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Preparation for Birth into an O_2-Rich Environment: the Antioxidant Enzymes in the Developing Rabbit Lung

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Summary

To determine if some specific "preparation for birth" occurs in the developing lung to help assure its successful adaptation to a comparatively O_2-rich world at birth, we measured the activities of the antioxidant enzymes in the developing lungs of rabbit fetuses from 10 d before parturition to several days after birth. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP) activities showed similar maturational patterns with significant increases in activity, compared with earlier gestational levels, during the last 3–5 d before birth. During the final days *in utero*, SOD and CAT activities increased by ~110% and lung GP activity by ~200%. There were no parallel changes in lung O_2 consumption demonstrable over this same prenatal period.

Abbreviations

CAT, catalase
GP, glutathione peroxidase
RDS, respiratory distress syndrome
SOD, superoxide dismutase

"I that am rudely stamp'd . . . unfinish'd, sent before my time into this breathing world, scarce half made up. . . ."
Richard III, Shakespeare

High concentrations of O_2 are toxic to the lungs of all species, including man. The principal biochemical defense against O_2-induced lung damage is generally accepted to be the antioxidant enzymes—SOD, CAT, and the GP system—and the lipid membrane constituent, Vitamin E (8, 15, 17, 41). Studies from our own and other laboratories have established positive correlations between relative resistance to hyperoxia and increased levels of some or all of the pulmonary antioxidant enzymes (4, 8, 15–17, 41). It was noted a few years ago that SOD, CAT, and GP activities were all higher in the lungs of newborn rats than in the lungs of 2-d premature rat fetuses (43). In another study, SOD activity in the lungs from a small sampling of premature infants (26–32 wk gestation) ($n = 3$) was found to be substantially lower than the SOD activity in the lungs of full-term newborns ($n = 4$) (1). These findings, combined with the fact that at birth the newborn leaves a hypoxic uterine environment (fetal arterial P_{O_2} ~20–25 mm Hg) (7, 29) to enter a relatively hyperoxic 21% O_2 environment, have made us wonder if some specific "preparation for birth" occurs in the developing lung to help assure its successful adaptation to a comparatively O_2-rich world at birth. To examine the question, we have measured the activities of the antioxidant enzymes in the developing lungs of rabbit fetuses at intervals from 10 d before parturition (gestation period = 31.5 d) to several days after birth. We report here the changes in these protective enzymes that occur over this extended developmental period.

MATERIALS AND METHODS

Animals. Pregnant California-strain rabbits were obtained from K-W Farms, Tice, FL. The exact breeding times (± 1 h) were provided by the supplier.

All the fetuses used were delivered on the same day by hysterotomy under ketamine:xylazine anesthesia (90 mg/kg:10 mg/kg) (Ketalar, Parke-Davis, Morris Plains, NJ; Rompun, Payvet Division-Cutter Labs, Shawnee, KS). All the pups used were sacrificed by an overdose of sodium pentobarbital administered intraperitoneally. The timed-gestations were interrupted at 21, 22, 26, 28, and 30 d. The pups used were <1-h old, 3–4-d-old, and 5–6-d-old. Adult does used for lung enzyme studies were sacrificed by pentobarbital overdose; the great vessels in the abdomen were then cut to exsanguinate the animals.

Biochemical studies. The methods used for lung preparation and for the biochemical assays—SOD (EC1.15.1.1.) (32), CAT (EC1.11.1.6.)(24), GP (EC1.11.1.9.)(34), DNA (35), and protein (38) content of the lung—have been previously described in detail (18). Briefly, the lungs were rapidly perfused free of blood with cold isotonic phosphate buffer (0.1 M potassium phosphate, 0.15 M potassium chloride, pH 7.4) via the pulmonary artery. The lungs were then weighed and homogenized in cold buffer (0.005 M potassium phosphate, pH 7.8) (1:25,w/v) with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY). Enzyme assays were done on the lung homogenates that had been frozen overnight. The SOD assay (32) measures the rate of reduction of cytochrome c at 550 nm, in the presence of 10^{-5} M cyanide (to inhibit cytochrome oxidase activity). Both cytosolic Cu-Zn SOD and mitochondrial Mn-SOD are measured by this method. CAT, present in the cytosol and peroxisomes of cells, was measured by the rate of reduction of H_2O_2 substrate, spectrophotometrically at 240 nm (24). GP activity was assayed spectrophotometrically at 340 nm by the rate of oxidation of NADPH (34). The assay mixture for measurement of this cytosolic enzyme includes cumene hydroperoxide as primary substrate, with sodium azide added to inhibit contributing activity from CAT enzyme. Purified enzymes and purified calf thymus DNA and bovine serum albumin used as standards for the assays were obtained from Boehringer-Mannheim, Indianapolis, IN (GP), Cal-Biochem, La Jolla, CA (SOD), and Sigma Co., St. Louis, MO (all other reagents).

