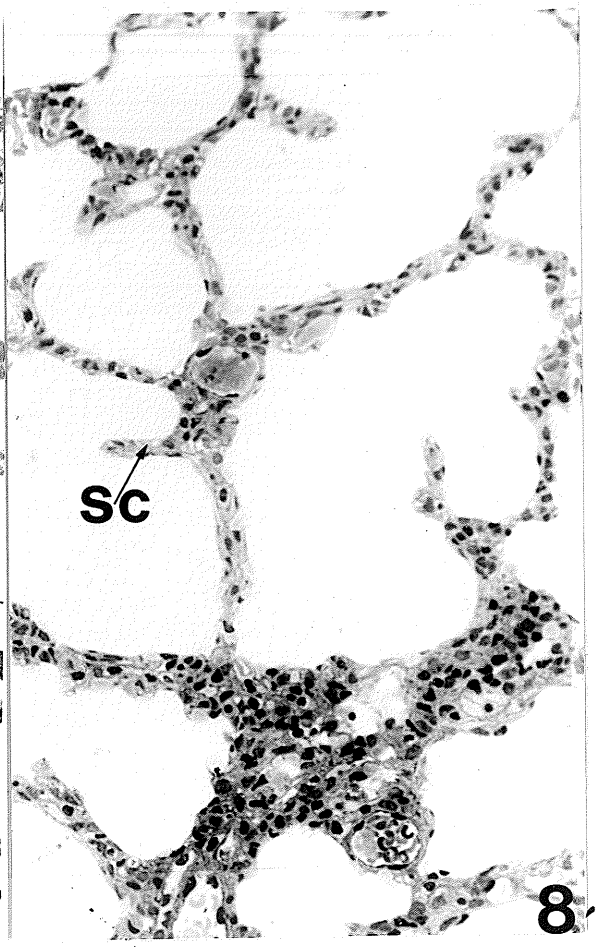
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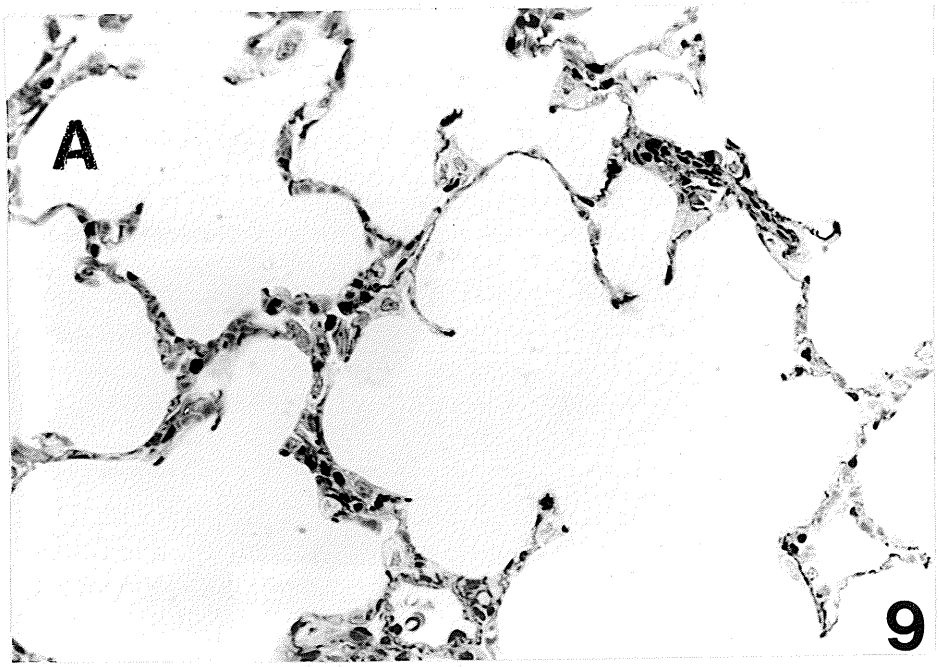
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B. Morphology of the Developing Lung--Starvation

FETAL On prenatal day 17, the lungs of fetuses whose maternal parent had been deprived of all solid nutrients from day 13 of gestation, did not show any noticeable morphologic changes from the control from day 17 of gestation. The lungs showed the identical pattern of tubules lined by cuboidal epithelial cells, with distended pale staining cytoplasm, lying in a loosely arranged mesenchyme. The epithelial cells show many mitotic figures and the mesenchyme contains some developing vascular elements (fig. 10). At days 18 and 19 the lung retains its tubular appearance and is quite similar to control lungs of the same age.

On prenatal day 20 the lumens of the "glandular" tubules expand to give a more distended appearance to the lung (fig. 11). The cytoplasm of the epithelial lining cells is vacuolated indicating large quantities of glycogen in the cytoplasm, as will be demonstrated later. On day 21 of gestation the terminal saccules in the starved lung do not appear to be as well expanded as they do in the control (fig. 12). The septum in malnourished lung appears to be thicker due to increased cellularity. The majority of the epithelial cells are still cuboidal in shape complete with pale staining cytoplasm. One day before birth the lungs of the malnourished fetuses do not appear to be as well developed as their counterparts in the control series. The lungs do not appear as expanded as in the controls, with the lumens of the airways not as distended (fig. 13, compare to fig. 5).

As noted earlier in the control lungs at day 21 some septal walls are thinning out, but this is not apparent in the lung from malnourished fetuses where the septal walls are uniformly thickened and cellular. There would also appear to be some delay in cytodifferentiation of the cuboidal epithelial cells into attenuated type I and granular type II alveolar cells since most of the epithelial cells are still cuboidal in shape.

POSTNATAL One day postpartum the lungs show the presence of some expanded smooth walled saccules with the primary septa being very thick and cellular (fig. 14); the lung is not as expanded as it was in the control situation (fig. 6). The total morphological picture at parturition would indicate that the starved lung is not as advanced in development as the control lung. There is not much progress in development of the lung during the few days following birth with only slight changes observed, such as a tendency toward greater expansion of the air saccules. At day 5, the primary saccules begin to distend with the flattening of the cuboidal epithelium and the subsequent differentiation into type I and type II epithelium (fig. 15). The septal walls become thinner and less cellular than those observed the previous 4 days.

One week after birth the starved lung still appears to be lagging behind the control lung in total development (fig. 16). At this time the control lung shows considerable septation with secondary crests subdividing the primary saccules (fig. 9). The formation of alveoli in

starved animals still appears to be retarded at this point. However, by day 10 the growth and development of alveoli has progressed rapidly with secondary crests subdividing saccules into alveolar sacs. The lung still morphologically does not appear to be as mature as the control lung (fig. 17). Over a period of a few days, the process of septation proceeds rapidly with formation of alveoli with the thinning of the septal walls so that it appears that there is no significant differences morphologically between the control series and the starved series at 14 days postpartum.

Figures 10 - 17. Methacrylate Sections Silver Methanamine Stain.

Figure 10. Day 17 prenatal starved, lung is similar to control showing air tubules (T) with mitotic figures (M), x400.

Figure 11. Day 20 prenatal starved, tubules are more distended, x400.

Figure 12. Day 21 prenatal starved, a very thick cellular septum with many large pale cuboidal cells (arrows) seen lining the terminal air saccules (TAS), x400.

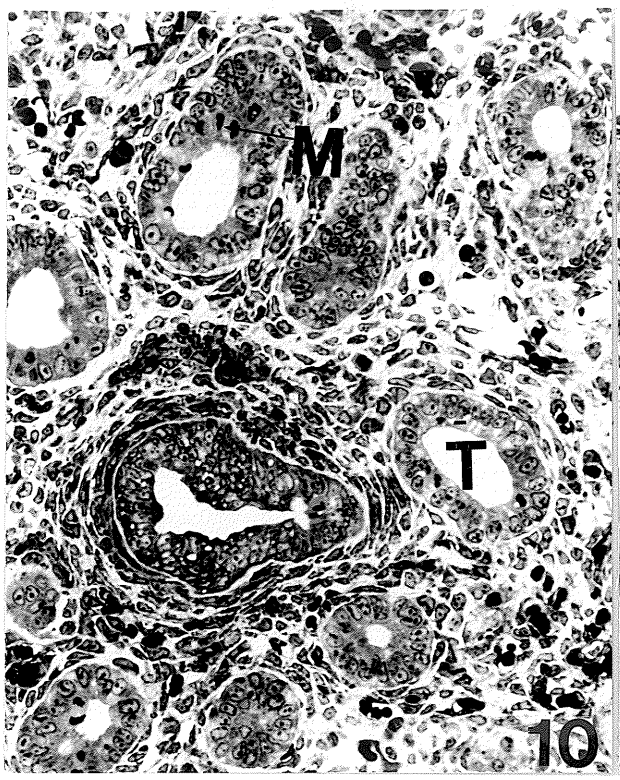
Figure 13. Day 22 prenatal starved, terminal air sacs (TAS) do not appear to be as expanded as in control, x350.

Figure 14. Day 1 postnatal starved, primary septa (PS) are thick and irregular with less expansion of saccules as compared to controls, x400.

Figure 15. Day 5 postnatal starved, considerable extension of the primary saccules (S) with thinner septal walls, x400.

Figure 16. Day 7 postnatal starved, a few secondary crests (SC) are seen developing, x400.

Figure 17. Day 14 postnatal starved, many more secondary crests (SC) are developing and alveoli (A) are rapidly forming, x400.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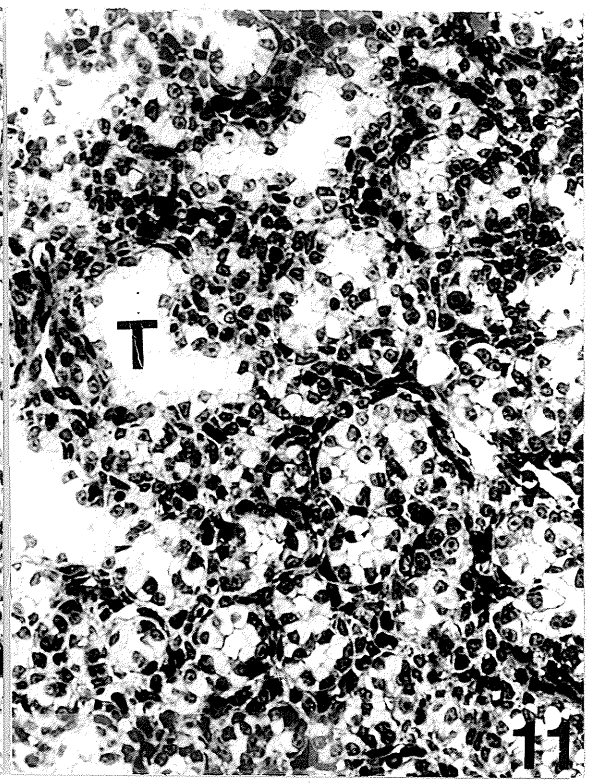
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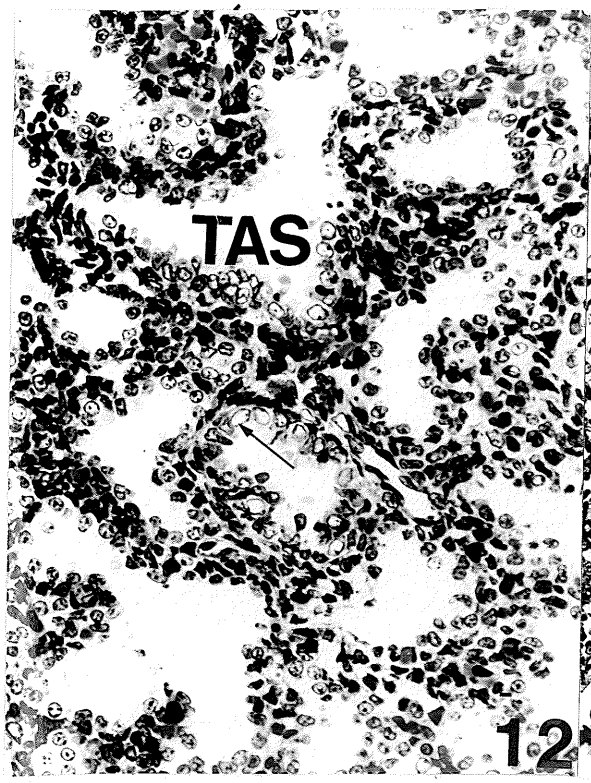
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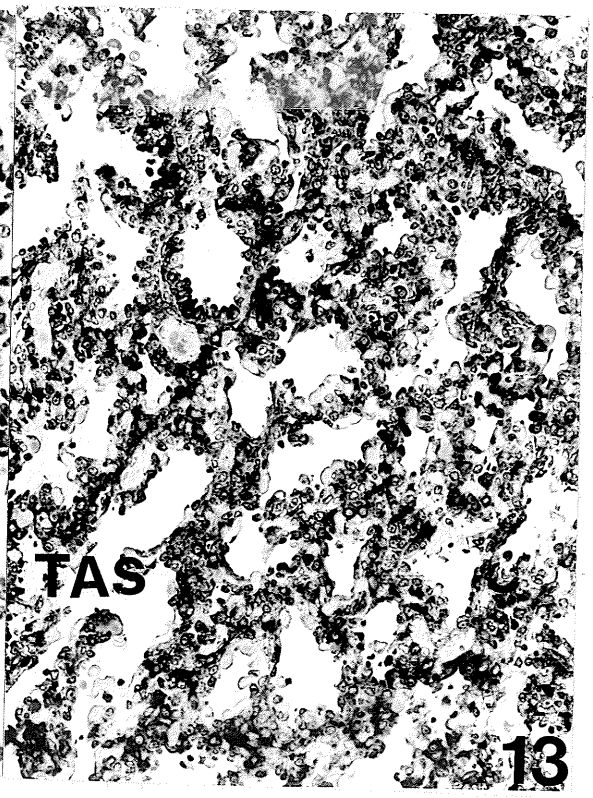
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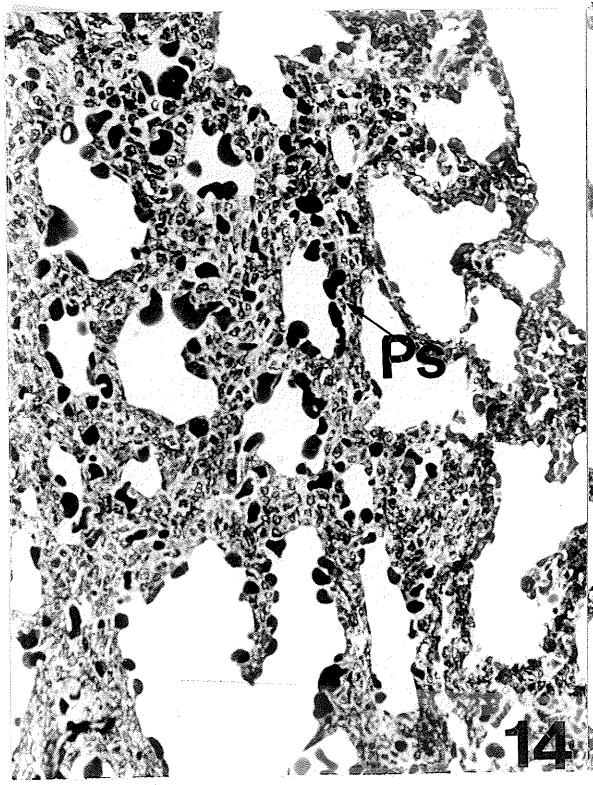
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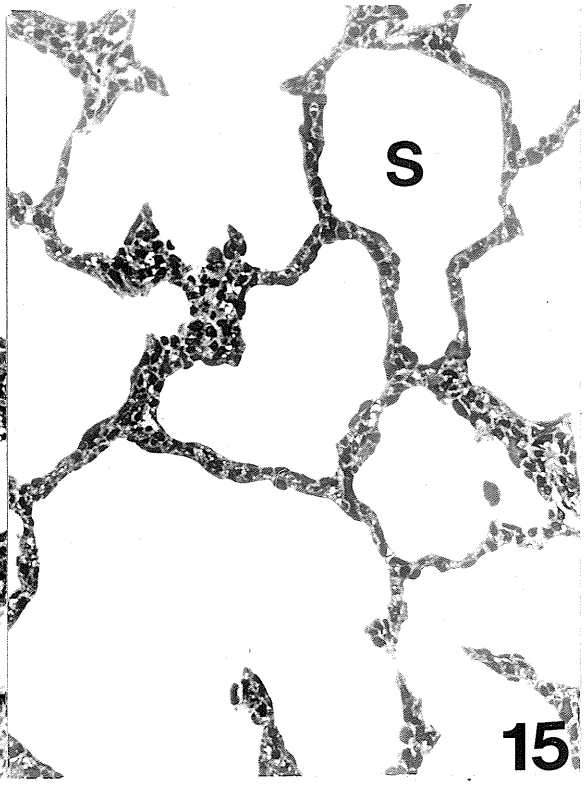
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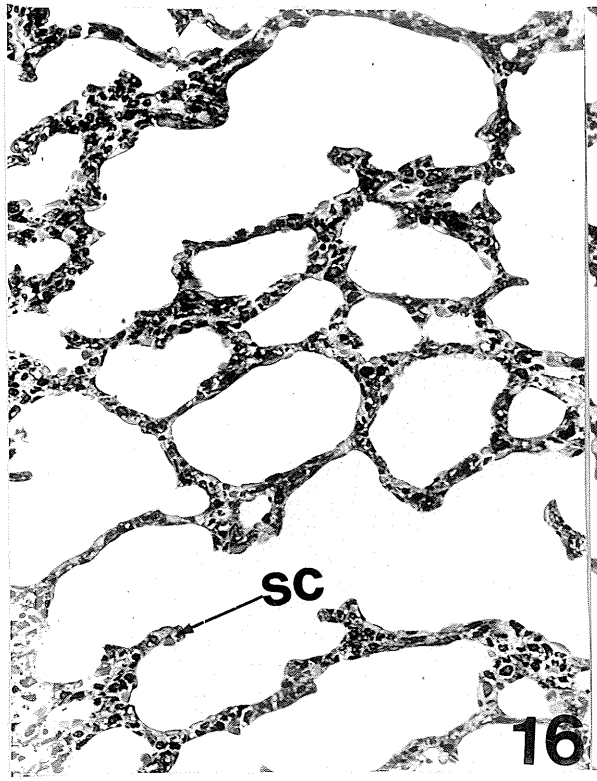
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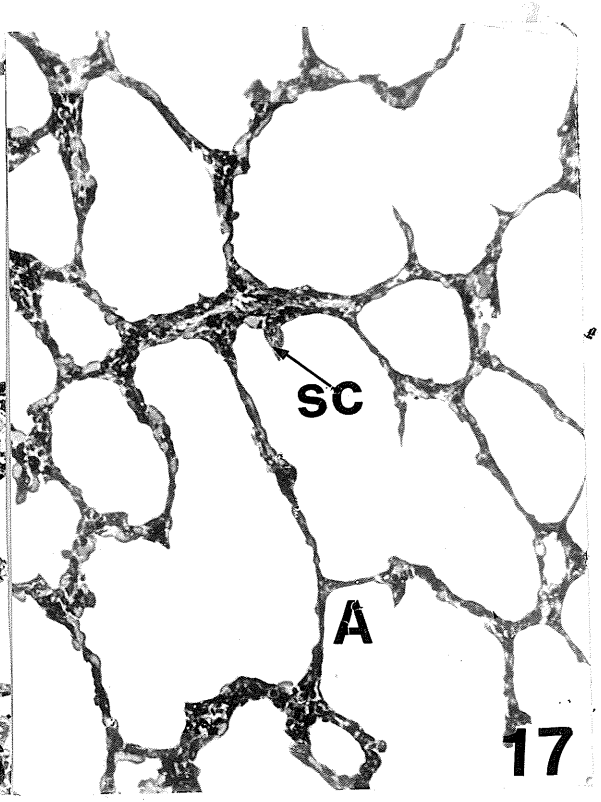
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C. Glycogen--Control

FETAL At the beginning of study on glycogen content, day 17 of gestation, the fetal rat lung consists of branches of the bronchial tree lined by a single layered cuboidal epithelium (fig. 2). On P.A.S. stained lung sections, these cuboidal cells are shown to contain large amounts of cytoplasmic glycogen (fig. 18). On day 18 there appears to be an increase in the number of tubules in the mesenchyme but no detectable change in the glycogen content of the cuboidal cells and several of the branching tubules remain solid with no obvious lumens (fig. 19). At day 19 a few of the epithelial cells appear to be flattening out, although the majority of the cells remain cuboidal with their cytoplasm distended with glycogen. As the cells attenuate they appear to lose their copious amounts of cytoplasm glycogen, and while most epithelial are positive, the mesenchyme of the lung has remained conspicuously negative (fig. 20).

Further expansion of the airways at day 20 is accompanied by a further increase in the attenuation and cytodifferentiation of the cuboidal cells. This early differentiation coincides with a noticeable decrease in the cytoplasmic glycogen content of the maturing epithelial cells (fig. 21). The mesenchymal cells still do not show any evidence of significant glycogen content at this point. The morphologic changes seen on day 21 are quite marked as noted earlier with increased cytodifferentiation of the epithelial cells and consequently a decreasing cuboidal epithelial cell population and a decrease in their glycogen

content. A few areas in the mesenchyme now appear to contain some glycogen (fig. 22). Just prior to birth on day 22, the airways become noticeably expanded, the epithelial cells having undergone considerable differentiation with a sharp decline in the amount of epithelial glycogen. Mesenchymal glycogen is accumulating so as to produce a noticeable shift of the glycogen distribution from the differentiating epithelial cells to the mesenchymal cells (fig. 23).

POSTNATAL With the onset of respiration there is still a further expansion of the lung tissue with the primary saccules showing considerable distention, at day 1 postpartum (fig. 24). The accumulation of glycogen within the mesenchymal cells and its almost complete absence from the epithelial cells appears to be practically complete by 1 day postpartum and remains largely unchanged during the first 10 days after birth when there is extensive accumulations of glycogen in the mesenchyme (fig. 25). Little change is seen in the glycogen pattern during the third and fourth weeks after birth with considerable mesenchymal glycogen present. Glycogen gradually disappears from lung tissue as it matures until it is not significant in the adult lung.

