In Vivo Protein Secretion by Lung

EVIDENCE FOR ACTIVE SECRETION AND INTERSPECIES DIFFERENCES

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Abstract The present study is an attempt to determine (a) if the lung actively secretes protein into the surface-active fraction of lung lavage returns; (b) if there are interspecies differences in this secretory activity; and (c) if the amount of nonradioactive protein in the lavage surface-active fraction shows interspecies variation.

I found that pilocarpine stimulates the release of radioactive protein into the lavage surface-active fraction of rabbits and that this pilocarpine effect is completely blocked by atropine. Inhibition of lung oxygen consumption by iodoacetate is associated with a dose-dependent inhibition of the pilocarpine-induced secretion. Microtubules may be involved in this secretory process because colchicine inhibits the pilocarpine effect.

Of the radioactive protein in the total surface-active fraction (tissue plus lavage returns), a greater percent appears in the lavage surface-active fraction at 2 and 4 h, after a pulsed injection of [U-14C]leucine, in the mouse than in the rat, which in turn has a greater amount than the rabbit. There is also a difference in the amount of nonradioactive protein per square meter of alveolar surface area in the lavage surface-active fraction of different species: mouse > rat > rabbit > cat > dog. The amount of nonradioactive protein per square meter of alveolar surface area in the lavage surface-active fraction is directly proportional to the species respiratory rate; the log of the nonradioactive protein in the lavage surface-active fraction is inversely proportional to the log of the species alveolar diameter.

I conclude that the lung actively secretes protein into the lavage surface-active fraction, that this secretion is under neurohumoral regulation, and that respiratory rate and alveolar size may influence this secretory activity and the amount of protein in this surface-active fraction.

Introduction

Current concepts indicate that the lung is lined by a duplex extracellular layer that contains a lipoprotein(s) (pulmonary surfactant). Because of its surface tension-lowering effect, pulmonary surfactant allows alveoli to remain open at low transpulmonary pressures (1–3). To produce this extracellular layer, lung cells must be able to synthesize and secrete molecules that are components of this extracellular material.

A large body of work has shown that the lung can utilize various substrates, such as glucose, acetate, palmitate, and glycerol, to form phospholipids (4–11). Lung slices can incorporate radioactive glucose into glycoproteins (12) and radioactive leucine into protein (13), and more specifically into protein found in a surface-active lung fraction (14). Thus, it seems clear that lung tissue possesses the ability to synthesize components of pulmonary surfactant.

In addition, studies have provided kinetic evidence for intracellular protein transport in lung (13) and radioautographic work has shown that protein (15, 16) and lipid (16) transport take place in the granular pneumocyte, the cell believed to be the site of synthesis of surfactant. Newly synthesized radioactive protein is released by lung slices, and this release is inhibited by low temperature or cyanide and stimulated by epinephrine, which indicates that this release is an active secretory process (13). In vivo studies are consistent with
a precursor-successor relationship between cellular lipid (17-21) and protein, including protein in a surface-active fraction (22), and similar fractions obtained by lung lavage.

The present study was undertaken to examine factors that influence the appearance of radioactive protein in a surface-active fraction obtained from lung lavage returns and hence, presumably, from the internal surface of terminal lung units. In particular I wished to examine the influence of a cholinomimetic alkaloid (pilocarpine), its antagonist (atropine), a metabolic inhibitor (iodoacetate), and a disruptor of microtubules (colchicine) on the release of protein into a surface-active fraction obtained by lung lavage. I also wished to determine if there is any difference in the amount of protein in the lavage surface-active fraction per square meter of alveolar surface in animals of different species.

METHODS

**Animals.** I used male New Zealand albino rabbits (Ginrich Animal Supply, Frederickburg, Pa.), male Long-Evans descent hooded rats (Blue Spruce Farms, Inc., Alta- mont, N. Y.), male black mice, strain C57BL/6J, (Jackson Laboratories, Bar Harbor, Me.), and mongrel cats and dogs of either sex (Leach Kennels, Chase City, Va.). All were allowed food and water ad libitum. The animals were sacrificed by exsanguination after the i.p. injection of sodium pentobarbital, 50 mg/kg. Before the lungs were removed from the chest, the left atrium was cut and the pulmonary artery perfused with cold 0.15 M NaCl until the lungs appeared bloodless.