O_2-consumption studies were done on fresh lung slices from fetal and neonatal rabbits according to previously described protocol (22). Approximately 100 mg (wet wt) of 1-mm thick lung slices (McIlwain Tissue Chopper, Brinkmann Instruments, Westbury, NY) were placed in a stirred, temperature-controlled

(37°C) cuvette containing 3 ml Krebs Ringer solution (5 mM glucose) buffered to pH 7.4 with 10 mM Hepes. The mixture was equilibrated with air. Oxygen consumption was measured with a YSI Oxygen Analyzer (Yellow Springs Instruments, Yellow Springs, OH) connected to an Omniscribe strip chart recorder (Houston Instruments, Austin, TX). Lung tissue O_2 consumption was calculated and expressed as microliters O_2 consumed per hour per microgram DNA. All samples were run in duplicate or triplicate.

For statistical analyses of results, Student's unpaired *t* test was used with a level of $P < 0.05$ chosen to indicate significant differences.

RESULTS

Figure 1 shows the activities of SOD, CAT, and GP expressed as enzyme units per mg DNA. As can be seen in the Figure, the maturational pattern for each of the antioxidant enzymes was essentially similar, with significant increases in activity above earlier gestational levels occurring during the last 3–5 d before birth. In Fig. 2, the data is expressed as percentage change in specific activity of the three enzymes compared with the values measured at 21–22 d gestation. During the final days *in utero*, the SOD and CAT activities increased approximately 110% and lung GP activity increased by 200%. After birth, all of the enzymes increased to even higher levels. Adult levels of the enzymes (from lungs of the does) were as follows (mean ± 1 SD):

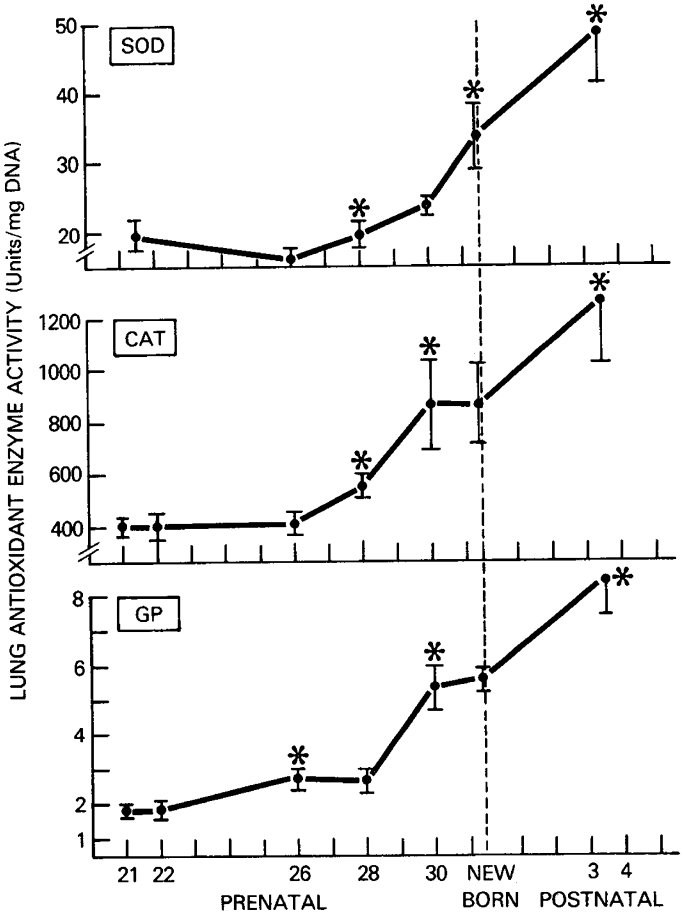

Fig. 1. Antioxidant enzyme activity (U/mg DNA) in lungs of fetal and neonatal rabbits. Values for superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP) are mean activity levels from three to four rabbit lungs at each age point tested. *Bars* represent ± 1 SE and *$P < 0.05$ *versus* preceding age point value. All three enzymes show pattern of significant increases in activity between d 26–28 of gestation and birth (31.5 d).