D. Glycogen--Starvation

FETAL The lungs of fetuses from malnourished rats showed the presence of glycogen in the cuboidal epithelial cells lining the tubules. On day 17 and 18 of gestation, the glycogen was seen occupying the cytoplasm of the cuboidal epithelium similar in location and intensity to the control study (fig. 26). At day 19 of gestation the pattern of glycogen accumulation remains very similar to the control of the same intrauterine age with most of the glycogen seen in the cytoplasm of the cuboidal epithelium and a paucity of glycogen observed in the mesenchyme (fig. 27).

However, several changes were observed from day 20 to birth in the lungs of fetuses from nutritionally deprived rats. The epithelial cells of the fetuses deprived of nutrition showed the presence of glycogen in the cytoplasm (fig. 28) without the marked decrease in quantity observed in the control study. This indicates that glycogen utilization may have been impaired, causing the epithelial cells to retain more of the glycogen than normal. Although some cytodifferentiation occurs, most epithelial cells are cuboidal and are still laden with glycogen at days 21 and 22 of gestation (fig. 29). Mesenchymal glycogen is not prominent at this stage of development in the malnourished rat lung.

POSTNATAL One day postpartum reveals a relatively large number of cuboidal cells still retaining glycogen in their cytoplasm

(fig. 30). This was not a conspicuous feature of the one day old control lung which had lost almost all of the epithelial glycogen by this time (fig. 24) and, although the number of epithelial cells with glycogen greatly declines, it is still a prominent feature of the postnatal lung (fig. 31). Glycogen can still be detected in the epithelial cells at 3 days after parturition. A gradual decline of glycogen occurs over the following week to 10 days after birth, when elimination of epithelial glycogen was completed. Mesenchymal accumulations of glycogen does not appear in the postnatal lung until after day 3 postpartum, but by day 10 it accumulated similar to control lungs of the same age (fig. 32).

Figures 18 - 32. Methacrylate Sections Stained with P.A.S. to Show Glycogen.

Figure 18. Day 17 prenatal control, cytoplasm of cuboidal epithelium of tubules (T) are filled with "dense" glycogen, x400.

Figure 19. Day 18 prenatal control, an increased number of tubules (T) are seen, cytoplasm also filled with glycogen, x400.

Figure 20. Day 19 prenatal control, the tubules (T) are expanded with most cuboidal epithelial cells filled with glycogen, x400.

Figure 21. Day 20 prenatal control, expanded tubules (T), increased cytodifferentiation of cuboidal epithelium resulted in a decrease in cytoplasmic glycogen, x400.

Figure 22. Day 21 prenatal control, a marked decrease in epithelial glycogen content seen, with a few mesenchymal cells, now appearing positive, x400.

Figure 23. Day 22 prenatal control, the cuboidal epithelium has lost most of its glycogen; mesenchymal glycogen now accumulating, x400.

Figure 24. Day 1 postnatal control, expansion of the primary saccules (S) with no glycogen in the epithelial cells but glycogen present in the mesenchyme (Me), x400.

Figure 25. Day 10 postnatal control, glycogen is found exclusively in the mesenchymal cells, x350.

Figure 26. Day 18 prenatal starved, similar to fig. 18, the tubular (T) epithelium is filled with glycogen, x400.

Figure 27. Day 19 prenatal starved, the pattern of glycogen distribution is the same as in the control, x400.

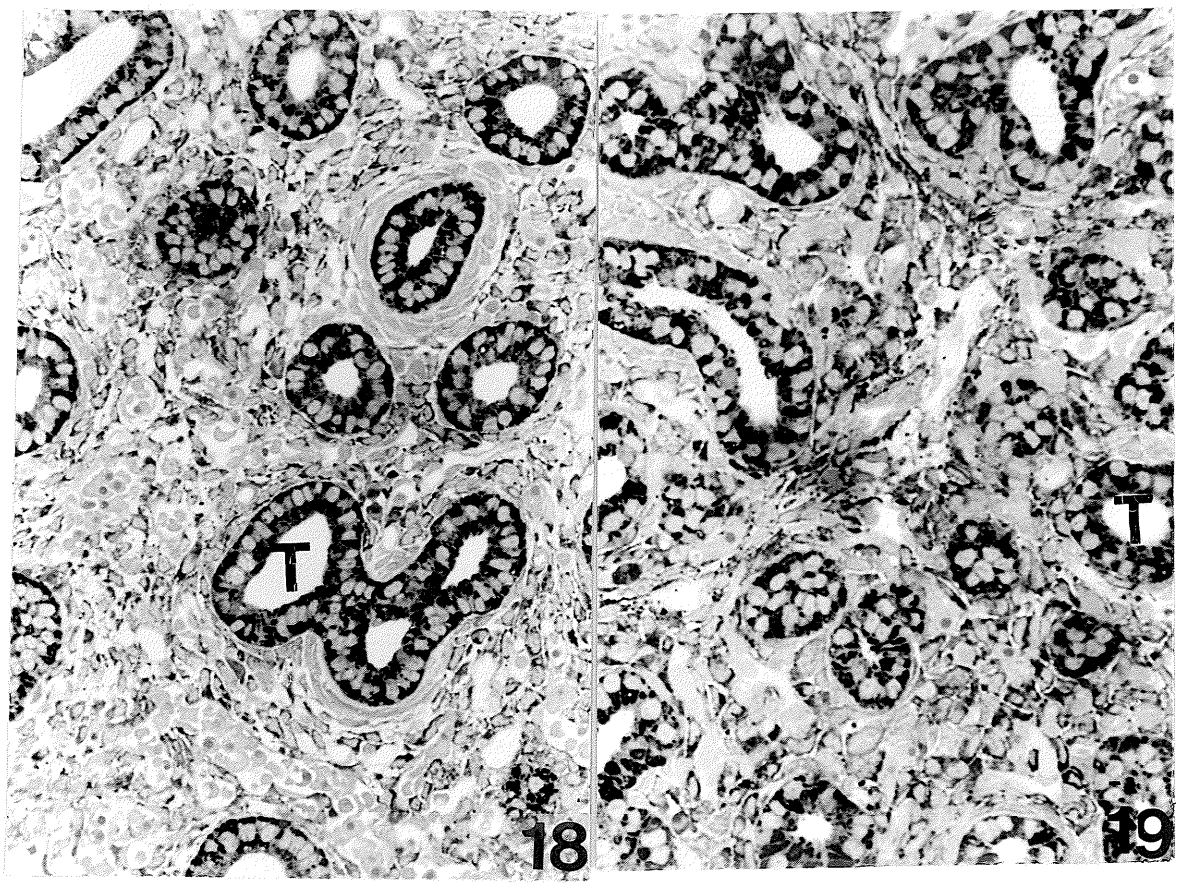
Figure 28. Day 20 prenatal starved, glycogen has not disappeared as rapidly as controls and is present in the tubular epithelium, x400.

Figure 29. Day 22 prenatal starved, many epithelial cells still cuboidal and still contain abundant glycogen, x400.

Figure 30. Day 1 postnatal starved, the lung still has many large cuboidal epithelial cells with conspicuous glycogen after birth, x400.

Figure 31. Day 3 postnatal starved, several epithelial cells still contain glycogen, little mesenchymal glycogen is observed, x400.

Figure 32. Day 10 postnatal starved, epithelial cells show no glycogen, glycogen now contained in mesenchymal cells, x400.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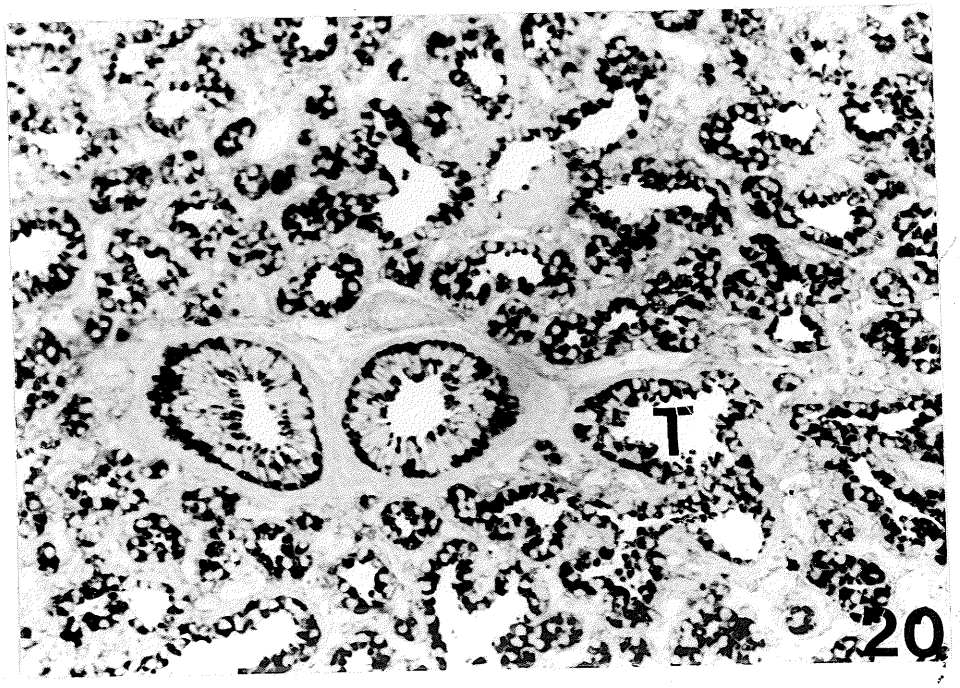
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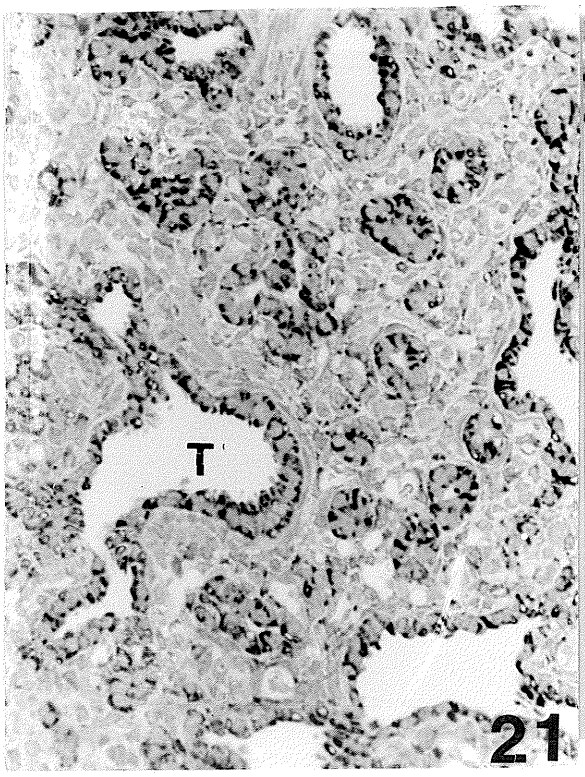
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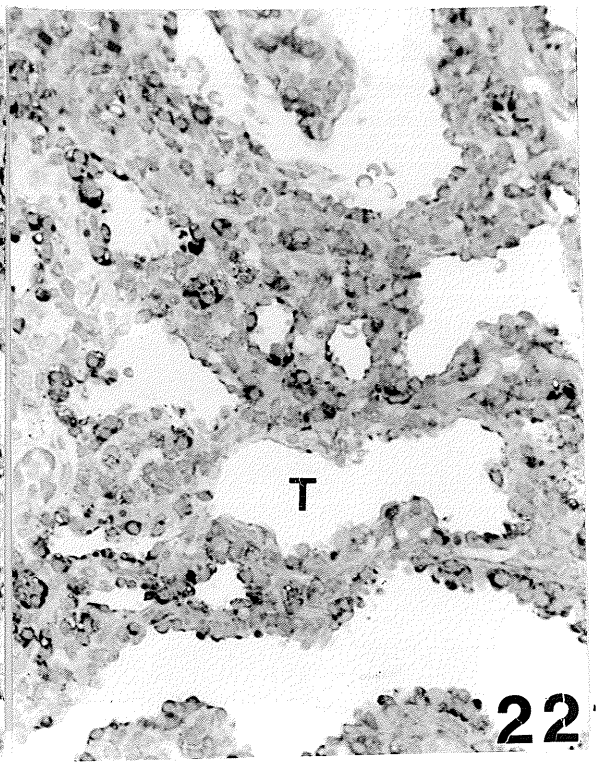
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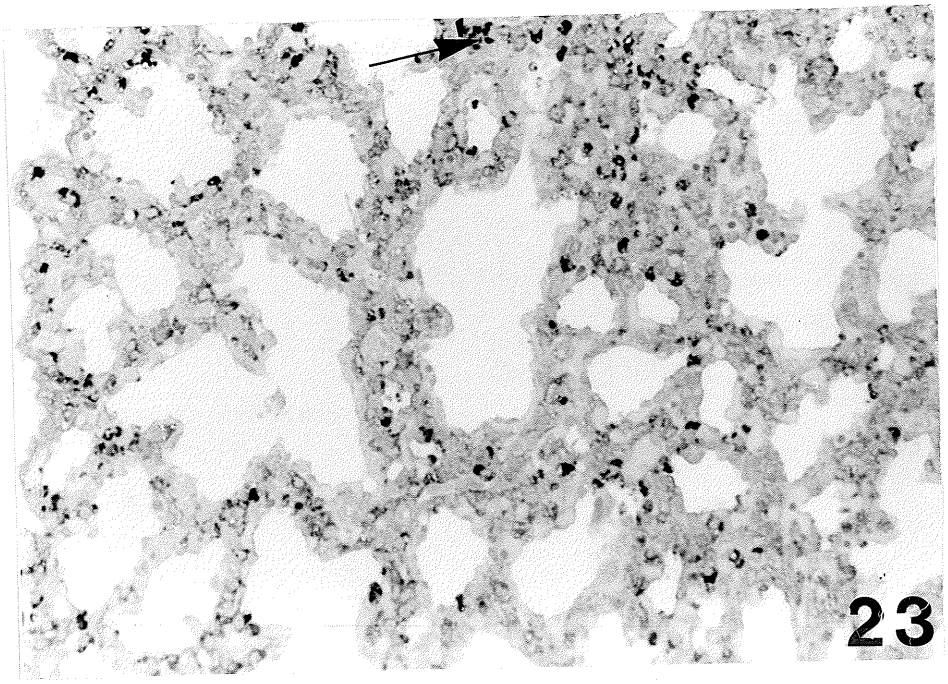
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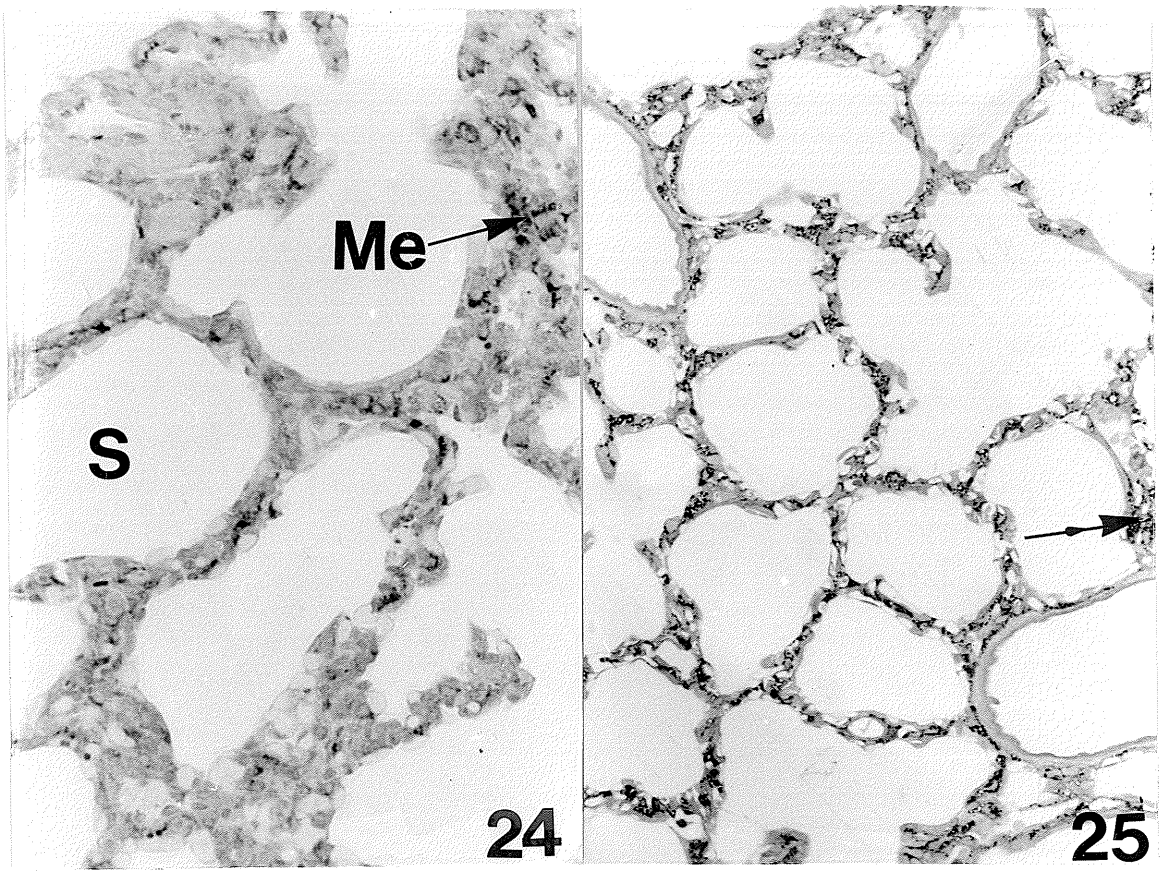
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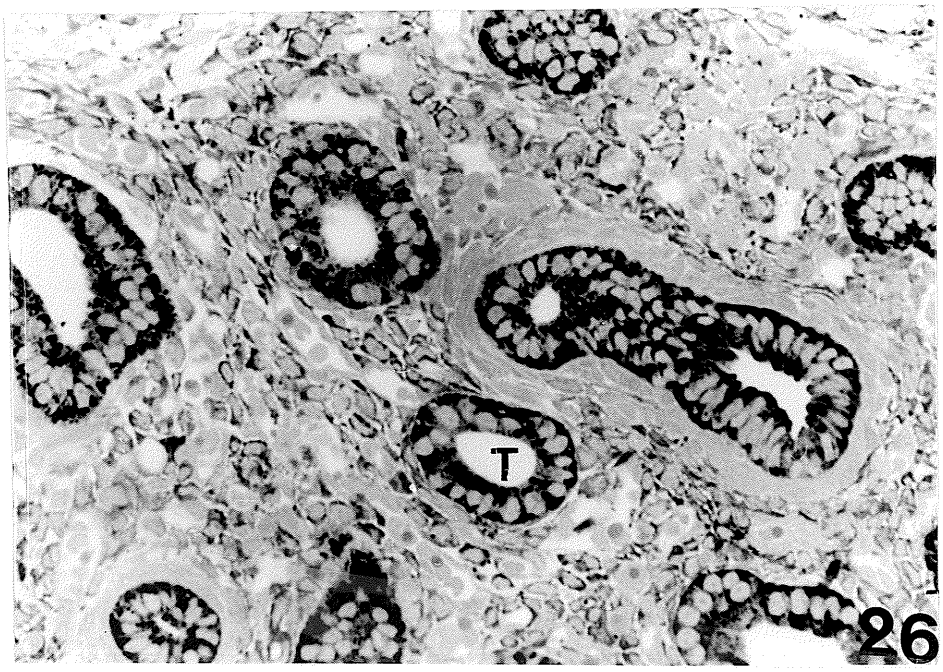
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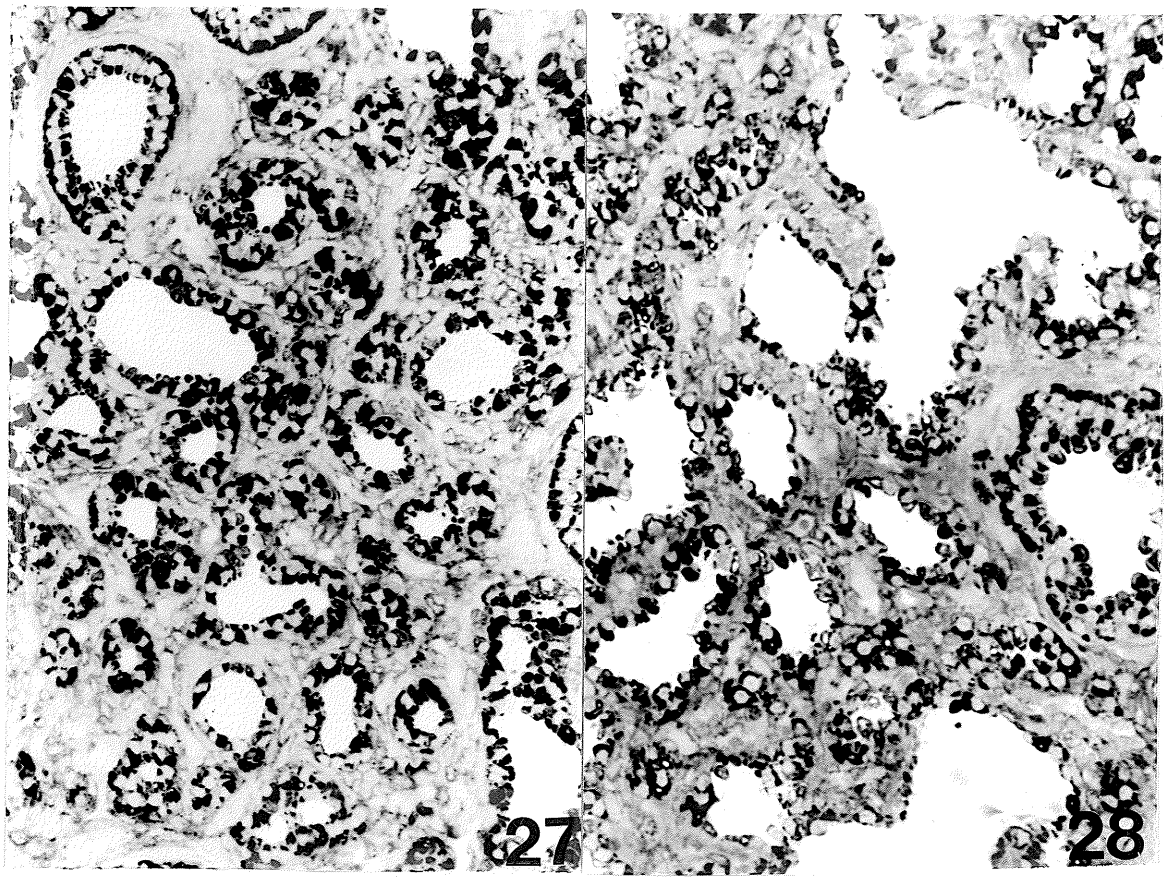
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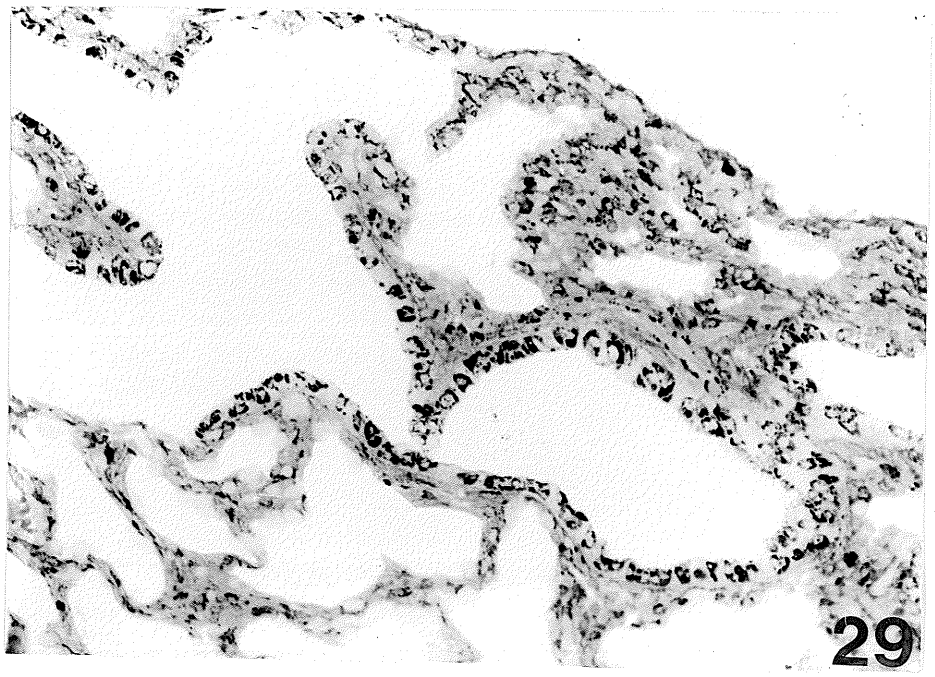
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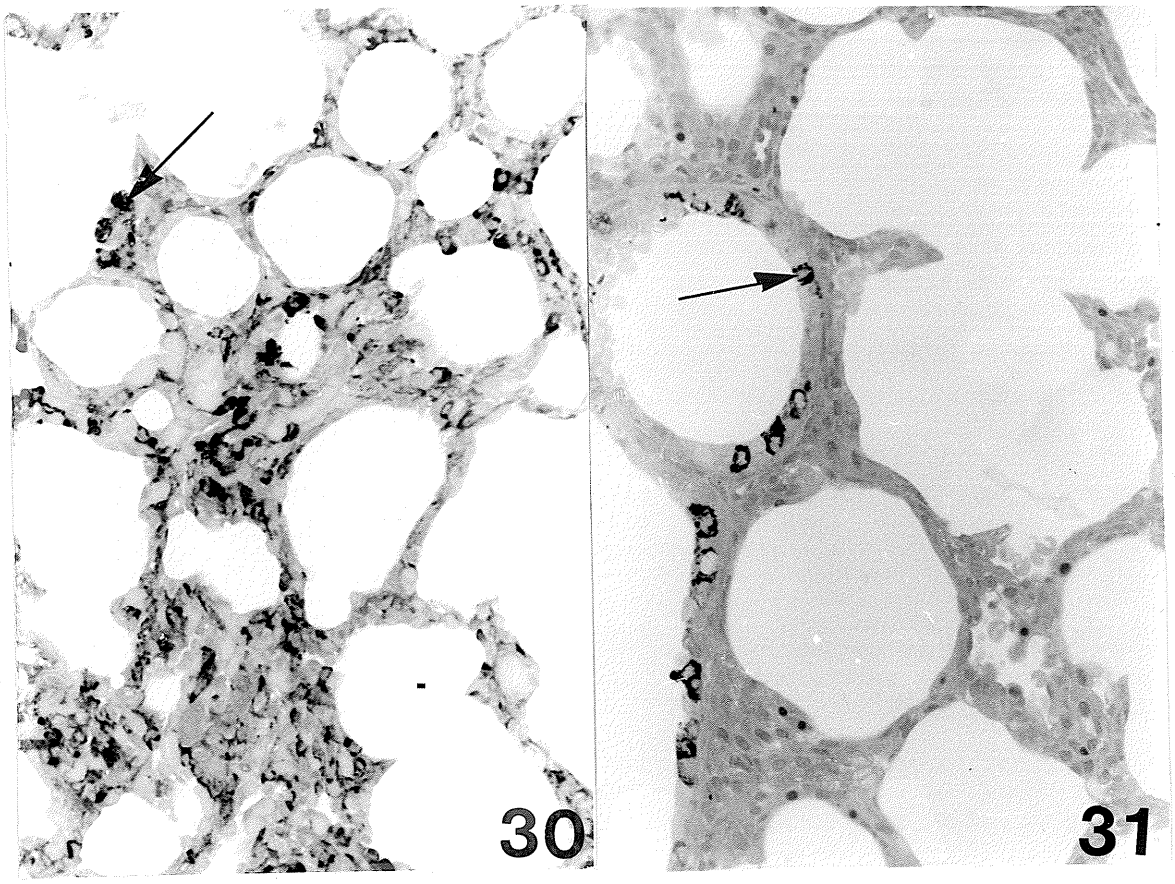
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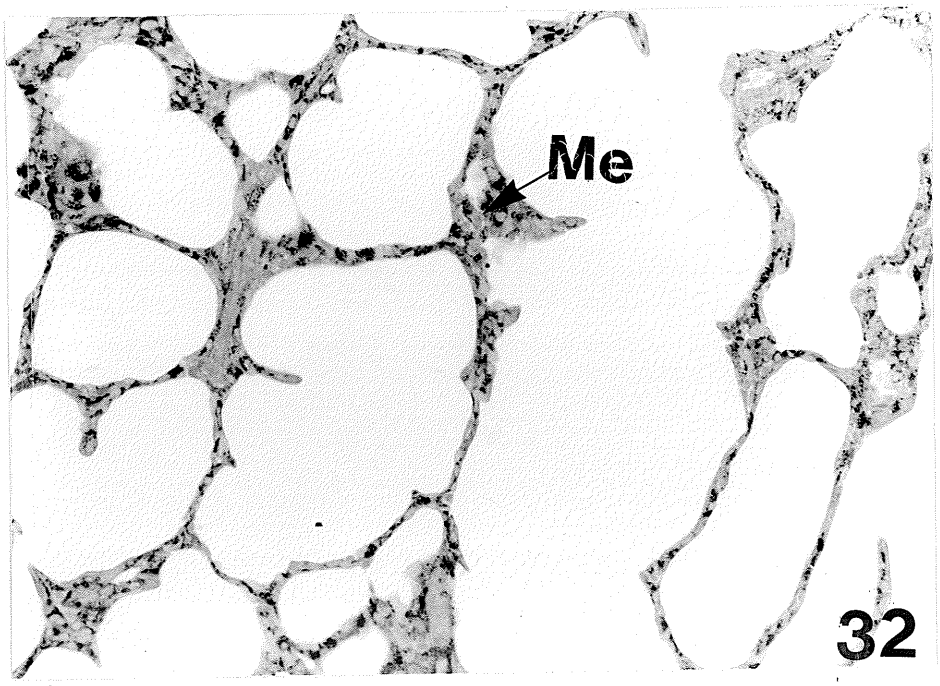
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E. Acid Phosphatase--Control

FETAL Acid phosphatase activity is first observed in prenatal lungs at approximately the 18th day of gestation and appears to be limited to the cuboidal epithelium lining the prominent respiratory tubules (fig. 33). On day 19 the distribution and reactivity is not changed, but there is a slight increase in the number of positively reacting tubules resulting in an overall net increase in the number of positive cells. Two days prior to parturition, the lung has a more expanded appearance, with many tubules occupying the shrinking mesenchyme. An increase in positive cells is observed probably due to vastly increased numbers of respiratory tubules. Enzymatic activity is confined largely to the cuboidal epithelium of the tubules on day 21; the tubules are expanded and many strongly positive cells are observed (fig. 34). On the last day of gestation, there is an apparent increase in the total number of positive cells with enzymatic reactivity located mostly in the epithelial cells, though a few non-epithelial cells also show some enzymatic activity.

POSTNATAL Acid phosphatase activity observed shortly after birth is not significantly altered from that seen at day 22 of gestation and the location of reactivity remains essentially epithelial in nature (fig. 35). On the second day changes in distribution of positive cells, in the actual type of positive cells, are observed. For example, at 5 days postpartum, the first reactive macrophages are observed in the lung, although the majority of the positive cells observed still appears

to be epithelial (fig. 36).

At day 7 postnatally, an increase in the number of reactive macrophages is observed although the total number of positive cells remains approximately constant. This trend appears to continue and at day 10 postpartum a noticeable increase in the number of positive macrophages is observed, as well as an intensification of the reactivity (fig. 37). The lung two weeks after birth is more mature with the development of alveoli. Acid phosphatase activity can now be seen in type II epithelium and in alveolar macrophages (fig. 38). A similar appearance is observed after the 3rd and 4th weeks of life; the lung interstitium is much thinner allowing more precise identification of the cell types. The total number of positive cells appears to remain constant and divided approximately equally between the type II alveolar cells and the alveolar macrophages.

F. Acid Phosphatase--Starvation

FETAL Acid phosphatase activity is greatly reduced in lungs of fetuses obtained from maternal rats on starvation diet. On day 18 of gestation, a weak reaction is seen in the cuboidal epithelium lining the respiratory tubules (fig. 39). The reaction product is much less dense in comparison to the control lung, but is still located in the same type of cell. It also appears that the total number of positive cells is reduced from the control.

There is a very gradual development of enzymatic reactivity in the late fetal stages of lung development. On each succeeding day of gestation to day 21, a slight increase in number and reactivity of positive cells is observed. The location of activity is identical to the control lungs, i.e. in the cuboidal epithelium lining the tubules (fig. 40). One day prior to parturition, the lung shows no significant increase in acid phosphatase development; it is obvious that acid phosphatase development is much slower in starved fetal rats than in the control.

POSTNATAL Shortly after birth, a slight increase in the total number of positive cells is observed along with a more intense reaction product in the positive epithelial cells. Activity gradually increases over the next few days, although the starved rat lungs still lag behind controls of the same time period. At day 5 postpartum, a sudden and sharp increase in the reactivity of acid phosphatase is seen in the developing lung. The total number of positive cells increases

dramatically with a more intense reaction product noted in the positive cells (fig. 41). The total amount of reactivity is still lower than in the control animals but the location and intensity of acid phosphatase content are similar, with positive epithelial cells and macrophages seen. Fromone week postpartum the increase in the total number of positive cells continues, indicating rapid enzymatic development which would appear to be increasing more rapidly in malnourished rats than it did in control rats from the same periods. At day 10 postpartum acid phosphatase levels in the starvation group of neonates are almost equal to that seen in the control group. Activity is observed in both type II alveolar cells and alveolar macrophages (fig. 42). The final examination made for acid phosphatase on the malnourished neonatal lung was performed on animals 2 weeks old. The acid phosphatase levels at this time appeared to be similar to control lungs. The number of positive cells and the ratio between epithelial and non-epithelial cells were similar for both groups (fig. 43).

Figures 33 - 43. Frozen Sections Showing Acid Phosphatase Activity, as Dense Black Deposits.

Figure 33. Day 18 prenatal control, the respiratory tubules (T) show an intense acid phosphatase reactivity in the epithelium, x400.

Figure 34. Day 21 prenatal control, increased number of respiratory tubules show an intense acid phosphatase reaction, x400.

Figure 35. Day 1 postnatal control, moderate acid phosphatase reactivity is seen in the epithelial cells (E) of the developing terminal air sacs (TAS), x400.

Figure 36. Day 5 postnatal control, reaction product is seen in epithelial cells (E) and in macrophages (M), x400.

Figure 37. Day 10 postnatal control, several darkly staining macrophages (M) are seen while epithelial cells (E) present do not appear to have as intense a reaction, x400.

Figure 38. Day 14 postnatal control, reaction product is seen in cuboidal epithelial cells (E) and macrophages (M), x400.

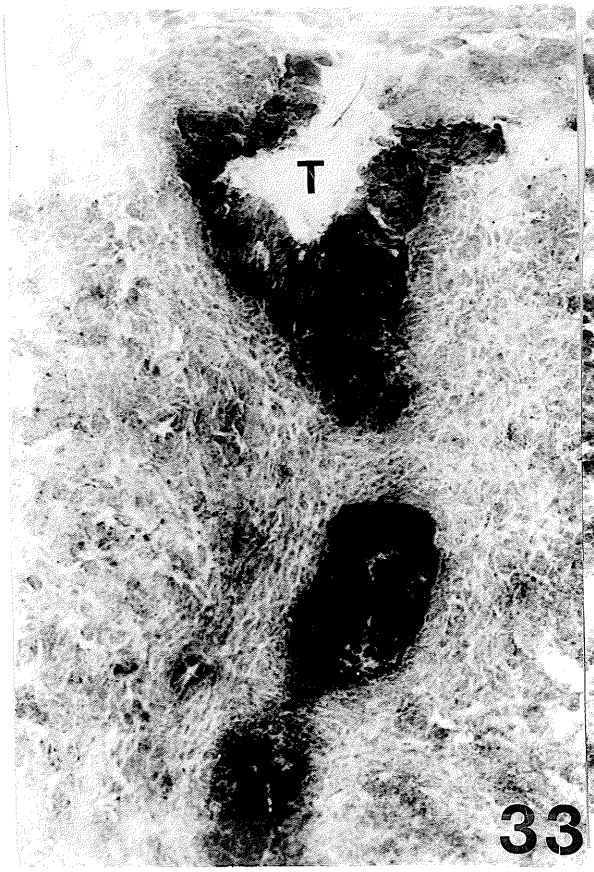
Figure 39. Day 18 prenatal starved, weak reaction product is seen in the respiratory tubule (T), x400.

Figure 40. Day 21 prenatal starved, weak reaction seen in the epithelial cells lining the tubules (T), x400.

Figure 41. Day 5 postnatal starved, increased number of reactive epithelial cells (E) with dense product, x400.

Figure 42. Day 10 postnatal starved, numerous intensely reacting alveolar epithelial cells (E) and macrophages (M) are seen, x400.