SOD, 76 ± 10 U/mg DNA (125% higher than newborn lung levels); CAT, 2095 ± 390 (↑150%); and GP, 16.9 ± 4.2 (↑200%). That the elevations in antioxidant enzyme activities which occurred during the last few days before birth were not merely a consequence of decreases in the amount of DNA, is indicated by the progressively increasing lung DNA content measured throughout the gestational period examined: mean values for the 21–22-, 26-, 28-, 30-d, and newborn rabbit lungs were, respectively, 0.82, 1.17, 3.48, 4.08, and 4.40 mg DNA/lung. When the enzyme activities were expressed per g lung weight, or per mg lung protein, a very similar late maturational pattern of increasing activity was observed. Only the absolute values differed from the data illustrated in Figures 1 and 2.

Superimposed on the antioxidant enzyme activity changes in Figure 2 is another curve which represents the changes in lung lecithin concentration (mg/g dry wt) during the latter part of gestation. These data, abstracted from studies of Farrell (11), and converted into percentage change from the earliest gestational age point value, reflect the time course of maturation of the lung surfactant system. It is apparent from the Figure that there is a close similarity between the developmental pattern of the surfactant and the antioxidant enzyme systems in the fetal rabbit lung.

Figure 3 shows the O_2 consumption of the lung slices from the fetal and neonatal rabbits. We wanted to determine whether prenatal changes in O_2 consumption would parallel prenatal changes in lung antioxidant enzyme levels, but the results shown in Figure 3 did not support this hypothesis. The fetal lung O_2 consumption was found to vary little with gestational age. Values for the last 10 d *in utero* ranged between 75–125 μl O_2 consumed per hour per microgram DNA. It was only after birth that any dramatic increase in O_2 consumption by the lungs was apparent.

DISCUSSION

The prenatal changes in lung antioxidant enzyme levels found in the rabbit are consistent with those observed in a more limited study in the fetal and newborn rat (43). The results of both of these animal model studies are, in turn, consistent with a human lung study, which showed lower antioxidant enzyme (SOD) activity in premature compared with full-term infants' lungs (1). When these findings are combined with the information that the principal non-enzymatic antioxidant factor, vitamin E, is deficient in prematurely born compared with full-term newborns, it seems reasonable to consider the late fetal changes in the systems that serve to protect the lung from hyperoxia as a "preparation for birth" phenomenon. The increase in the antioxidant defense capacity of the lung appears to be one of the necessary *in utero* maturational changes that help to assure successful adaptation of the lung to its new environment and its new respiratory function at birth.

The chronology of maturation of the lung antioxidant enzyme system appears to be quite similar to the prenatal maturation of the surfactant system of the lung. Many studies of various species, including man, have demonstrated the sequence of morphologic and biochemical changes occurring in the lung during the last 10–20% of gestation that lead to normal surfactant production and secretion (11, 14, 28, 37). These similar developmental patterns of the protective antioxidant and the surfactant systems (in the rabbit) raise the interesting question of whether the increase in the antioxidant enzymes in late gestation occurs in response to the same types of hormonal stimuli that are believed to govern the onset of accelerated surfactant synthesis. Although adrenal glucocorticoids appear to be the primary hormones that stimulate activity of the synthetic enzymes involved in surfactant production, a variety of other hormones have also been demonstrated to influence surfactant synthesis, including thyroxine, estrogen, and β-adrenergic agonists (11, 13, 14, 25, 26, 36). An important question in future studies is whether the same hormonal agents act to stimulate increased synthesis of cytosolic and/or mitochondrial SOD and cystosolic CAT and GP. Eren-