Figure 43. Day 14 postnatal starved, a more mature looking lung shows the presence of strongly reacting macrophages (M) and type II epithelial (E), x400.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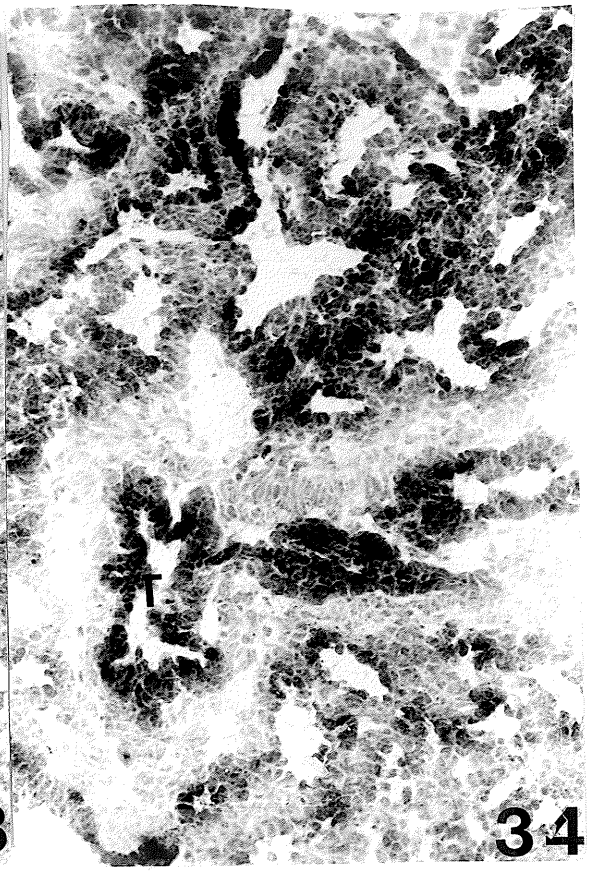
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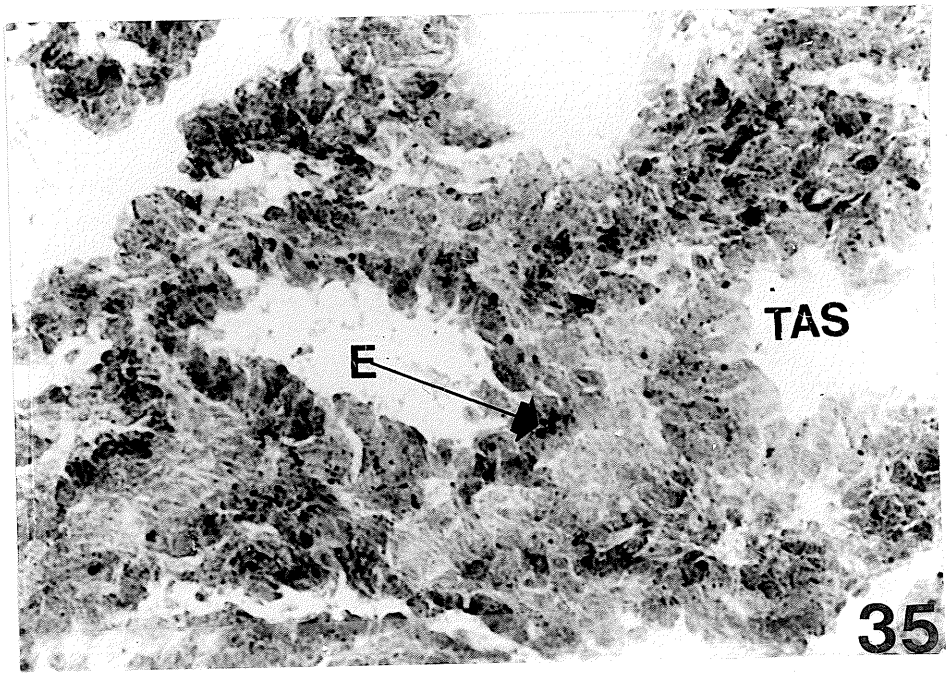
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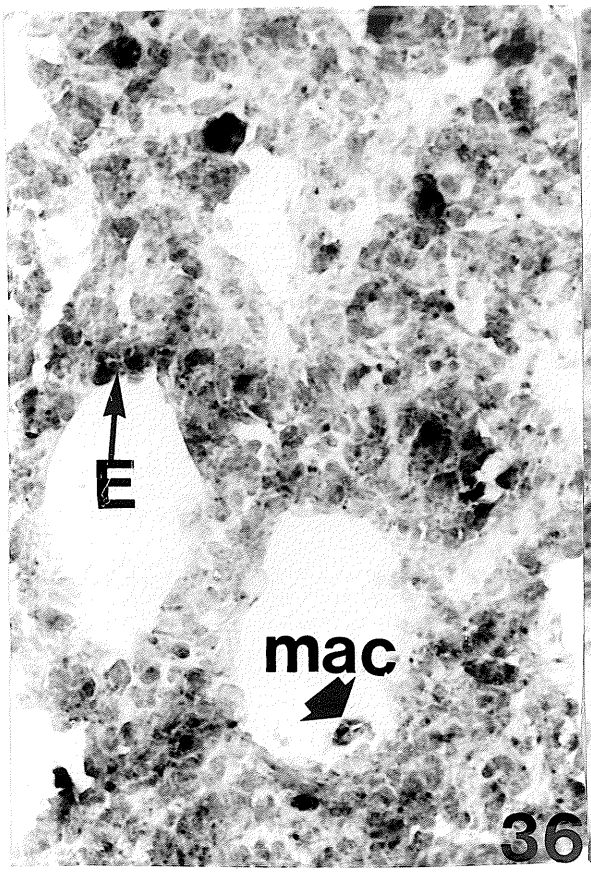
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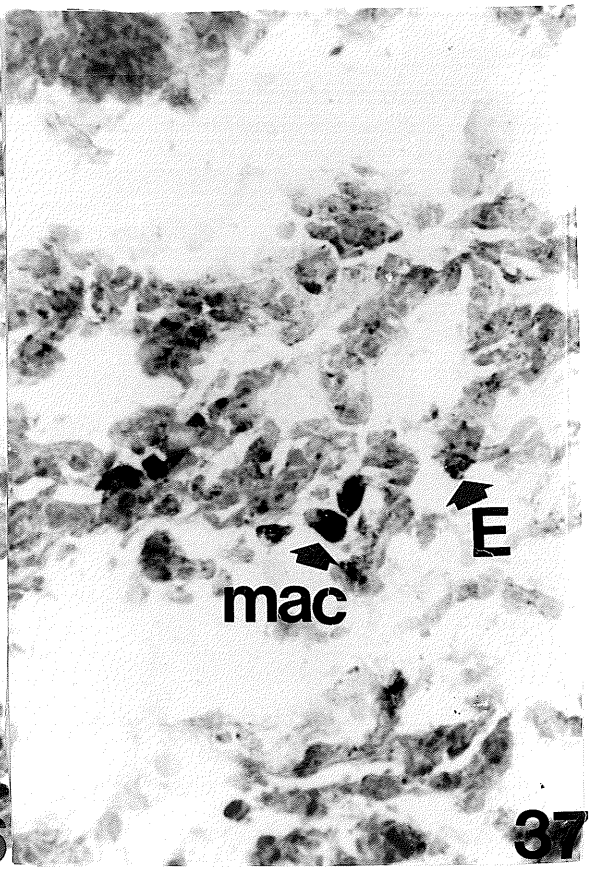
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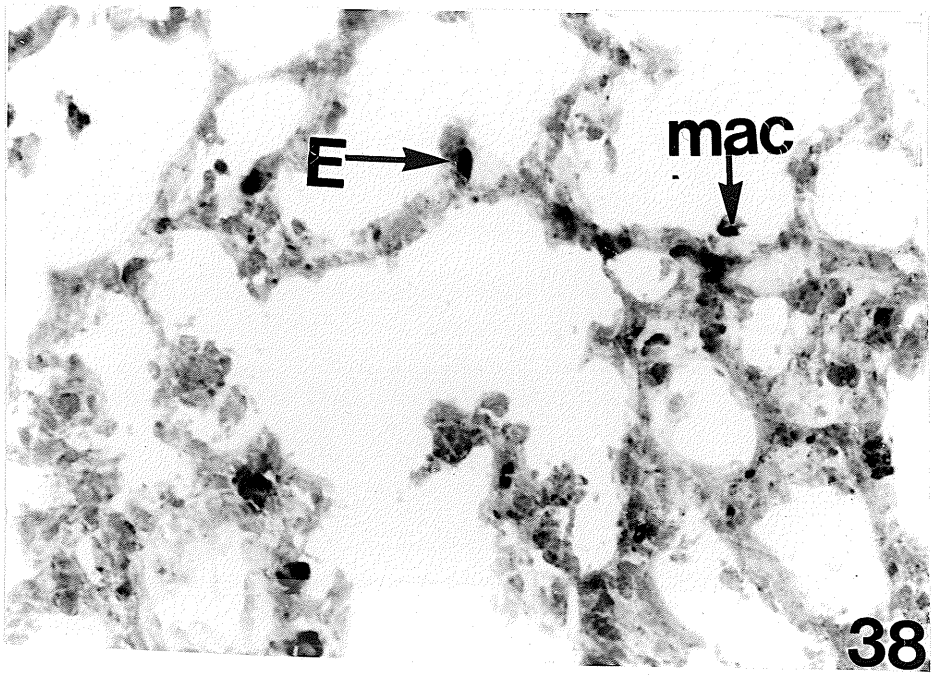
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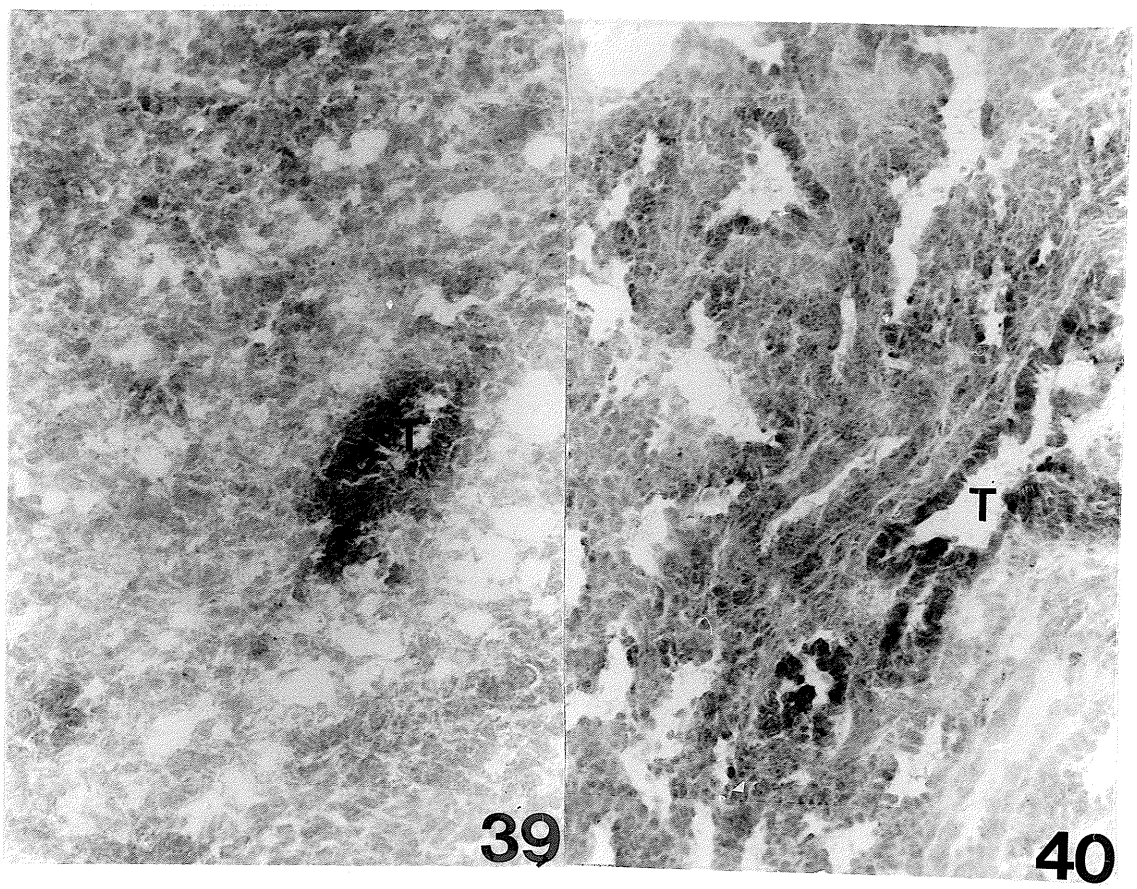
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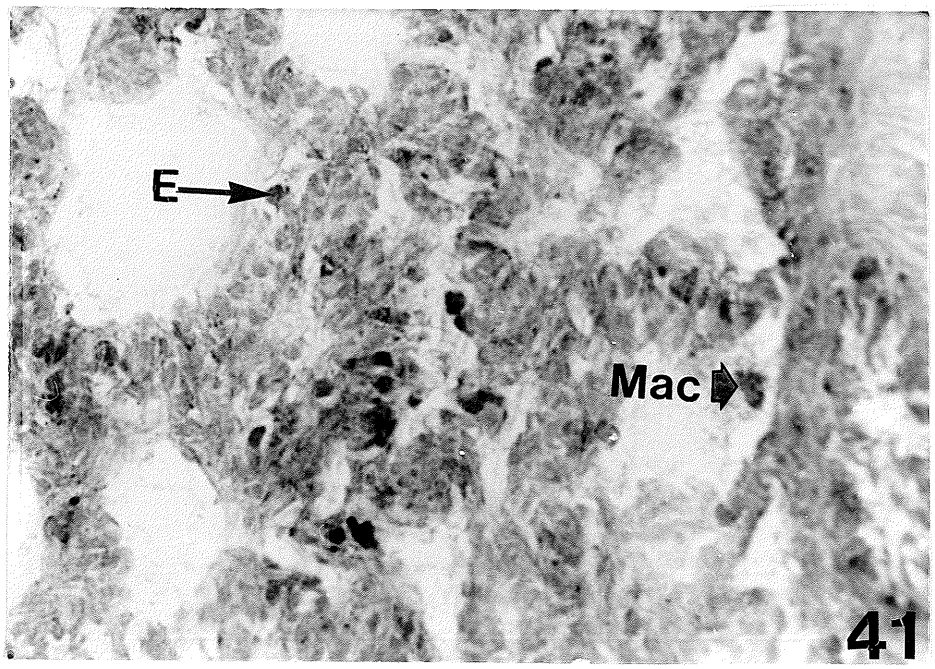
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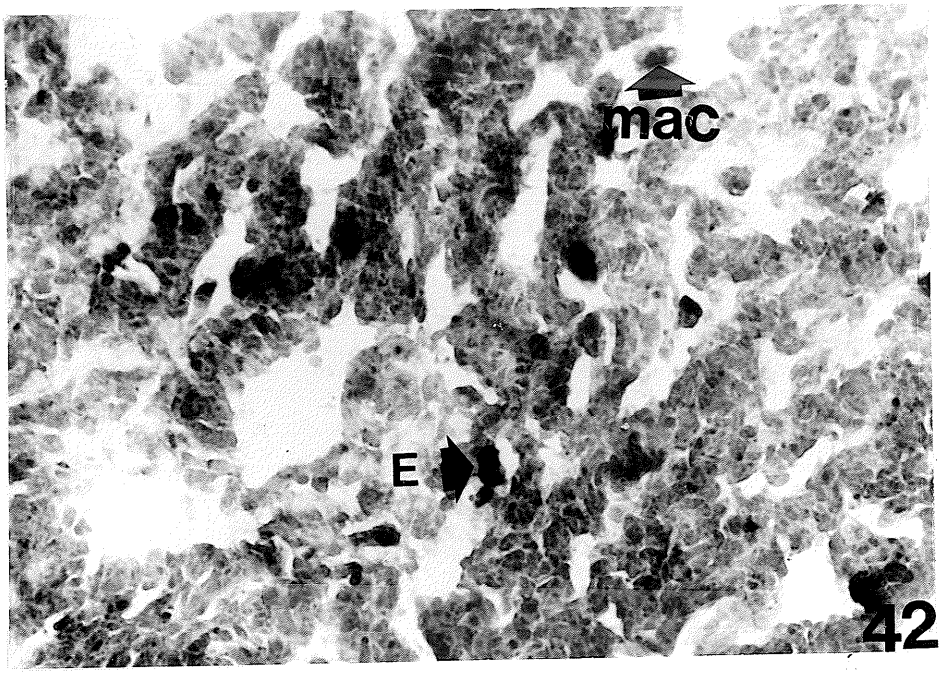
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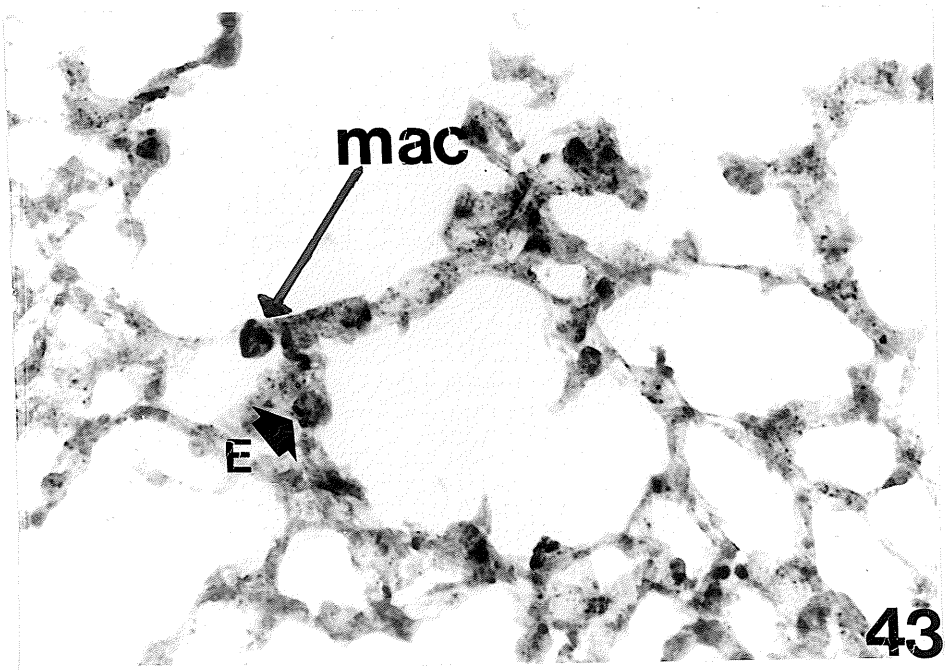
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ELECTRON CYTOCHEMISTRY

It is necessary to firmly establish the identity of the acid phosphatase positive cells in both groups of animals and to identify the ultrastructural location within the positive cells. Lung tissue to be examined was taken from fetal lungs at day 22 of gestation and neonatal lung at day 10 postpartum.

A. CONTROL

In prenatal lungs, acid phosphatase is observed primarily in the type II alveolar cells. This cell type is characterized by its location, microvilli and lamellar bodies (fig. 44). A high power micrograph of the cytoplasm of a type II cell clearly demonstrates the ultrastructural location of acid phosphatase in prenatal lung (fig. 45) with the dense reaction product seen at the periphery of some of the lamellar bodies and in some of the multivesicular bodies. An occasional macrophage has been detected with some limited acid phosphatase activity in their lysosomal bodies (fig. 46).

Postnatally at day 10, a similar enzymatic location is seen as has just been described. Many more macrophages are obvious postnatally than were seen prenatally and the intensity of the reaction is increased in both type II cells and macrophages. In type II cells activity is located at the periphery of some of the lamellar bodies and in some of the multivesicular bodies (fig. 48).

B. STARVATION

Prenatally, some type II cells are positive for acid phosphatase activity. The reactivity appears in a similar location to that seen in the control lung and acid phosphatase is present in the multivesicular bodies with lesser amount observed around the lamellar bodies (fig. 49). No macrophages were seen at this time in the lung. At day 10 postpartum the acid phosphatase activity is well developed in type II cells as well as in macrophages. The ultrastructural location of the reactivity is around the lamellar bodies and in multivesicular bodies of the cuboidal type II alveolar cells, similar to control lung (fig. 50).

Figures 44 - 50. Acid Phosphatase Reaction, Lead Method, Unstained Sections, Electron Microscopy.

Figure 44. E.M. Day 22 prenatal control, type II cell with acid phosphatase reactivity around lamellar bodies (LB) and in multivesicular bodies (mvb), x15,890.

Figure 45. E.M. Day 22 prenatal control, cytoplasm of a type II cell showing dense and phosphatase reaction product at the periphery of lamellar bodies (LB) and in multivesicular bodies (mvb), x61,250.

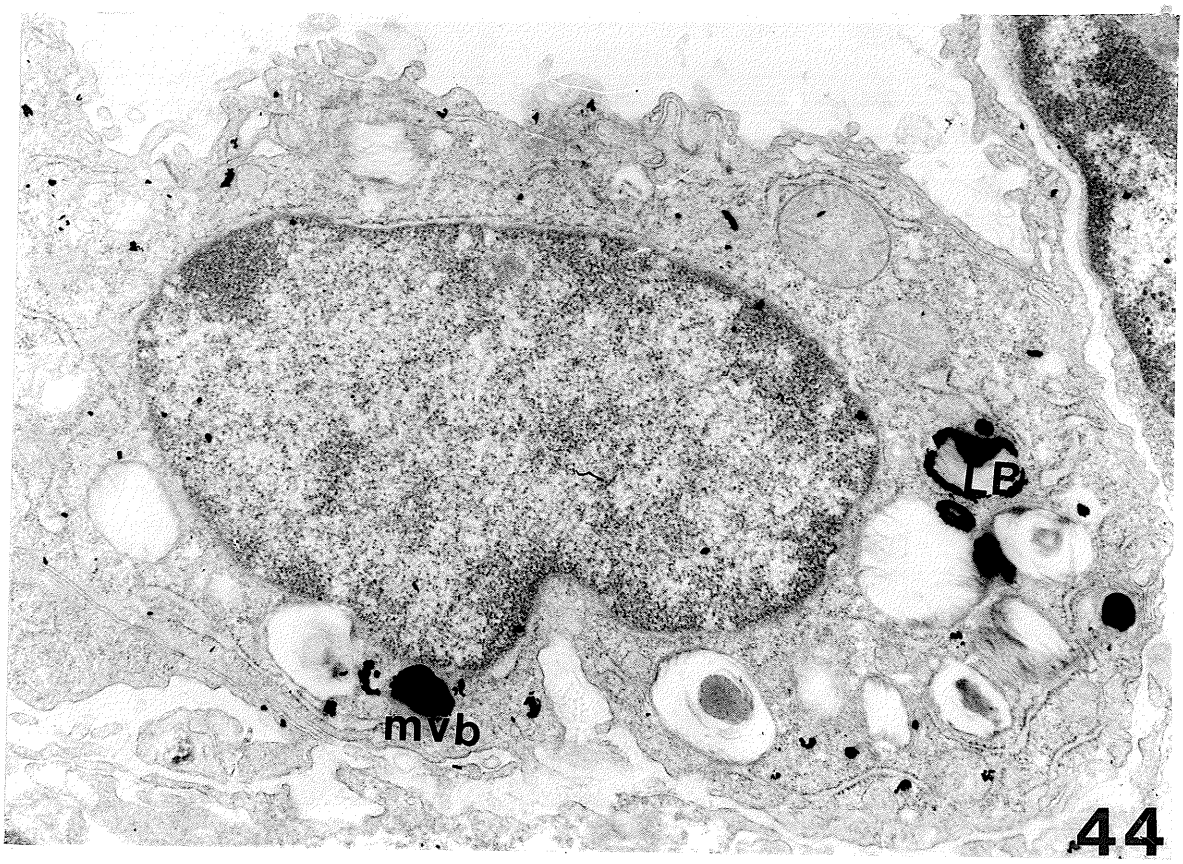
Figure 46. E.M. Day 22 prenatal control, macrophage showing dense reaction product in lysosomal bodies (L), x15,890.

Figure 47. Day 10 postnatal control, EM showing a macrophage (Mac) with many reacting lysosomal bodies (L) and a type II alveolar cell with acid phosphatase reactivity around the lamellar bodies (LB), x22,610.

Figure 48. E.M. of type II cell ten days after birth showing an intense acid phosphatase reaction at the periphery of lamellar bodies (LB) and in multivesicular bodies (mvb), x29,750.

Figure 49. E.M. Day 22 prenatal starved, a type II alveolar cell showing a slight reaction product for acid phosphatase in multivesicular bodies (mvb) with no reaction product seen around the lamellarbodies (LB), x22,610.