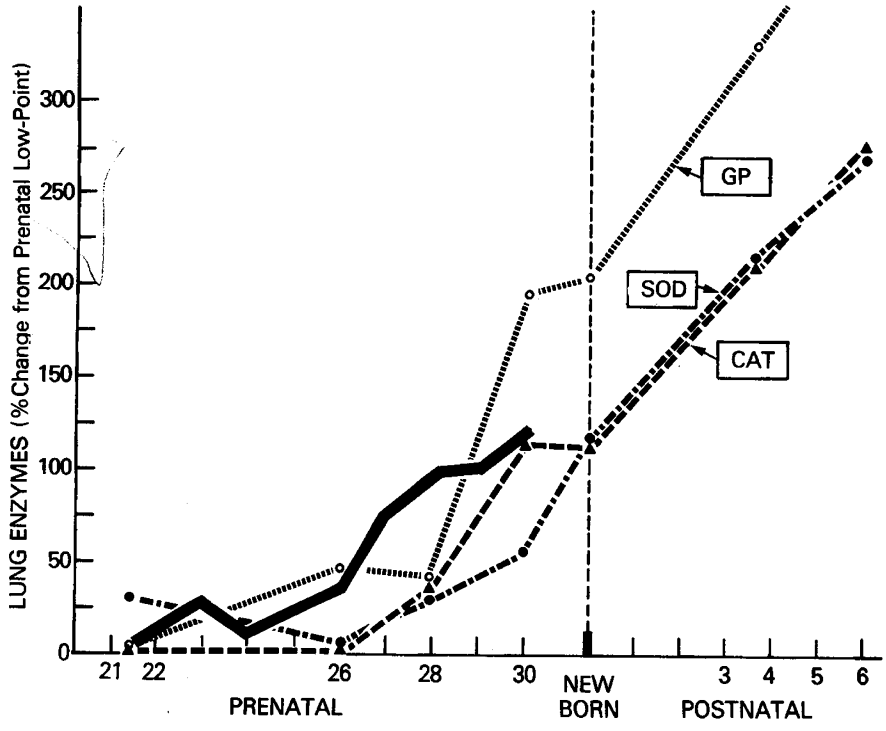

Fig. 2. Antioxidant enzyme activity in lungs of fetal and neonatal rabbits (data from Fig. 1) expressed as percentage increase in activity above the baseline value, which is the lowest activity measured over the gestational period tested. Mean values for superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP) (from three to four rabbit lungs per age point) show pattern of marked increases during the last few days of gestation (SOD and CAT increase ~110% and GP increases ~200%). Postnatally, all the antioxidant enzymes show continued elevations in activity. Also represented in the Figure, by the *thick solid-line curve*, are the percentage changes in lung lecithin concentration (mg/g dry wt), adapted from data from Farrell (11).

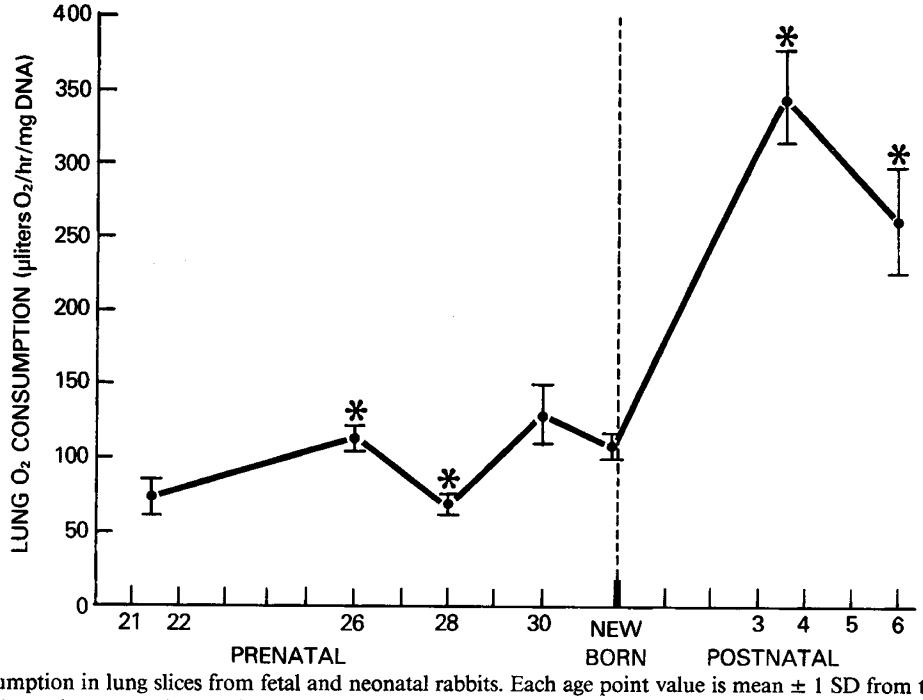

Fig. 3. Oxygen consumption in lung slices from fetal and neonatal rabbits. Each age point value is mean ± 1 SD from two to four rabbit lungs. *$P < 0.05$ compared with previous age point. Unlike the antioxidant enzymes (Figs. 1 and 2), there is no definite pattern of progressively increasing values for O_2 consumption during the last several days before birth.

berg *et al.* (9) found low SOD activity in the lungs of fetal sheep made hypothyroid earlier in gestation; SOD levels were restored to control fetal levels by thyroid hormone treatment. After birth, lung antioxidant enzyme levels do not appear to be modified by either thyroxine or glucocorticoid treatment (44).

We can hypothesize that the prenatal increases in lung SOD, CAT, and GP activities may be related to the late prenatal differentiation of the type II pneumocyte, which is the cell source of surfactant production (5, 27, 28, 42). This hypothesis is predicated primarily on the evidence that the type II cell is also

the alveolar lining cell most resistant to hyperoxic exposure (3, 10, 30). High levels of activity of the antioxidant enzymes in the late-maturing type II cells could help account for their relatively marked tolerance to O_2-induced damage compared with the other cell types in the gas exchange region of the lung (type I cells, endothelial cells).

There is some experimental evidence to support the concept that the normal stimulus for increased synthesis of the antioxidant enzymes is an intracellular increase of O_2^-, H_2O_2, and lipid peroxides, the substrates which these enzymes detoxify (19, 20, 23). These reactive species are normal products of O_2 consumption and aerobic metabolism in the cell. Their concentrations are believed to rise considerably under hyperoxic conditions, and under conditions associated with increased O_2 consumption (19–21, 23, 31). For this reason, we prepared lung slices from fetal and neonatal rabbits and measured their O_2 consumption to try to determine whether significant prenatal increases in lung O_2 consumption occurred, for this could provide some biochemical mechanism to explain the augmentation of antioxidant enzyme levels just before birth. The results of this study (Fig. 3) did not support this hypothesis; thus, elucidation of the stimulus (hormonal? genetic?) for the late prenatal rise in the antioxidant defense system necessitates future study.

As would be expected, the more prematurely born the human infant, the greater are the chances for immaturity of the surfactant system, and the greater the incidence of hyaline membrane disease or RDS (2, 11, 12, 14). If the lung's innate defenses against hyperoxia are also deficient in the very premature newborn (1), this could contribute to the overall pathology seen in the RDS infant's lung. Premature infants with low levels of antioxidant enzymes may be ill-adapted to deal with the 21% alveolar O_2 level that they are suddenly exposed to at birth. In fact, it has been suggested that RDS itself is a manifestation of O_2-induced lung injury (4, 6, 33, 39). This idea has not received much attention in the recent literature because the development of RDS in the premature infant has been correlated so closely with evidence for markedly deficient lung surfactant, which alone can lead to serious gas exchange problems and life-threatening hypoxemia in these tiny newborns. Low levels of lung antioxidant enzymes may assume greater clinical importance when, within hours after birth, the more seriously ill premature infants begin to require vigorous respiratory support with supplemental O_2 therapy. In these situations, if the premature infant is deficient in the defensive lung enzymes required for maximal protection from 21% O_2, then exposure to the considerably higher O_2 concentrations which may be required clinically, would be very likely to prove toxic to his compromised lung. Hyperoxia-induced lung damage could contribute significantly to morbidity and influence the eventual clinical outcome in these infants.