Figure 50. E.M. Day 10 postnatal starved, a dense reaction product is seen around lamellar bodies (LB) and in multivesicular bodies (mvb) of a type II cell, x22,610.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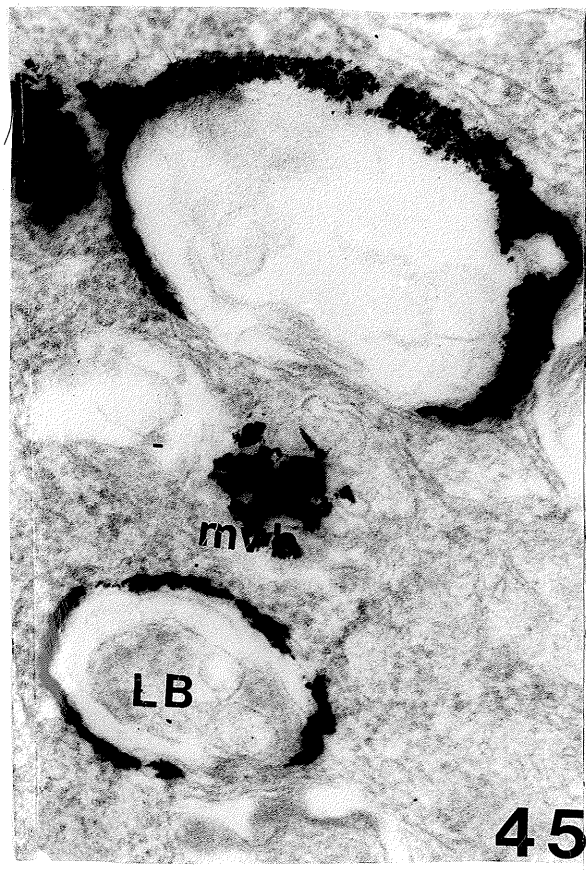
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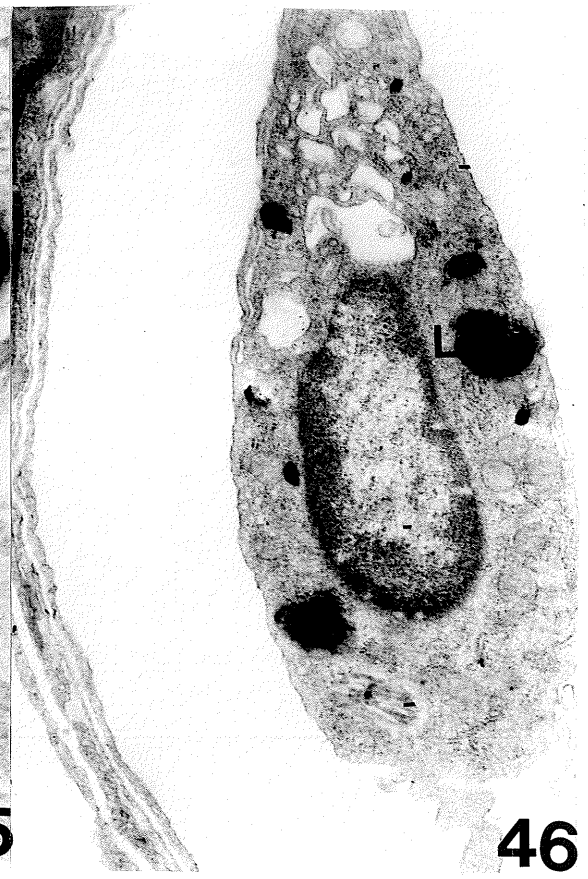
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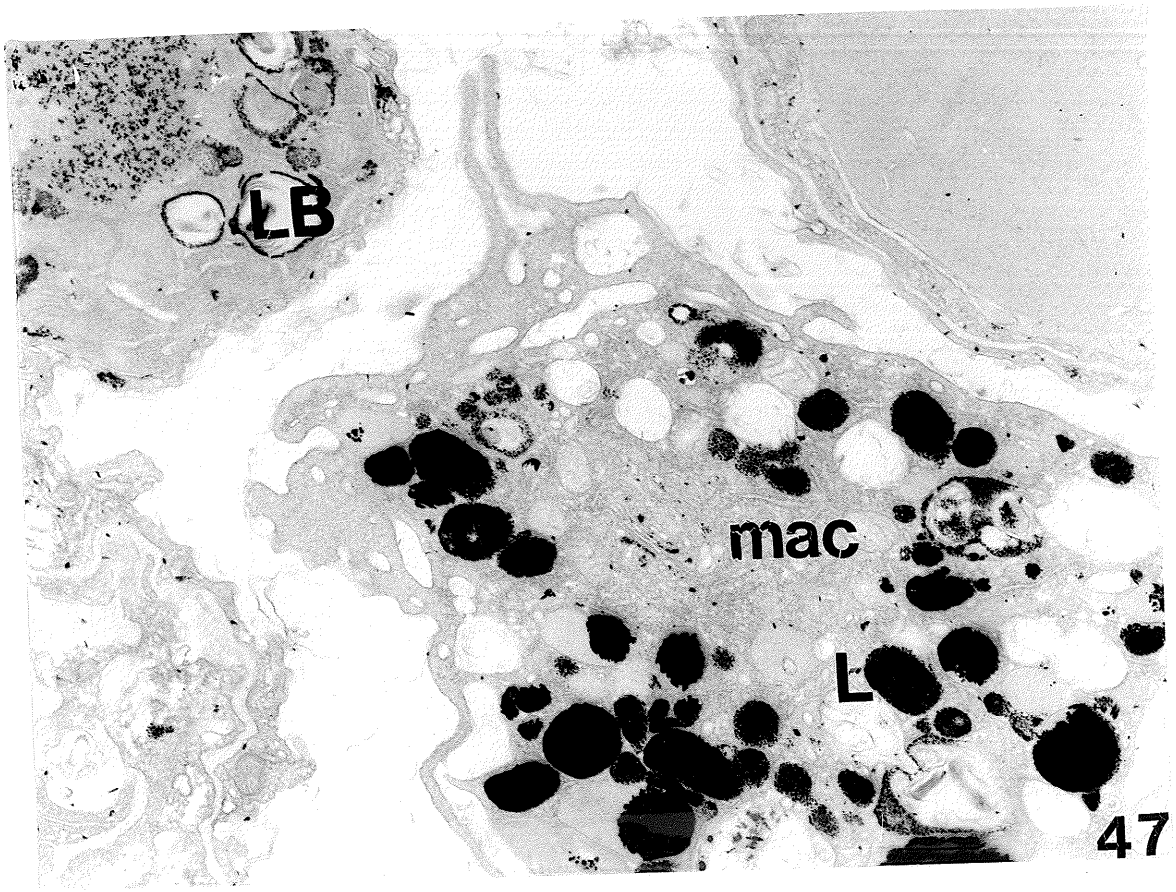
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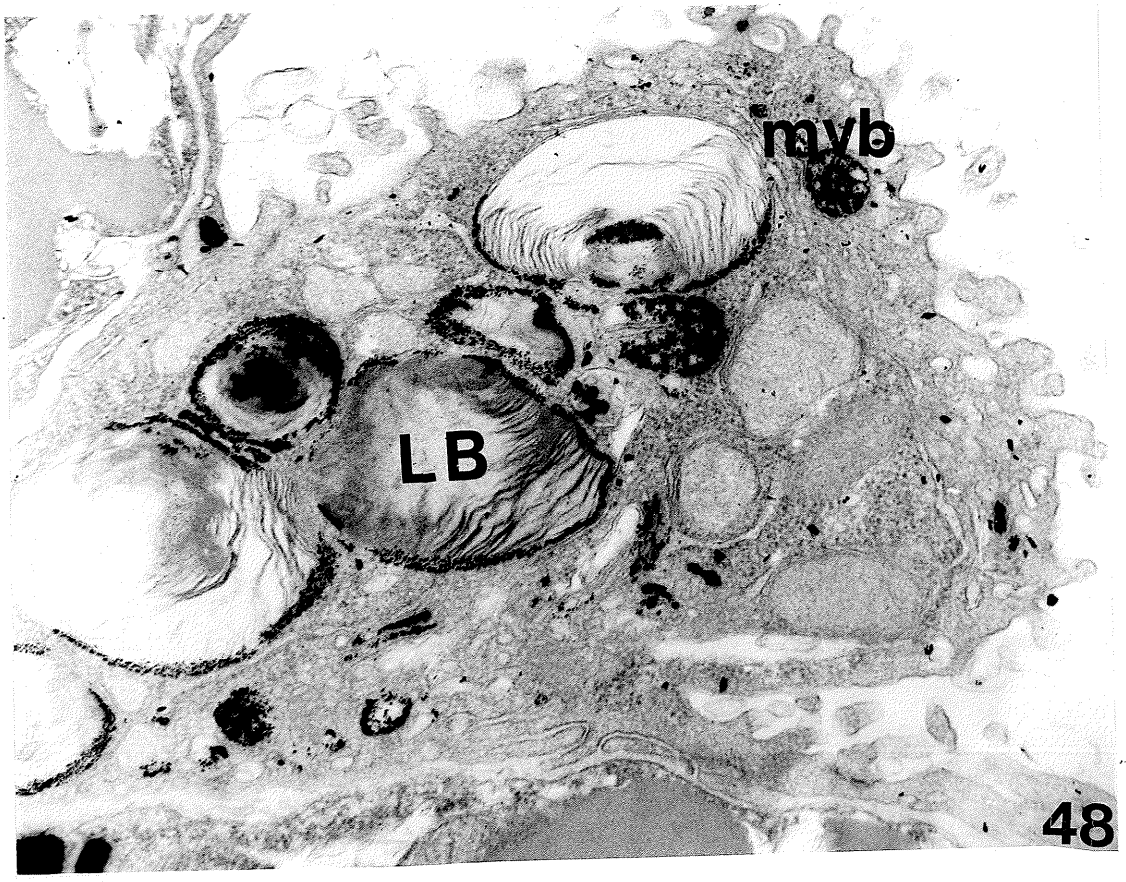
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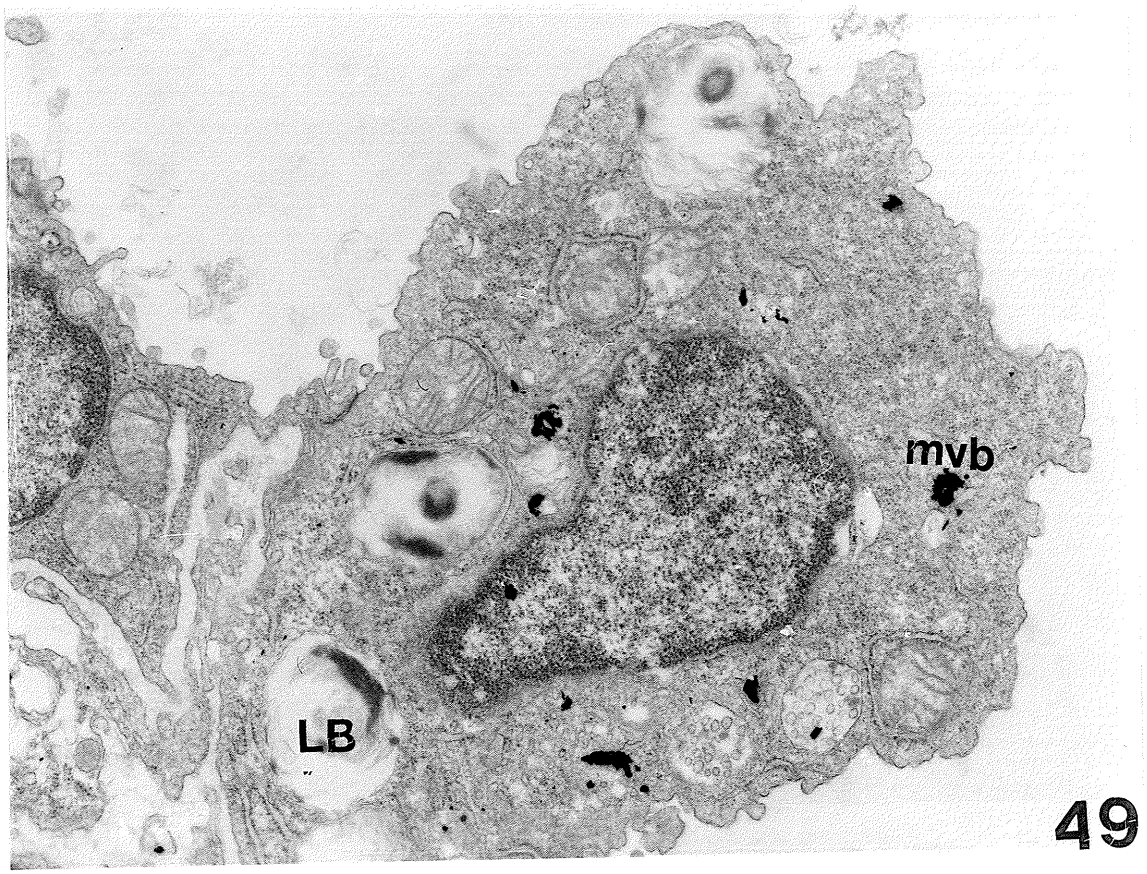

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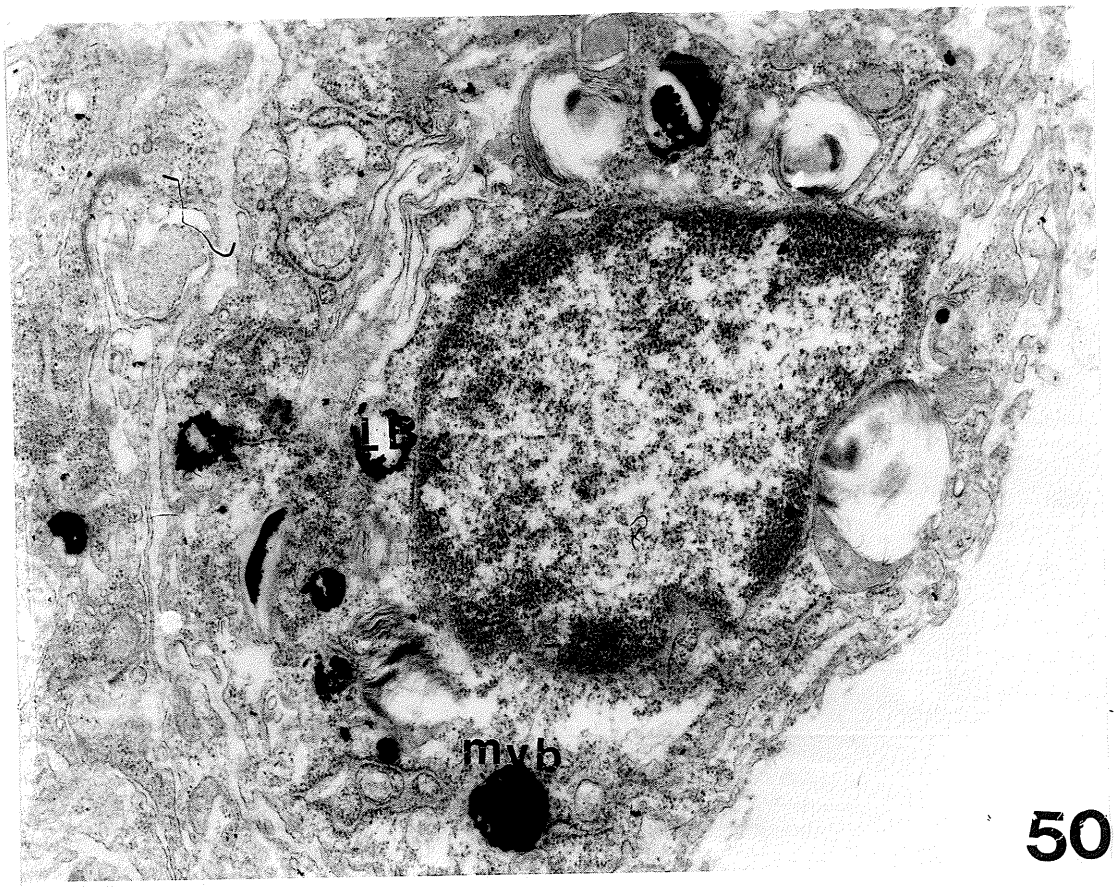

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CELL COUNTS ACID PHOSPHATASE

A chart (fig. 51) was prepared to illustrate the relative activity levels of acid phosphatase observed at regularly timed intervals in both the control and starved rats. A total of 1,500 cells was counted on each time at 400x and the percentages of positive cells was plotted against time.

CONTROL The control group, represented by the solid line, contains initially a low level of acid phosphatase activity which changes rapidly after day 18 of gestation. A dramatic increase is noted in activity between day 18 and 22 at which time the rate of increase slows. By postnatal days 2 and 3 the level of acid phosphatase activity appears to reach a maximum and does not show any significant change up to four weeks postpartum.

STARVATION The starvation group is represented by the broken line (fig. 51). Initially there is a very low level of acid phosphatase activity and the percentage of positive cells on day 18 of gestation is much less than in the control group. On subsequent prenatal days, the level of acid phosphatase activity remains very low with only a very slight increase observed at parturition. Postnatally, a slight increase in the level of acid phosphatase activity is observed up to day 3 postpartum, after which a spectacular rate of increase in the percentage of positive cells is seen. This increase continues to day 10 postnatally when the level of acid phosphatase activity approaches the control level, and by two weeks postpartum, levels of activity are very similar between control and starvation groups.

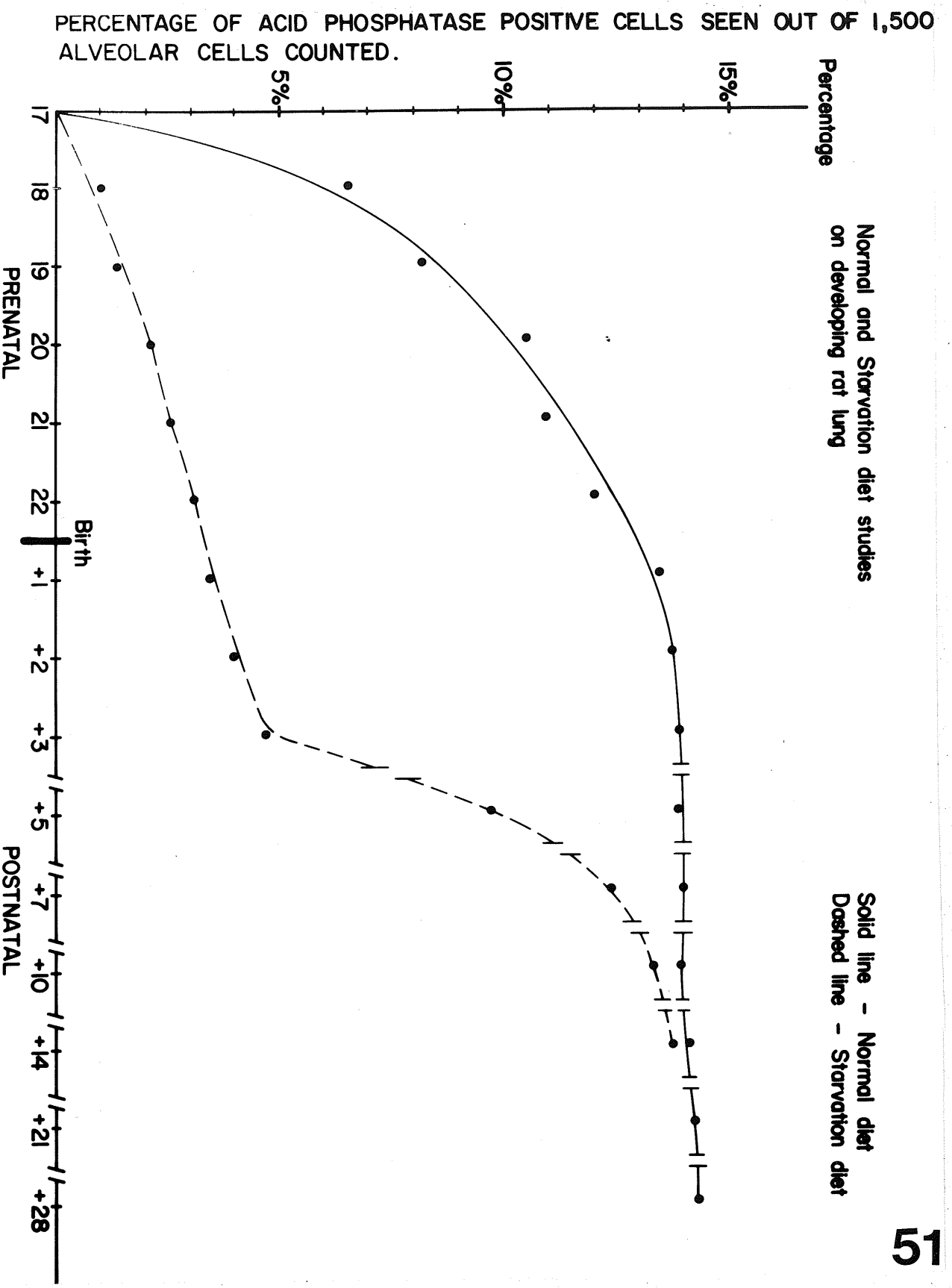


Figure 51. Percentage of acid phosphatase positive cells seen at various times in the control and starved

ACID PHOSPHATASE DIFFERENTIAL CELL COUNTS

Although the identity of all positive cells could not be established in the histological preparations, it was possible to determine the relative proportion of positive epithelial and positive non-epithelial cells observed and the various sequenced times used in the control and starvation groups.

CONTROL

FETAL The control group chart illustrates the number of positive epithelial cells and non-epithelial cells in 1,500 lung cells at each time (fig. 52). On day 18 of gestation, the vast majority of positive cells are epithelial in nature and as pregnancy progressed the number of positive cells increased but the ratio between epithelial and non-epithelial does not change significantly. The maximum reactivity in fetal lung is observed on day 22 and as earlier mentioned almost all the reactivity is confined to epithelial cells.

POSTNATAL In the two days after birth, an increase in reactive cells is observed in both the epithelial and non-epithelial fractions; however, the relative proportions between the two fractions is not greatly altered.

At day 3 postpartum a significant change is detected in the distribution of acid phosphatase activity. The relative proportion of positive cells observed in the epithelial and non-epithelial group appears

to alter, with an increase of activity seen in the non-epithelial cells (fig. 52). This shift of reactivity from epithelial to non-epithelial fractions continues on day 5 and the first positive alveolar macrophages can be positively identified at this time. It is also interesting to note, at this time, that the proportion of acid phosphatase positive cells remains constant (fig. 51).

On day 7 the changing pattern of reactivity continues at an accelerated rate and continues through days 10, 14, 21 and 28 postpartum. On day 28, it appears that the relative proportions of positive epithelial and non-epithelial cells are approximately equal. At this stage the positive non-epithelial cells are predominantly macrophages with a few positive interstitial cells.

STARVATION

FETAL The differential cell chart prepared for the starvation group illustrates the relative proportion of positive epithelial and non-epithelial cells from day 18 of gestation up to day 14 postpartum (fig.53)

There was very slight acid phosphatase activity observed on day 18 in both groups of cells. This limited reactivity is evident right up to the time of birth and is confined almost entirely to the epithelial cells. The level of reactivity seen on day 22 is far less than the level earlier observed in the control group.

POSTNATAL In comparison to the control group, acid phosphatase activity is greatly reduced before birth and shortly after birth. 3 days after parturition an increase in activity is first observed in the starvation group; this increase is noted in the epithelial cells. On days 5 and 7, a further significant increase is observed in the number of positive cells with a shift in relative reactivity from epithelial to non-epithelial cells, though not as marked as in the control group.

At day 10, when the lung reaches a level of activity similar to the control, there is a considerable shift in the distribution pattern of acid phosphatase activity from epithelial to non-epithelial cells. The final examination made on the starvation group was performed two weeks after birth. At this time the malnourished animals show a distribution of the enzyme between epithelial and non-epithelial similar in proportion to day 14 in control animals. The non-epithelial cells seen are almost all macrophages.

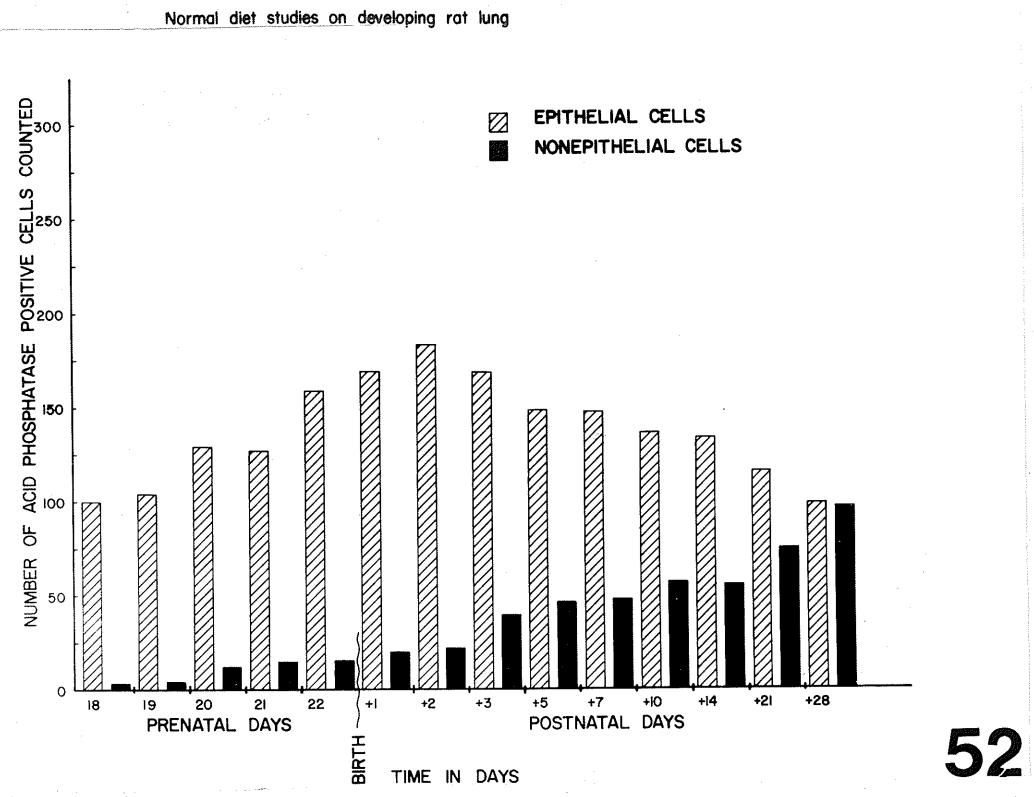


Figure 52. A chart comparing the number of positive epithelial cells and positive non-epithelial cells seen at sequenced time intervals during control experiment.

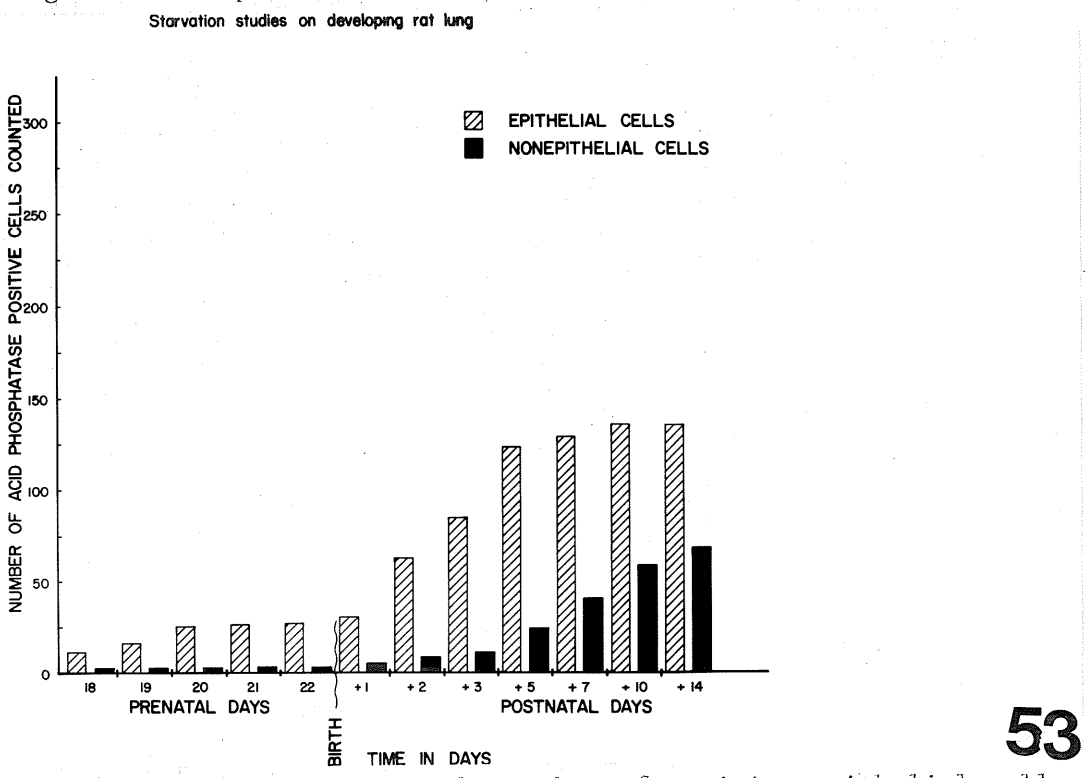


Figure 53. A chart comparing the number of positive epithelial cells and non-epithelial cells observed at sequenced time intervals in the starvation experiment.

PEROXIDASE ACTIVITY

CONTROL

FETAL Peroxidase activity is not abdundant in fetal rat lung; the first evidence of any significant activity is observed on day 18 of gestation, when a number of randomly scattered positive cells are seen (fig. 54). A gradual increase is noted in the number of positive cells from day 20 of gestation to just before birth (fig. 55). As the airways develop, many of the positive cells seen appear to be epithelial.

POSTNATAL The increase in peroxidase activity observed in the perinatal period continues for a period of two weeks after birth. At day 3 postpartum, the intensity of the reaction was somewhat stronger than prior to parturition with most of the activity present in the epithelial cells (fig. 56).

At two weeks postpartum, the total number of positive cells appears to reach a maximum level. Some of the positive cells can be identified as type II alveolar cells because of their location in the alveoli, while other positive cells appear to be in the interstitium or blood vessels though their exact identity can not be determined (fig. 57). The pattern of reactivity does not change significantly in the 3rd and 4th weeks of neonatal development. The alveolar macrophages do not appear to contain any significant quantities of peroxidase at any time during this study.

STARVATION

FETAL The lungs of fetuses removed from malnourished rats contain considerably fewer cells with peroxidase activity. A few cells show a weak reaction product at day 18 (fig. 58), with a very gradual increase in numbers of positive cells on subsequent prenatal days. By day 20 a few more mildly reactive positive cells are observed in prenatal lungs but the number of positive cells appears to be considerably lower than that observed in the control group. A continuous but very slight increase in activity is detected in the prenatal period (fig. 59). There is, however, still a decided reduction in the level of peroxidase activity as compared to the control lungs at similar times.

POSTNATAL After birth, peroxidase activity beings to increase at an accelerated rate and at 3 days postpartum a marked increase can be detected in the number of positive cells (fig. 60), though still considerably less than seen in the control. Peroxidase activity rises markedly at approximately one week postpartum and this sharp increase continues to day 14 after birth. At this time the peroxidase level in the starved lung is approximately equal to those of the control lung. At 2 weeks postpartum many of the positive cells can be identified as epithelial (fig. 61).

Figures 54 - 61. Frozen Sections D.A.B. Peroxidase Stain Showing Peroxidase as a Dark Deposit.

Figure 54. Day 18 prenatal control, peroxidase reactivity is seen in scattered locations, x400.

Figure 55. Day 22 prenatal control, positive cells are increased in number and intensity, x400.

Figure 56. Day 3 postnatal control, many intensely reacting peroxidase cells are seen, x400.

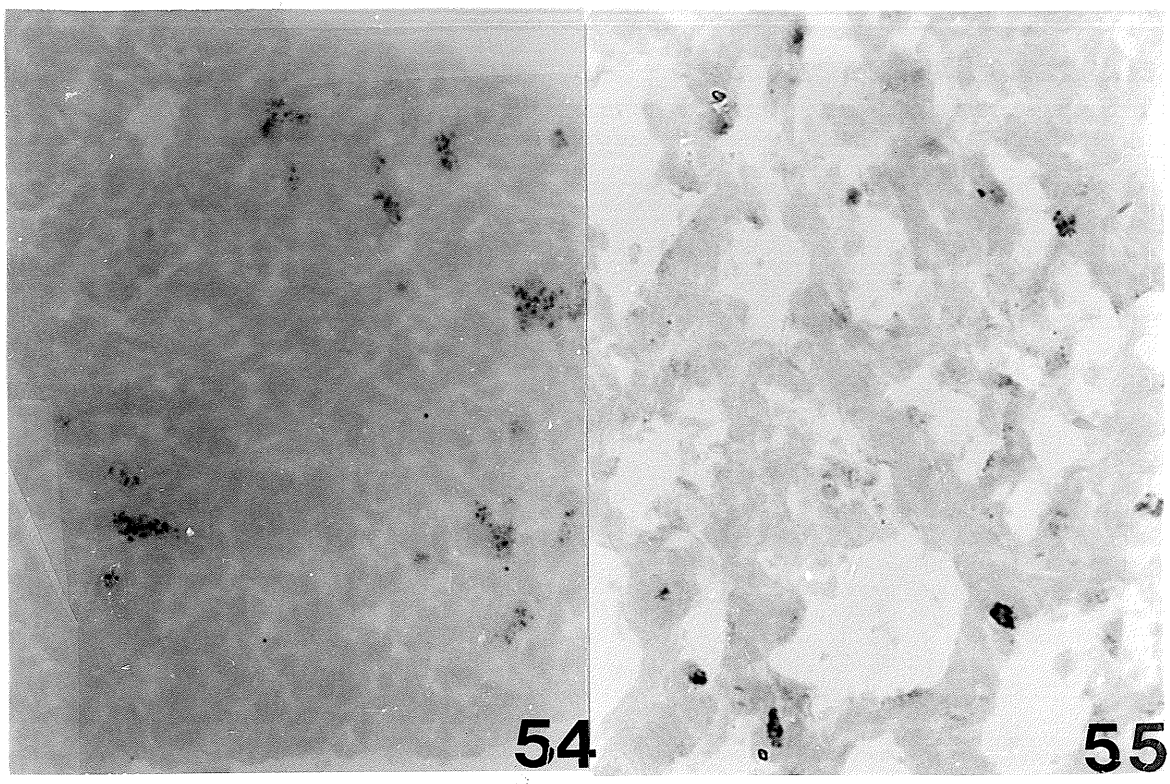
Figure 57. Day 14 postnatal control, a mature appearing lung shows the presence of many densely reacting peroxidase cells, some clearly epithelial, x350.

Figure 58. Day 18 prenatal starved, a few weakly reacting cells can be discerned, x400.

Figure 59. Day 22 prenatal starved, a few more mildly reactive peroxidase cells can be seen, x400.

Figure 60. Day 3 postnatal starved, several moderately positive cells are scattered in the lung, x400.

Figure 61. Day 14 postnatal starved, many densely positive peroxidase cells are observed, some epithelial (E), x400.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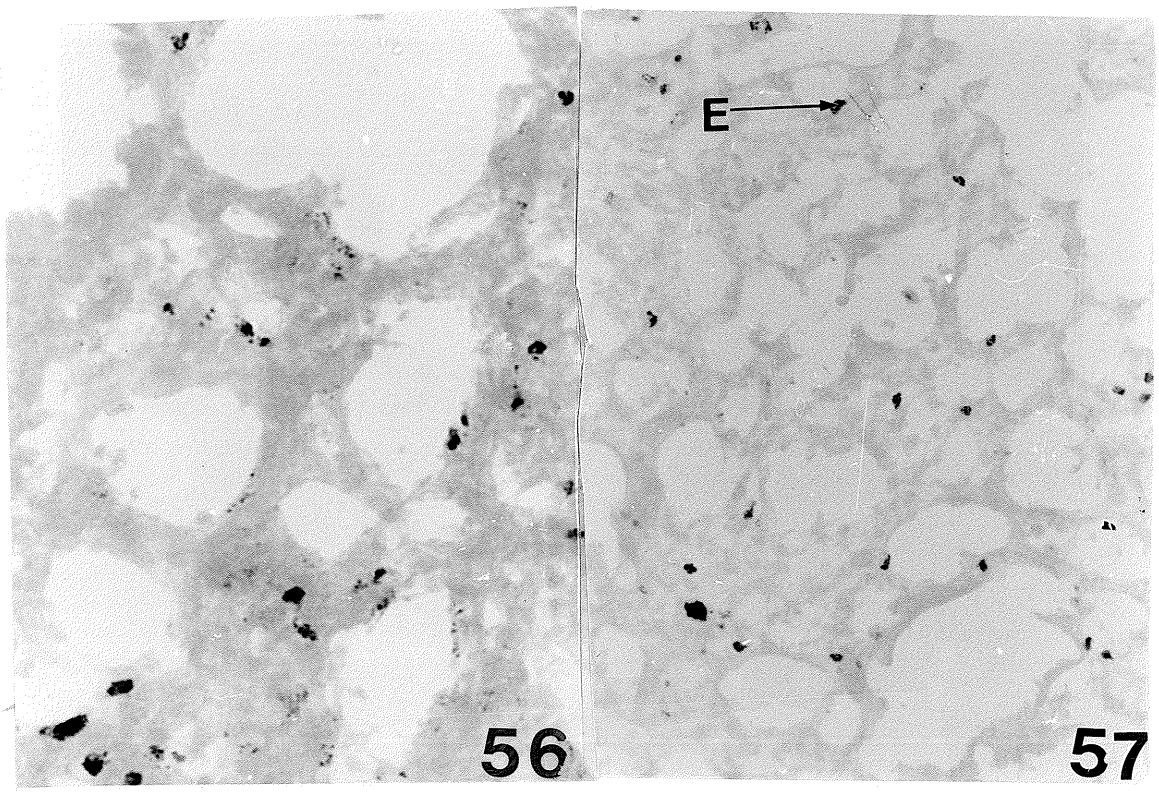
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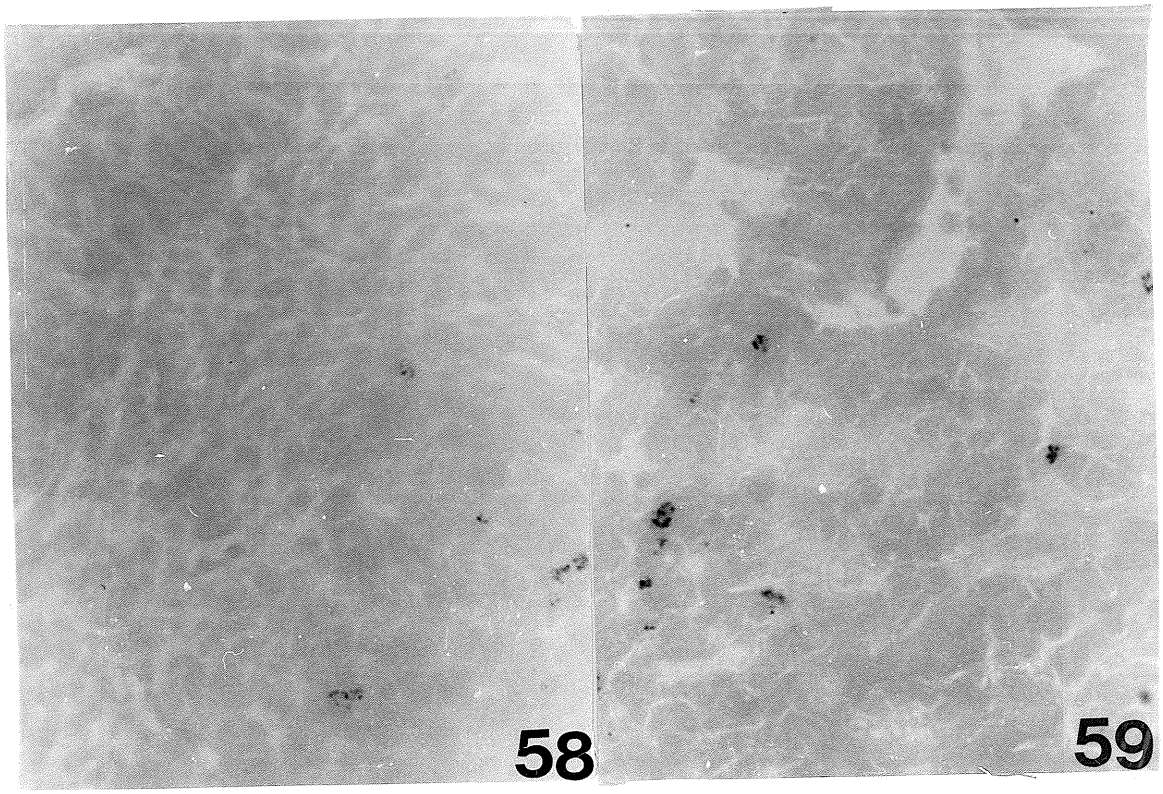
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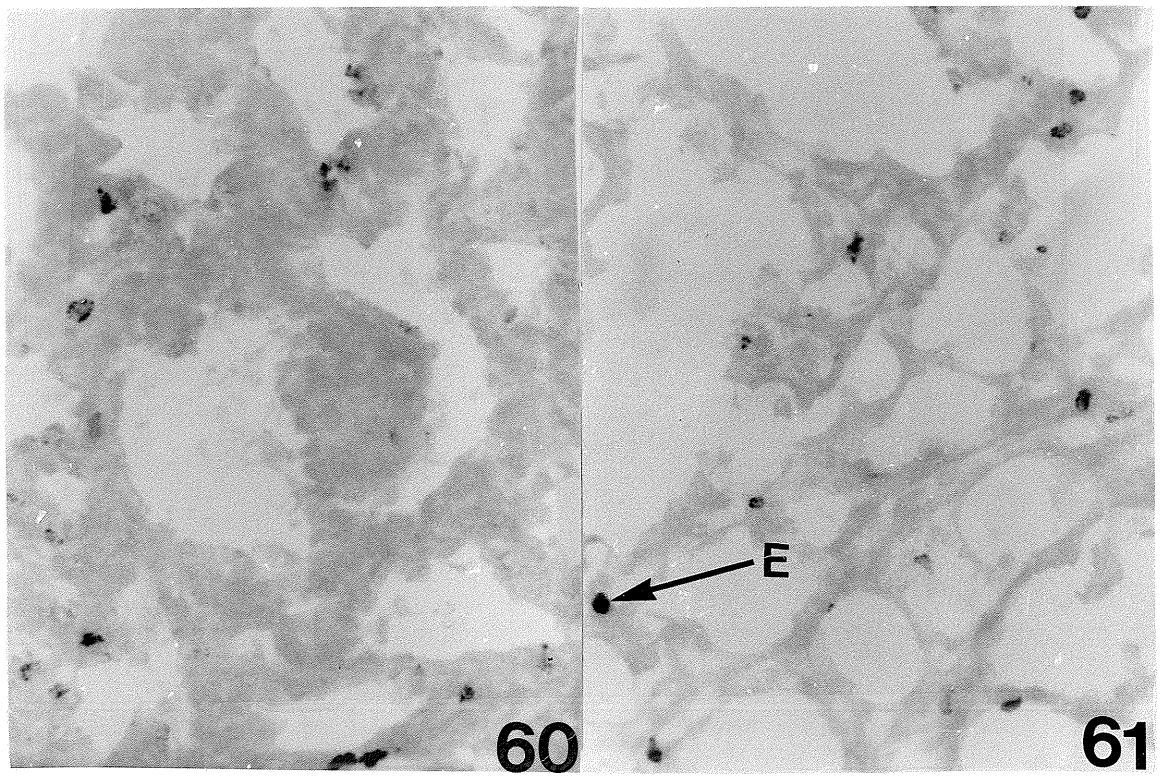
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ELECTRON CYTOCHEMISTRY

In order to firmly establish the identity of the peroxidase positive cells and to learn the ultrastructural location of peroxidase activity it was necessary to utilize electron cytochemistry. Lung sections from day 22 of gestation and day 10 postpartum were used for this part of the study.

CONTROL

In prenatal lungs, reaction product is mainly located in type II alveolar cells. The enzyme product is localized in small rounded structures called peroxisomes (fig. 62). A few positively reacting leucocytes are also observed in the developing vascular system; the leucocytes show the presence of many strongly reactive granules in their cytoplasm (fig. 63).

On day 10 postpartum a similar peroxidase pattern is observed, with reaction product located in leucocytes trapped in the pulmonary capillaries and type II alveolar cells (fig. 64). Peroxidase activity in type II cells is found in the small rounded peroxisomes with no activity observed in lamellar bodies.

STARVATION

In prenatal and postnatal lungs of malnourished rats, ultrastructural location of peroxidase and intensity of reaction are similar

to that observed in the control lungs. The type II alveolar cells and polymorphonuclear leucocytes are the sites of peroxidase activity. In the epithelium of both the day 22 fetal lung (fig. 65) and 10 day postnatal lung, peroxidase activity is located in small vesicular peroxisomes in the type II cells; no activity is associated with the lamellar bodies (fig. 65).

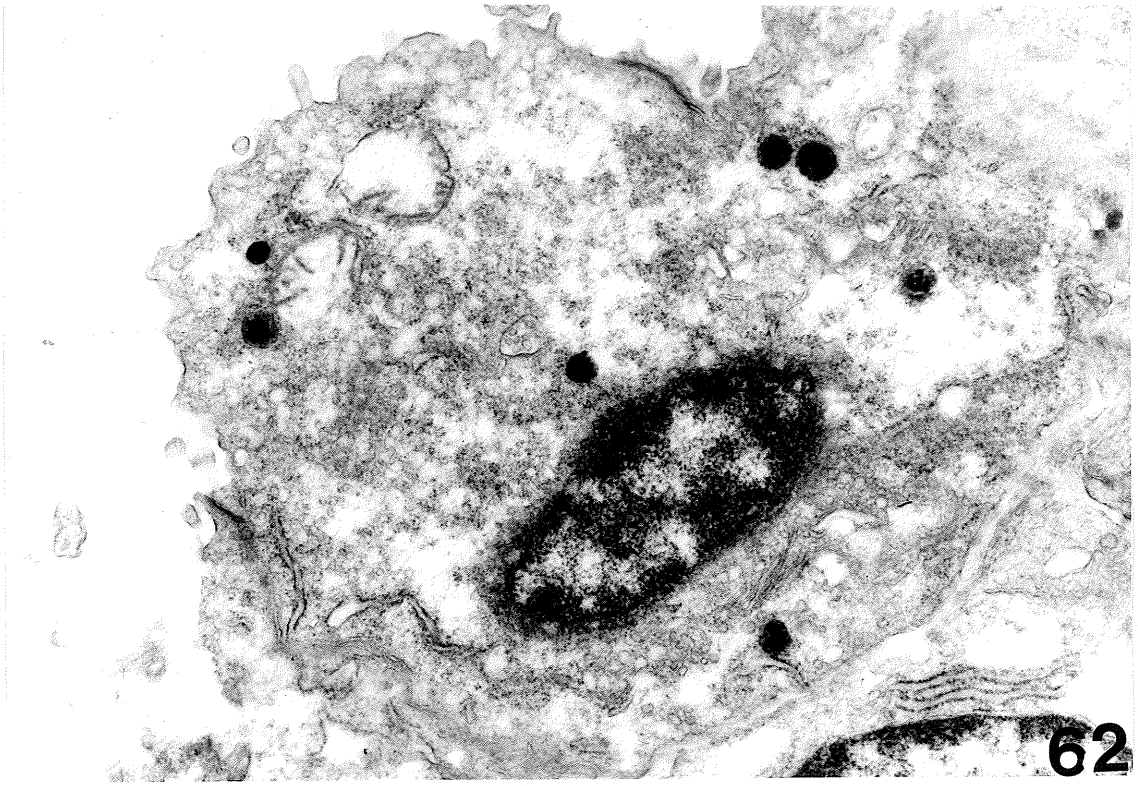
Figures 62 - 65. Electron Micrographs D.A.B. Peroxidase Reaction, Unstained Sections.

Figure 62. Day 22 prenatal control, type II alveolar cell showing D.A.B. positive bodies in the cytoplasm, x22,610.

Figure 63. Day 22 prenatal control, a leucocyte showing a dense reaction product (Per) in its granules, x61,250.

Figure 64. Day 10 postnatal control, numerous strongly positive peroxisomes (Per) are located in the cytoplasm of a type II alveolar cell, x22,610.