In a recent NIH-sponsored workshop on bronchopulmonary dysplasia, the idea was discussed that a considerable part of the lung pathology in sick premature infants with RDS may indeed be due to superimposed O_2-toxicity (40). No reason was provided, however, to explain the RDS infant's seeming susceptibility to O_2-induced lung complications. The answer to this important question is very likely a complex one, but we think it is reasonable to suggest that at least part of the explanation for the susceptibility of the infant with RDS to the damaging effects of hyperoxic treatment could be the interruption of the normal development of the antioxidant defensive capacities of the lung due to premature birth.

In conclusion, as is the case with the well studied surfactant system of the developing lung, the antioxidant enzyme system of the rabbit lung shows a rapid rise in activity only late in gestation. These prenatal increases in biochemical activity in both systems appear to be important "preparations for birth" that will allow the newborn's lung to assume efficient functioning in the relatively O_2-rich environment that greets it at birth. The prematurely born may be handicapped in its ability to tolerate hyperoxic exposure and to resist serious O_2-induced lung pathology.

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A Comparison of the Sensitivities of Neonatal Ovine Pulmonary and Femoral Arteries to *l*-Norepinephrine Stimulation

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Summary

We compared the concentration-response relationship of intralobar pulmonary arteries to *l*-norepinephrine with that of femoral arteries from newborn lambs. In addition, the effect of inhibition of the neuronal and extra-neuronal uptake mechanisms on these concentration-response relationships was examined.

Concentration-response curves on 10 intralobar pulmonary arteries were performed with and without inhibition of the uptake mechanisms. Uptake inhibition shifts the curve to the left; thus, the $-\log EC_{50}$ with uptake inhibition (6.63 ± 0.15) is greater than that without uptake inhibition (6.13 ± 0.14). Similar curves on 10 femoral arteries demonstrated that the $-\log EC_{50}$ with and without uptake inhibition (6.20 ± 0.13 and 6.00 ± 0.04, respectively) are not statistically different.

Because, in the presence of intact uptake mechanisms the concentration of *l*-norepinephrine in the α-receptor microenvironment is less than that placed in the organ bath, the appropriate comparison of pulmonary and femoral arterial response to *l*-norepinephrine stimulation requires uptake inhibition in both vessels. The concentration-response curve with uptake inhibition for the intralobar pulmonary artery is significantly to the left of that for the femoral; the $-\log EC_{50}$ for the pulmonary vessel (6.63 ± 0.15) is greater than that of the femoral vessel (6.20 ± 0.13). The intralobar pulmonary artery *in vitro* is more sensitive to *l*-norepinephrine stimulation than is the femoral.

Persistent pulmonary vasoconstriction is a serious, often life threatening problem in the neonatal period (5, 6, 21, 25, 26). There is evidence which suggests that α-adrenergic constriction may be involved in the control of the neonatal pulmonary circulation (4, 14). Tolazoline, an α-adrenergic antagonist, has been used successfully to manage some babies with persistent pulmonary vasoconstriction (11, 17) although the therapeutic benefit of this drug also could be related to its histaminergic effects (10). Much remains to be learned about the basic physiology and pharmacology of the newborn pulmonary vascular α-adrenergic system. Little is known about the relative sensitivities of various vascular beds in the neonate to α-adrenergic stimulation. Differences in sensitivity of different vascular beds to adrenergic agonists such as l-norepinephrine could have physiologic consequences. As an initial approach to this problem, we compared the concentration-response relationship to l-norepinephrine of intralobar pulmonary arteries with that of femoral arteries from newborn lambs. In addition, as a prerequisite to this study, the effect of inhibition of the neuronal and extra-neuronal uptake mechanisms on these concentration-response relationships was examined. Differences between neonatal pulmonary and femoral arteries were found in both the concentration-response relationship and the effect of inhibition of the uptake systems.

MATERIALS AND METHODS

Fourteen neonatal lambs (age, 2–8 d; average 4 d) were anesthetized with chloralose (50 mg/kg; Sigma Chemical Co., St. Louis, MO). Femoral arteries were placed in Krebs-bicarbonate buffer; pH 7.4, bubbled with 95% O_2–5% CO_2. The left lung was removed, and the pulmonary arterial tree was dissected to the