Figure 65. Day 22 prenatal starved, peroxidase activity is located in scattered peroxisomes (Per) of the type II alveolar cells, x15,890.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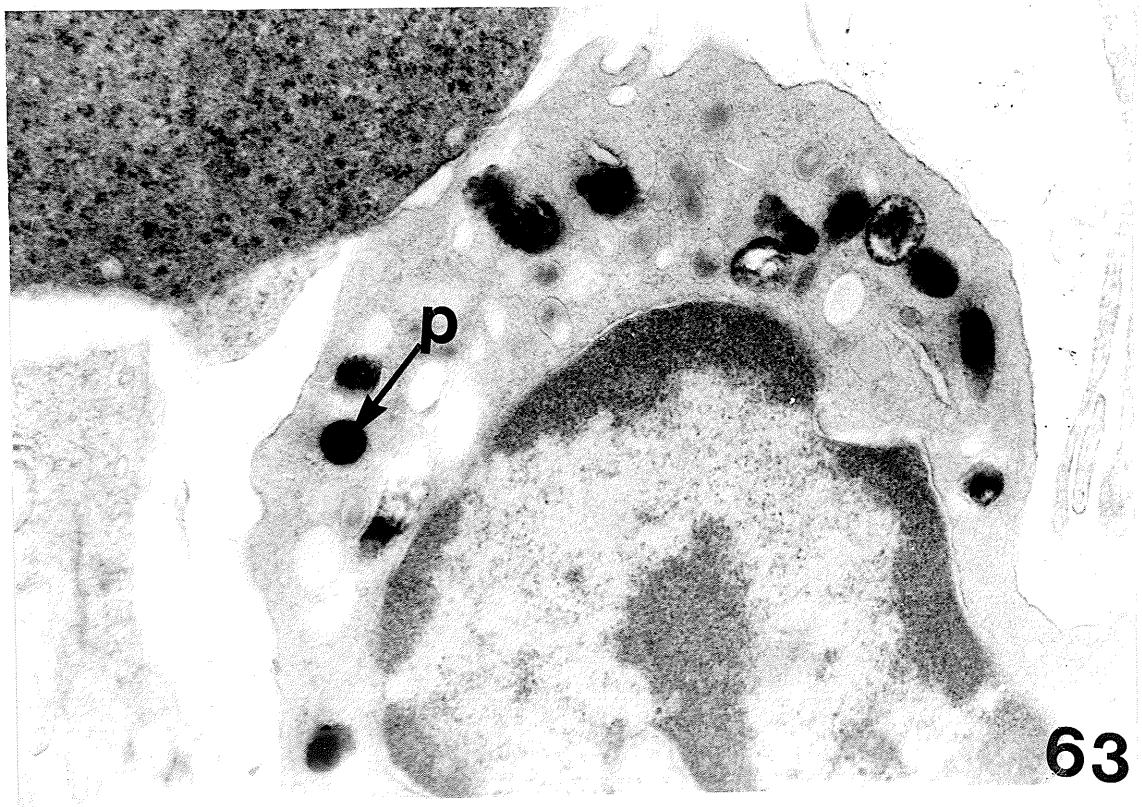
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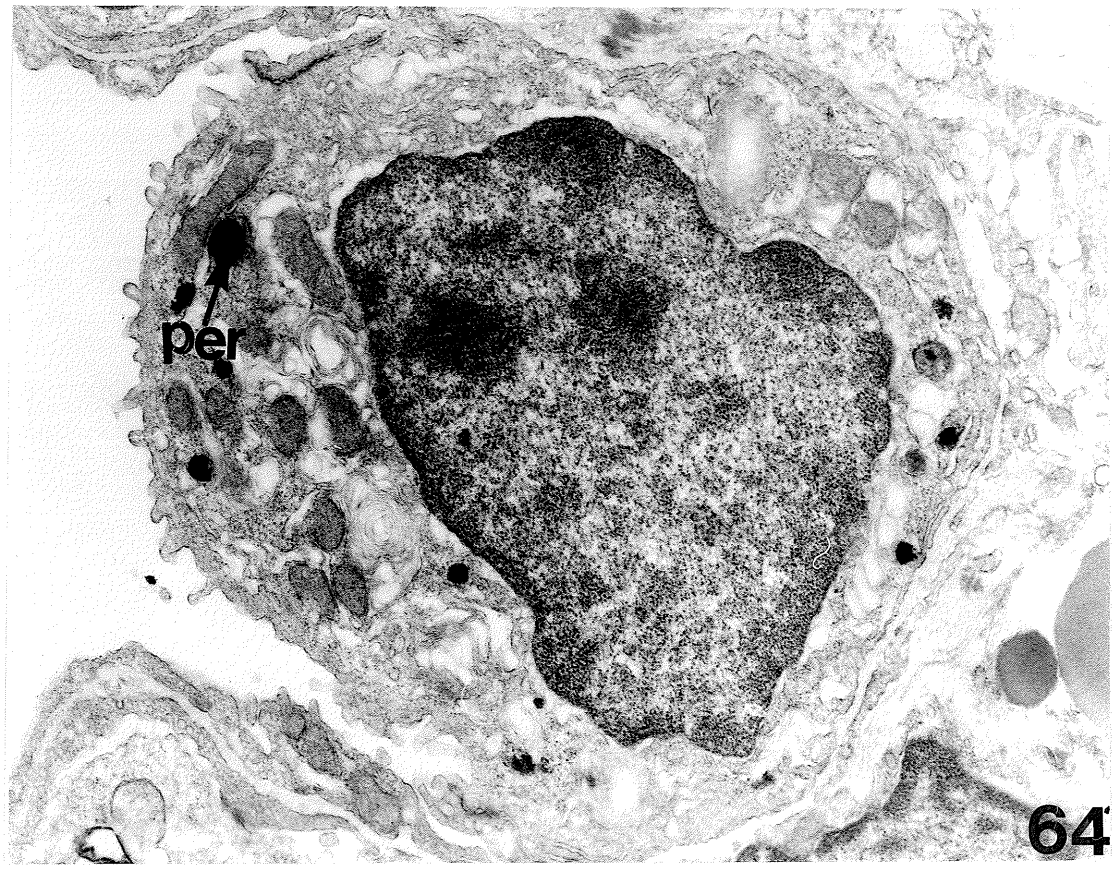

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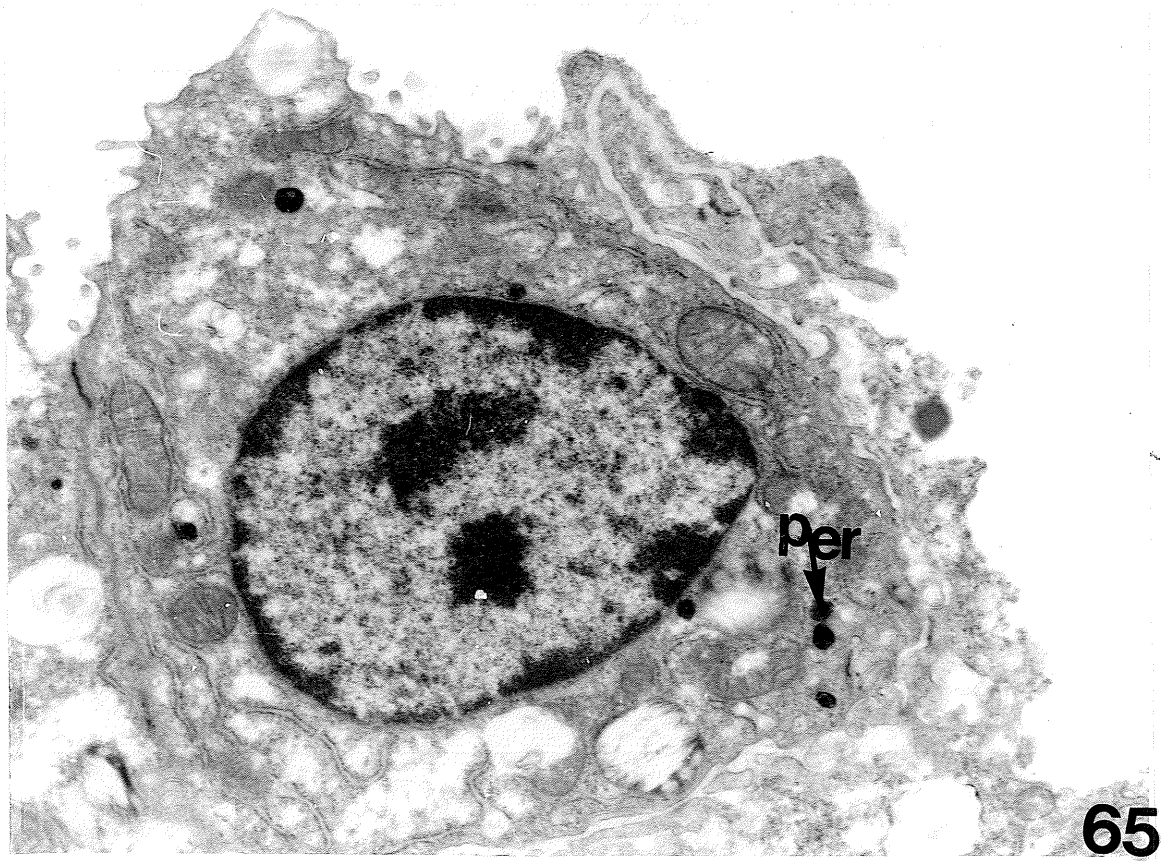

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I. PEROXIDASE CELL COUNTS

A chart (fig. 66) was prepared to illustrate the relative amounts of peroxidase activity observed at the various time intervals in control and starvation experiments. The total number of positive cells counted in 15 random high power fields (x400) was plotted against time in days.

The control group shows initially a very low incidence of peroxidase activity followed by a moderate increase from day 20 to the time of birth. The perinatal period is further marked by increasing peroxidase activity continuing to day 10 postpartum. At this time the peroxidase level peaks and remains constant up to four weeks after parturition.

In the starvation group, an even lower peroxidase activity level is seen initially than observed in the controls. Peroxidase activity is also much lower in the perinatal period and there is a more gradual increase than in the controls up to day 5 postpartum when a sharp increase in activity is observed. The number of peroxidase positive cells increases rapidly to reach control values by 2 weeks after birth.

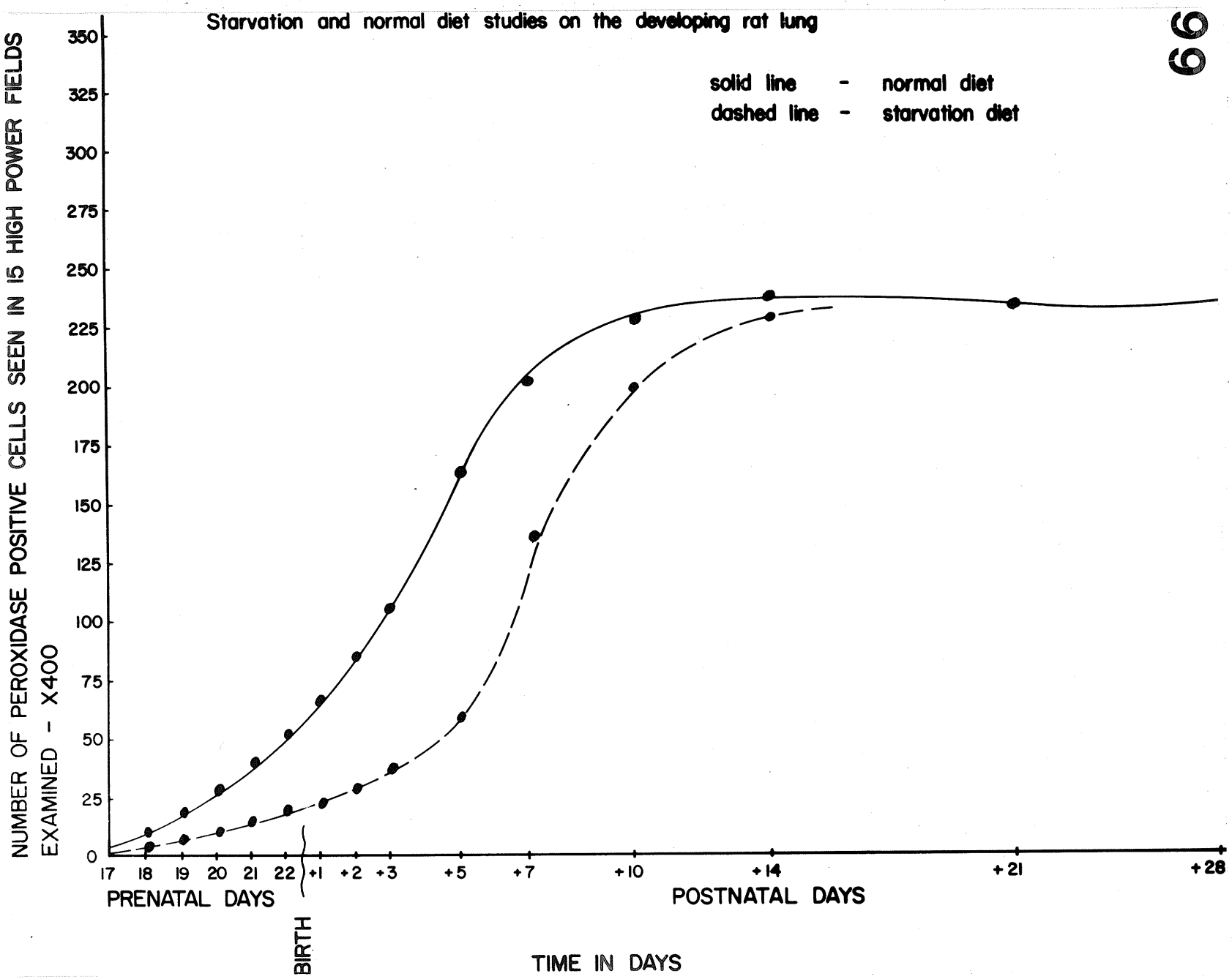

Figure 66. The number of peroxidase positive cells observed during the control and starvation experiments.

DISCUSSION

PRENATAL DEVELOPMENT OF THE LUNG

NORMAL In normal fetal lung, growth and cellular development follows a well recognized sequence of events which results in the production of a functional blood-air barrier by birth (69,70,74). Normal lung development has been studied by many investigators using a wide variety of parameters (13,23,29,68,71,72,83); this study has employed morphological, histochemical and ultracytochemical techniques to demonstrate lung development from the late fetal to postnatal juvenile stages.

Growth in the late fetal stage, in particular from day 17 to day 20 of gestation, is largely confined to cellular proliferation (69). Cellular mitosis appears to be mainly in the cuboidal epithelium found lining the rapidly growing and expanding air tubules. Autoradiographic studies in rats have revealed that the percentage of cells in D.N.A. synthesis is at a maximum of 12 percent on day 18 of gestation, a value which declines very rapidly to 3 percent by parturition (23). Similarly, 50 percent of the total of labelled cells observed are epithelial on day 20 with this value falling dramatically as the fetus reaches full term. Fetal rat lung examined on days 17 and 18 in the present study confirmed this information by showing the presence of many mitotic figures in the undifferentiated cuboidal epithelium lining the rapidly growing tubular airways. The cuboidal epithelium in addition to mitotic figures contained copious amounts of cytoplasmic glycogen as previously suggested by Sorokin and

O'Hare. These are probably interrelated findings (11,70). The high mitotic rate starts to decline rapidly after day 20 of gestation coincident with the sudden disappearance of glycogen from the cuboidal cells, suggesting that glycogen is necessary to provide energy anaerobically for mitosis in the cuboidal epithelium.

After day 20 cellular proliferation is no longer the most conspicuous morphological feature observed in the fetal rat lung as increasing evidence of cellular differentiation was observed, to replace proliferation as the major form of development. This evidence substantiates the biological reciprocal relationship between proliferation and differentiation observed in animal cells. This strongly suggests that cellular proliferation must precede subsequent cytodifferentiation and the ultimate production of a functional blood-air barrier. After a burst of mitotic activity, the cuboidal epithelium embarks upon a phase of cellular differentiation, which must take place before a functional lung can develop. The earliest evidence of cytodifferentiation in the cuboidal epithelium is the appearance of lamellar bodies simultaneously with the development of surface activity within the lung. The morphological presence of lamellar bodies and physiological development of surfactant coincide with one another and commence at approximately day 20 of gestation in the fetal rat lungs, which suggests their relationship (69,70,72,84). Cytodifferentiation also involves the flattening and attenuating of cuboidal epithelium cuased by the approximation of developing vascular channels next to the tubular epithelium; this phenomena can also be seen with the light microscope commencing at approximately day 20.

The exact mechanism of this attenuation of the cuboidal tubular epithelium is not completely understood, but it may be related to a physical stretching process as new capillaries are formed and bulge into the airways placing a great physical stress on the epithelium (23). These newly formed squamous or type I cells first appear in the lung after the period of maximal epithelial cell division is completed, which would suggest that they do not arise as a result of mitosis of their own kind. Rather it has been convincingly shown in a serial morphologic and cytodynamic study that type I cells arise by a transformation of the cuboidal cells into a flat attenuated shape (23). Further evidence has been seen in lung injury experiments where type I cells are susceptible to injury from oxidant gases and are subsequently replaced by type II epithelium. These repair cells which undergo hyperplasia are then transformed in functional type I cells by a similar mechanism to that observed in the fetal rat lung (18,20-23). Results from these injury and repair experiments have clearly indicated that only two cell types actually participate in the regenerative phase; type II and endothelial cells (94), meaning type I squamous epithelium must be considered as a transformed or differentiated cuboidal type II cell. In recapitulation it is evident that cellular proliferation of the cuboidal epithelium in both fetal and injury models must proceed the process of cytodifferentiation into functional type I and type II cells.

In normal fetal rat lung development, cellular proliferation occurs between day 17 and day 20 with differentiation occurring between day 20 and parturition, as judged by the cellular morphology and

glycogen content. Several researchers have noted that cytodifferentiation commenced at day 20 and resulted in the production of type I and type II epithelium with the subsequent production of surfactant (69-72). The differentiation into type I and II cells within the primitive air sacs allows for the production of a functional air-blood barrier necessary to maintain life in the critical perinatal period. The development of an air-blood barrier can therefore be used as a morphologic criterion in determining the maturity of the lungs in the neonates. The appearance of surfactant and lamellar bodies would indicate differentiation of cuboidal to type II cells and are therefore markers which indicate the change in the fetal lung from glandular to the canalicular phase of development.

STARVED As earlier suggested normal prenatal lung growth is characterized by cell proliferation between day 17 and day 20 of gestation when a high rate of endodermal mitosis is reflected by large accumulations of glycogen within their cytoplasm (11,23,70,72). Subsequently as mitosis decreases and glycogen disappears, epithelial cell differentiation progresses from day 20 to parturition. The present experiments show that severe caloric restrictions imposed on the fetus via maternal starvation at a time when cellular hyperplasia is maximal in the fetus, caused a marked retardation on lung development and cellular maturation. Faridy has indicated that lung growth in the fetus is most adversely affected by complete food deprivation of the mother between gestation days 17 and 20. This time period corresponds directly to the period of maximum epithelial cell hyperplasia in the fetal lung. It was also observed that even after birth and a partial

recovery, that the lungs continued to remain smaller and hypocellular, following starvation of the mother during this critical growth period (84).

The rate of mitotic division is slowed by nutritional deprivation which causes a marked delay in cellular maturation, since cellular division precedes differentiation. The processes of epithelial cell division and differentiation are therefore both retarded by malnutrition. Dietary deprivation at this very vulnerable stage of growth in the fetus causes a partial curtailment of the normal growth and maturation pattern, which have functional implications. For example, there may be a reduction of the number of functional type II cells which would lead to a reduced level of surfactant secretion. In support of this theory, Faridy has shown a 35 percent reduction in surfactant content of neonatal lungs after maternal starvation during the gestation period of day 17 to day 21 (84). These results concur with the morphological evidence which indicated that maternal starvation greatly altered the normal cellular sequence by slowing mitosis and retarding cytodifferentiation of the epithelial cells ultimately results in an immature air-blood barrier in the critical perinatal period. In this fashion the newborn animals ability to function normally at birth may be jeopardized.

The neonatal rat lung is normally considered to be an immature organ at birth and does not contain any true alveoli (74). There is existing at this time, however, a functionally effective air-blood barrier which will allow respiration to take place in the newborn.

This may not be the condition which exists in the lungs of newborn from malnourished mothers; with the slowed mitotic rate and delayed maturation of the pneumocytes, it is apparent that the lungs may develop even more slowly than normal and result in an even more immature organ at birth. This could seriously risk the life of the fetus and lead to still birth or possible early neonatal death. In the present study it was observed that, at birth, there were fewer areas of well developed air-blood barriers in the starved animals as compared to controls. The rate of still birth was greatly elevated from normal as was the early neonatal death rate per se. However , it was interesting to note that even though starvation of the maternal rats results in delayed lung development there was no change in the length of the gestation periods, with births occuring around day 22 as normal. When the lungs after stillbirths and early neonatal deaths were examined histologically, they exhibited a similar morphological structure to lungs from survivors sacrificed at the same age. There did not appear to be any evidence of hyaline membrane disease, consequently the high death rate could not be directly attributed to lung failure. It would appear that after starvation there was underdevelopment and immaturity of several organ systems and the combination of all probably contributed to the high death rate (79).

Blackburn et al. observed that surgical decapitation of fetal rats on day 16 of gestation retards lung differentiation in a similar manner to maternal starvation (87). It was observed that the pneumocytes fail to under normal cytodifferentiation, while they still retain large volumes of cytoplasmic glycogen with very few lamellar bodies being developed. The synthesis of surfactant was reduced apparently in relation

to the low number of lamellar bodies produced in the pneumocytes (87). Similarly, the complete denial of nutriments results in a greatly reduced secretion of surfactant in the newborn rats (84), which would also appear to result from retarded differentiation of type II cells.

Gross et al. studied the effects of starvation on pulmonary surfactant production and they were able to demonstrate a significant inhibition of pulmonary surfactant production after acute dietary deprivation (85). The inhibition of surfactant secretion has been directly related to reduced levels of enzymatic activity concerned with its production and it was found that there was a sharp decrease in enzyme levels associated with surfactant secretion during starvation. This would then clearly establish the enzymatic basis for reduced surfactant production and/or secretion associated with starvation (85). The present study, as discussed later, supports these findings by showing reduced levels of enzymatic activity in animals exposed to starvation situations.

Blackburn concluded that retarded development of fetal lungs was related in some way to reduced levels of circulating hormones secreted by the pituitary-adrenal-thyroid axis (87). This reduced level of hormones may cause impaired glycogen utilization by the cuboidal epithelium and consequently inhibit surfactant secretion by allowing slower cytodifferentiation. He concluded that the impairment of biochemical differentiation was related to deficits in enzyme induction by specific pituitary-adrenal-thyroid hormones (87).

It has been suggested that steroid hormones may trigger the synthesis of ribonucleic acid which codes for particular proteins; which may be enzymes involved in the breakdown if glycogen with energy production and the biosynthesis of surfactant (74,94). It may be at this level that maternal starvation manifests itself in the retarded development of fetal rat lung. In support of the hormonal control mechanism of lung growth and development are several investigators (87,95). Adamson and Bowden showed that in organ cultures of fetal rat lung, the corticosteroid prednisolone decreases D.N.A. synthesis in the undifferentiated epithelium and at the same time accelerated the uptake of palmatic acid and the production of lamellar bodies in those cells, resulting in premature differentiation (95). This evidence suggests that the growth (cellular division) and maturation (cyto-differentiation) of the fetal rat lung are associated with an adrenal hormone mechanism. This hormonal mechanism causes cellular division to be retarded and subsequent differentiation to be accelerated which lends further support to the reciprocal relationship between mitosis and differentiation.

The evidence of these investigators helps explain the findings of the present study in which growth and differentiation of alveolar epithelium may be related to hormonal controls. Starvation imposed on pregnant rats could lead to hormonal insufficiencies experienced by the fetus. Hormonal insufficiencies as suggested by Blackburn may then lead to deficiencies in enzymatic activities necessary for normal cellular division and development.

POSTNATAL DEVELOPMENT OF THE LUNG

NORMAL The antenatal growth and development of rat lung has been investigated extensively by Burrie et al. and Thurlbeck (74-79). The results of the present study of control rats concurs with the findings of these investigators. At parturition, the lungs of rats are in what has been described as the canalicular phase of development, giving the lungs a relatively immature appearance at birth. The lungs are composed largely of undeveloped air saccules referred to as primary saccules. Primary saccules although immature, are capable of maintaining limited respiration since a functional blood-air barrier is present due to the presence of differentiated type I and II alveolar cells. Primary septa found between the saccules, present at birth, are composed of a thick double capillary network system. This pattern is characteristic of a lung in the canalicular phase and continues to be prominent in the neonate until approximately 5-7 days postpartum (74). Maturation of the lung or change from canalicular to alveolar phase begins with the outgrowth of buds or crests on the primary septa commencing at approximately 5-7 days after birth. The new growths or secondary crests continue to grow and divide the primary saccules into recognizable alveolar structures. Secondary crests are well developed by day 10 but have a rather thick appearance since they are still characterized by a double capillary network.

The major morphological changes observed after day 10 is the transformation of the thick capillary network in the septa by fusion

into much thinner single capillary networks in the lung septa. This remodelling results in the production of alveoli with a thin mature appearance, the alveoli then appear to undergo physical expansion and have adopted a mature alveolar appearance after 3 weeks of life.

STARVED In neonates deprived of normal nutrition it was observed in this study that there was a reduced level of growth which was further accompanied by a delay in differentiation. At birth, the lung was smaller and less mature than normal and still contained many undifferentiated cuboidal cells with large quantities of cytoplasmic glycogen. Morphologically, the lungs appear to be less well developed in the newborn malnourished rat; the primary saccules are more irregular and not as well expanded as the controls. Secondary crests, which serve as markers of maturation into the alveolar stage, do not appear as early as they did in the controls. Hence, the lung appears to be retarded in its normal development for some time, even after birth. This retardation could be a carry-over effect from the growth retardation experienced in the fetal malnutrition. The normal development of secondary crests may be related to the appearance of mesenchymal glycogen, and the slower start in development of malnourished rats may be due in part to delayed appearance of mesenchymal glycogen. It is believed that mesenchymal glycogen may be essential in early postpartum to supply energy for replication of mesodermal cells which are necessary for the development of secondary crests (77). The later appearance of mesenchymal glycogen is still an enigma but may be related to delayed epithelial maturity and the later disappearance of glycogen from epithelial cells.

The lungs of malnourished rats exhibit accelerated development from day 10 to two weeks postpartum and become similar in appearance to control lung of the same age. The present study, however, does not indicate whether there is any overall reduction in the size of the lung or of individual alveoli. The observed acceleration in development may be accounted for or may reflect the feeding of the neonate on the mother. This would deplete the maternal rats nutritional reserves but would increase the intake of necessary nutriments needed by the neonate for adequate growth. The mother rat would therefore be supplying young rats with nutrition and hence allowing some recovery from the earlier starvation effects. The maternal rat could therefore be buffering or protecting the neonate from any further nutritional damage. Several investigators have found that the effects of starvation could be partially reversed if the starvation did not occur during vulnerable periods of cellular divisions (81,83,84,97).

Caloric restrictions imposed during a critical period of growth when energy demands by the mitotic cells are the greatest would result in the inhibition of cellular hyperplasia. This decreased growth resulting from decreased caloric intake has been observed by several investigators (97,99,100). It has also been observed that postnatal feeding would partially reverse the effects of fetal starvation and in the present study this was observed and attributed to neonatal feeding on the maternal rat. Zamen has shown the growth rate of malnourished postnatal rats and control rats are approximately equal but the deprived rats did not entirely catch up. He also pointed out

that refeeding must take place before the normal period of cell hyperplasia has ended in order for growth to occur in malnourished rats (97). Lung growth or cellular hyperplasia was reported by Wennick et al. to level off between day 10 and day 14 postnatally (98). The nutritionally deprived rat probably accelerates its postnatal growth by mitosis to allow for organ size increase, however, after two weeks postpartum growth is again slowed as maturity approaches with protein synthesis and degradation coming into equilibrium (98).

The complete explanation for pulmonary underdevelopment of malnourished neonates is complex and probably involves the cumulative effects of several mechanisms interreacting with one another, such as:

1) The reduced level of pituitary, adrenal, thyroid hormones; similar to effects observed by Blackburn.

2) Proteins needed to provide amino acids for fetal protein synthesis are in deficiency and may account for reduced levels of enzymes and hormones.

3) Caloric reductions result in less substrate availability for energy production necessary for cellular division and cellular synthesis.

All these mechanisms would seem to contribute to the slow growth and retarded differentiation experienced by malnourished newborn rats.

GLYCOGEN CONTENT OF THE LUNG

EPITHELIAL CELLS Glycogen is very abundant in the undifferentiated cuboidal cells lining the pulmonary tubules from day 17 to day 20 of gestation. The significance of epithelial glycogen has been elucidated by Sorokin who emphasized that energy necessary for fetal development may be provided by anaerobic respiration before the liver assumes a glycogenic function (29). Pulmonary glycogen consequently is observed in regions where cellular division is most rapid and since anaerobic respiration yields energy less efficiently than aerobic respiration; much more substrate must be present in the cells using this type of glycolysis. Epithelial mitosis in prenatal lung,which obtains energy by anaerobic glycolysis,needs copious quantities of glycogen stored in the epidermal cells to supply the amounts needed by the dividing cells. Glycogen is needed in the prenatal lung until epithelial mitotic rates subside and glycogen rapidly disappears from epithelial cells at day 20 and continues as epithelial differentiation progresses. Glycogen disappeared from epithelium by birth and is not a prominent feature of the postnatal lung. Cellular proliferation has been observed to be the greatest between days 17 and 20 and this is when glycogen is also most abundant (23). The differentiation of epithelial cells commences at day 20 and coincidentally there is when glycogen begins to decline; hence there appears to be an inverse relationship between epithelial glycogen content and differentiation which could serve as an indicator of normal pulmonary development in prenatal rat lung.

In starved animals, glycogen disappears much more slowly from epithelial cells. The demonstration of abundant glycogen after day 20 with retention up to 1 week after parturition indicates that glycogen utilization is retarded by severe dietary deprivation. The abnormal retention of glycogen suggests that the mitotic rate of epithelial cells is slowed, as well as subsequent cell maturation. It was shown that development of a functional air-blood barrier at birth depends upon rapid mitosis of epithelial cells up to day 20 with subsequent depletion of glycogen coincident with cytodifferentiation (23). Dietary deprivation of pregnant rats causes retarded cellular division and differentiation in the epithelium of the fetal rats which resulted in abnormal retention of epithelial glycogen beyond the time of birth. The unusually large quantity of glycogen remaining in the epithelium after day 20 of gestation may reflect a depressed enzyme system required for glycogen utilization, since metabolism in general is depressed (72, 97,99,100). Using this as an index of epithelial immaturity, diminished function may be implied up to 1 week postpartum when glycogen is still present in some cuboidal epithelial cells of starved rats.

MESENCHYMAL CELLS Mesenchymal glycogen, which is conspicuously absent in the normal fetal lung between days 17 to 21, and increases slightly up to birth as the epithelial glycogen rapidly declines. There is a substantial increase in mesenchymal glycogen a few days after birth as the mitotic rate of the mesenchyme begins to increase (23,29,70,72). Maternal starvation which slows the processes of epithelial division and differentiation also adversely affects the

normal appearance of mesenchymal glycogen. This is not a feature of fetal lung and it appears later than normal at approximately 5 days postpartum in the neonate.

The presence of glycogen in the mesenchymal cells shortly after birth is likely related to the postnatal growth phase of the lung. Growth and development of the lungs has been divided into three phases (74-77): Phase one, which begins 24 hours after parturition and lasts up to day 4 deals largely with simple lung expansion with little mitotic activity. Phase two deals with cellular proliferation beginning at day 4 and continuing for about 10 days. In this phase, the immature primary saccules are divided by growing secondary crests to form alveolar structures. The mitotic activity of the mesenchymal cells of the crests concurs with appearance of mesenchymal glycogen associated with the rapid growth of the secondary crests. It appears that these three factors are directly inter-related and are necessary for normal lung development. Secondary crest formation is related to the growth of mesenchymal derivates (77) and is dependent upon a rapid mitotic rate in the mesenchyme; this results in increased energy demands and consequently the accumulation of glycogen. These results have been observed by several investigators (29,70,72,77) and concur with the findings of this study which demonstrated the presence of mesenchymal glycogen in control lungs from early in postnatal life up to about day 14.

The later appearance of secondary crests observed in malnourished animals resulted in later septation of the primary saccules

and slower lung maturation. The later development of secondary crests may be directly related to the later accumulation of glycogen in the mesenchymal cells which caused the slower division and retarded differentiation of the lung postnatally. This sequence of events in postnatal life may reflect in part the retarded epithelial development observed in fetus and neonate, since it appears that mesenchymal glycogen does not begin to accumulate until most of the epithelial glycogen has disappeared, thus indicating the two events may be interrelated.

ACID PHOSPHATASE ACTIVITY IN THE LUNG

EPITHELIAL CELLS--NORMAL

Acid phosphatase in the rat lung performs several important functions, without which the rat could not survive. One of its major roles is to act as a degradative lysosomal enzyme found in the alveolar macrophages (15). An equally important function, although not until recently as well established, is its close association with surfactant synthesis by the type II epithelial cell. Recent evidence based on morphological, cytochemical and autoradiographic findings by several investigators clearly demonstrate an intimate relationsip between this enzyme in lamellar bodies and surfactant synthesis by the type II cell (9,11,14,25,27,40).

Electron microscopic studies on the developing rat lung by O'Hare et al. have revealed that lamellar bodies could be identified in differentiating type II cells as early as day 21 of gestation (70). Simultaneously with this observation in type II cells is the secretion of surfactant (71) and the appearance of hydrolases in the type II cell. Hydrolases activity increases in parallel with type II cell maturation and surfactant secretion (13). This evidence would appear to unite all three phenomena and suggests that they are all inter-related with one another. In further support of this theory Adamson and Bowden, using autoradiographic methods, have shown that it is the perilamellar membranes of the type II cells which are the active sites of surfactant synthesis(27). Acid phosphatase activity has been demonstrated cytochemically at the periphery of lamellar bodies in the perilamellar membrane (25,28), suggesting the involvement of acid

phosphatase in surfactant metabolism. The observations of Meban, Etherton and Bothom suggest that acid phosphatase is involved in the synthesis of dipalmitoyl Lecithin (25,28) but its exact role has yet to be elucidated. It is believed to act as a hydrolytic enzyme cleaving intermediate substances in the synthesis of dipalmitoyl lecithin from palmitic acid (24). Thus acid phosphatase activity in the developing rat lung may serve as an indicator of type II cell maturity and surfactant synthesis.

Acid phosphatase activity in the fetal rat lung was first detected on day 18 and steadily increased as parturition approached probably reflecting an increased demand for surfactant by the developing lung. Enzyme activity continually increased postnatally up to approximately day 10 postpartum, when it levelled off and remained constant. This suggests that enzymatically the lung was competent and relatively stable by two weeks. Morphologically, the lung also appears to be relatively mature by this time as the lung enters a stage of equilibrium. Ultracytochemical observations of fetal lung at day 22 and day 10 postnatally confirm the ultrastructural location of acid phosphatase in the pulmonary cells. On day 22, activity was confined to type II cells with most of the reaction products seen at the periphery of the lamellar bodies and in multivesicular bodies. This enzymatic evidence supports Sorokin's hypothesis that the two structures are in some way related (11). Postnatally, E.M. observations revealed a similar cellular location for acid phosphatase activity with a dense reaction product found predominantly around the lamellar bodies and in multivesicular bodies of type II cells. In addition to type II cells,

alveolar macrophages show strong reaction products in lysosomal bodies, indicating the presence of a functional lysosomal enzyme system in the lung postnatally.

EPITHELIAL CELLS--STARVED

In the starvation experiments, acid phosphatase development is drastically reduced and retarded in the fetus and neonate. This evidence would establish the enzymatic basis for the inhibition of pulmonary surfactant synthesis caused by acute dietary deprivation. The effects of food deprivation in enzyme development involved in surfactant synthesis is likely due to several interacting causes. Changes in the nutritional status would drastically reduce the level of circulating substrate concentrations impeding energy production and reducing the availability of necessary ingredients needed for the production of enzymes (83,86).

Acid phosphatase activity was depressed prenatally and remained much lower than normal in the controls at birth. This might be related to the slower mitotic rate of the epithelium, with their lingering glycogen contents, resulting in a retarded differentiation, which manifested itself in reduced enzyme levels in the epithelial cells. Gross et al. showed that acute starvation would significantly reduce the enzymatic activities of the alveolar cells which would inhibit surfactant synthesis (85). Faridy has also observed changes in the development of the surfactant system in the fetus caused by a reduced nutritional status of maternal rat imposed between days 17 and 21 of gestation. He found a reduction of 35 percent in the surfactant secretion of the neonate after starvation conditions (84) which

substantiates the present study in which maternal starvation causes slower differentiation and reduced acid phosphatase levels which could lead to a depressed surfactant synthesis. Maternal malnutrition during pregnancy could therefore greatly influence the functioning of the neonatal lungs in the critical perinatal period.

Postnatally acid phosphatase levels remained much lower in malnourished rats as compared to controls of the same age. At one week postpartum the level of enzyme activity showed a sharp increase and it began to approach control levels by two weeks postpartum. This probably reflects the increased feeding the newborn was experiencing at the expense of the mother, with the incorporation of the nutrients the lung development approached normal (81,96,98).

Ultracytochemical examination at day 22 prenatally, revealed some acid phosphatase activity in the multivesicular bodies, but none was observed in the perilamellar membranes. The reduced amount of acid phosphatase activity within the type II cell along with an actual reduced number of maternal cells result in much lower enzyme levels observed at birth. This is reflected in the relative immaturity of the lung, which could influence the animals ability to function, and perhaps be partially responsible for the high early postnatal mortality rate. On day 10 type II cells appear to have a normal amount of acid phosphatase activity around the lamellar bodies and in the multivesicular bodies, as indicated by the cytochemical results. The sequential pattern of glycogen depletion in epithelial cells followed by enzymatic development and cellular maturation takes place at a

relatively accelerated rate after 5 days postpartum and equilibrates at approximately two weeks. The increased acid phosphatase activity revealed at day 10 postpartum is probably due to this accelerated maturation rate.

There appears to be a critical point reached in the maturation of the lung cells when they are able to undergo a series of biochemical events culminating in the production of a functional lung. If lung development is impeded by malnutrition, or by any abnormal cause, then the lung may be too immature to function normally which may ultimately compromise the neonate. The biochemical immaturity may be due to the decreased activity of enzyme systems which perform essential roles in the maintenance of normal pulmonary function.

MACROPHAGES

Alveolar macrophages exhibit a high level of acid phosphatase activity, which functions as a degradative enzyme system within lysosomal bodies (6.13,14). Macrophages engulf protein debris and foreign material and digest them with acid phosphatase which is sequestered in the membrane bound lysosomes. The earliest alveolar macrophages can be identified in control rats is just before birth. The presence of acid phosphatase would clearly indicate the relative maturity of the macrophage.

Acid phosphatase activity develops early in the alveolar macrophages at approximately day 21 of gestation and can be identified positively in the lung by day 5 postpartum. This suggests that neonatal

rats have some resistance to pulmonary infections in early life since some mature alveolar macrophages are present at birth. On day 10 postpartum there are many positive macrophages present with a large number of densely reacting lysosomal bodies, indicating a well developed defense mechanism in the normal lung. Malnutrition slows the development of lysosomal acid phosphatase and weakly reactive macrophages are not noticeable until day 5 postpartum. At birth no reactive macrophages are seen ultracytochemically, but by day 10 a few reactive macrophages are observed. However, there does appear to be a definite reduction in number of reactive cells compared to the control. The reduction in enzymatic activity suggests that their function in particular protection against newborn infections may be altered in the early neonatal period. This may mean that the malnourished neonate would have a weaker macrophagic defense system and may be more susceptible to pulmonary infections.

PEROXIDASE ACTIVITY IN THE LUNG

NORMAL Peroxidase activity in the developing lung has not been thoroughly investigated, although it has been identified and located within pulmonary tissue (56,57,73). The exact function of peroxidase is still an enigma, although several hypotheses have been advanced (64,65,73). DeDuve was able to demonstrate that peroxisomes increased their oxidative respiratory rate in direct response to ambient oxygen tension, which suggests that the peroxisomes within type II cells are concerned with an oxidative protective function (56). This evidence has been substantiated by several other investigators who found that breathing oxygen in high concentrations led to adaptative changes within the lung which enables the animal to tolerate very high concentrations of oxygen (59-63). Cross has postulated the presence of an adaptative anti-oxidant defense system which is developed in newborn animals to permit survival (62). Goeckerman observed that large volumes of hydrogen peroxide may be generated in response to high oxygen concentrations and peroxidases may protect the type II cells by breaking down the peroxide (101). Evidence that peroxidase may have a protective role is largely circumstantial and is involved in the degradation of hydrogen peroxide formed within the cells (65). e.g. (1) there is evidence that young animals have more peroxidase than adult animals (62) and young animals are more resistent to high partial pressures of oxygen (59-61). (2) during adaptation, certain oxidative enzyme systems are involved and levels rise with adaptation in the type II cells (62,63,64,66). (3) It has been demonstrated that type II cells are resistent to oxidative injury, are able to proliferate while

type I cells are very vulnerable, and it is the type II cell which contains peroxidases (27,62,63).

Oxygen toxicity studies done by Adamson and Bowden have shown that in the epithelium, only the type II will survive high concentrations of oxygen. Type II cells will then proliferate and repair the pulmonary damage done to type I cells and are eventually transformed into functional type I cells (16,17,22,23).

The present study has shown that type II cells do contain peroxidase activity within their peroxisomes and that type I cells contain no peroxidase activity. All this circumstantial evidence suggests that peroxidase may play a protective role in oxygen toxicity and shields the type II cells making them more resistent to oxidative injury.

The relationship of peroxidase activity to the development of type II cells was examined in this investigation and it was found that, in normal rats, peroxidase appears shortly before birth and increases through the early postnatal period to a maximum level by day 10. The development of peroxidase activity in type II cells coincides with the development of another enzyme system, superoxide dismutase, as shown by Autor et al. (102). Superoxide dismutase catalyzes the dismutation of the oxygen free radical into hydrogen peroxide which must be broken down to less toxic forms by peroxidase. It may well be that these two enzyme system cooperate to provide an antioxidant adaptative defense system (62,64,65,66,102).

Crapo has shown that superoxide dismutase increase parallels the development of oxygen tolerance observed in rats exposed to 85 percent oxygen for 7 days (66). Rosenbaum et al. have observed that catalase activity parallels oxygen tolerance developed in high concentrations of oxygen, while Chow and Cross both indicated that the presence of peroxidase may be a key mechanism in protection from hyperoxia (64,66). It was observed that neonates can adapt more rapidly to changing oxygen tensions than can adult animals making older rats more susceptible to injury (59). This evidence suggests that peroxidase which shows increasing activity associated with cellular maturation may be obligatory to the newborn lung for the provision of protection against the relative hyperoxia experienced at birth when it changes from anaerobic intrauterine to aerobic extrauterine life.

In the control study, on day 22 of gestation, peroxidase activity was visualized with E.M. in the peroxisomes of type II cells and not in lamellar bodies. On day 10 activity was observed in the peroxisomes of type II cells and in leucocyte granules but not in macrophages. The finding of no significant peroxides activity in macrophages concurs with observations made by Biggar (55), who also found that macrophases do not contain peroxidase.

In the starvation studies, peroxidase activity was depressed in the fetus and did not develop until near parturition. Postnatal activity was reduced but a burst of synthesis was observed at approximately one week and continued to day 14 where peroxidase activity approached control levels. The reduced level of enzyme activity after starvation illustrates the effect of delayed cellular differentiation

and slower metabolic rate culminating in reduced protein synthesis. This probably produces enzyme deficiencies in the immature lung. Morphologically the cells may appear normal but functional maturity, as assessed by enzyme levels, indicate that the cells may exhibit defective cellular functions in the critical perinatal period.

If the hypothesis of a protective role for peroxidases is correct, abnormally low pulmonary levels in the first week of life could render the malnourished newborn more susceptible to oxidant lung injury.

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