**Purification studies in rabbits.** These animals weighed 2.7±0.3 kg (mean±SD). To "pulse-label" lung protein, I injected 0.1 ml of l-[U-14C]leucine (17×10^-4 μmol; sp act 280 μCi/μmol) (New England Nuclear, Boston, Mass.), dissolved in 0.15 M NaCl (saline) into an ear vein. This was followed in 4 min by the intravenous injection of 3.2 μmol of l-[14C]leucine in 0.2 ml of saline. 56 min after the [14C]leucine injection, the rabbits were given either 1.0 ml of pilocarpine (3 mg/kg body wt in saline), or saline alone, i.p. This was repeated after an additional 30 min and the rabbits were sacrificed 30 min after this second injection. In some experiments atropine sulfate (4 mg/kg body wt) in 0.1 ml of saline or 0.1 ml of saline alone was immediately given by vein after each injection of pilocarpine. In other experiments I injected colchicine (0.5 mg/kg body wt) in 0.1 ml of saline, or 0.1 ml of saline i.v. 16 h before the pulse-labeling procedure.

After the rabbits were sacrificed and their lungs perfused with cold saline, the lungs were removed intact from the chest, separated from the thoracic contents, and lavaged with cold saline containing 10^-4 M Tris at pH 7.2. In preliminary experiments I lavaged each pair of lungs 10 times with 50 ml of fresh saline each time. After each injection of 50 ml into the clamped trachea, the clamp was removed and the lung emptied by its own recoil and gentle kneading. When the results of these preliminary experiments were known, I performed all rabbit experiments with five lavages of 50 ml each.

After the lung lavages I removed all the large bronchi and vessels and then minced and homogenized the lung in cold saline-Tris solution by 30 passes of a motor-driven Teflon pestle in a glass homogenizing vessel. To obtain the surface-active fraction, the lung homogenate and the lung lavage material were then separately subjected to the isolation procedure previously used (14), a modification of the method described by Klein and Margolis (23). Carrier albumin was added to the lavage and tissue surface-active fraction and protein was precipitated with trichloroacetic acid (TCA). The precipitated material was extracted with lipid solvents and hot TCA and assayed for radioactivity as previously described (24, 25).

To determine the percent distribution of radioactive protein between the lavage and tissue surface-active fractions, I divided the radioactivity in disintegrations per minute in the protein of the lavage surface-active fraction by the combined radioactivity of the lavage and tissue surface-active fractions and multiplied this figure by 100 to convert it to a percentage.

This approach requires that the labeling of the protein in the tissue surface-active fraction occur as a discrete short pulse and that continued incorporation of the label be prevented. To ascertain if this was the case I performed preliminary pulse-labeling experiments as described above; rabbits received an i.v. pulse-chase and rats an i.p. pulse-chase. The animals were then sacrificed at 0, 60, and 120 min after the injection of the chase of [14C]leucine (n=3 for rats and rabbits at each time). The protein-specific radioactivity of the surface-active fraction for rats at these times was 440±10, 367±8, and 368±15 dpm/mg protein; for rabbits the figures were 107±5, 90±10, and 87±7 dpm/mg protein, respectively. These studies show the pulse-labeling procedures are effective because there isn't any rise in the protein-specific radioactivity of the surface-active fraction after the i.v. or i.p. injection of nonradioactive leucine.

Since pilocarpine stimulates secretion by tracheobronchial glands (26), I sought to examine the possibility that radioactive material secreted by tracheobronchial mucus glands might have contaminated the surface-active fraction. I therefore pulse-labeled rabbits with radioactive leucine, gave them pilocarpine as described above, and then sacrificed them. I ligated the right and left main bronchi and lavaged the trachea and main bronchi with the same volume of saline used to lavage the entire lung in the intact lung experiments. This tracheal lavage material from the pilocarpine-treated radioactive rabbits was thoroughly mixed with the surface-active fraction isolated from nonradioactive rabbit lungs. I then reisolated the surface-active fraction and assayed it for radioactive material that was insoluble in cold and hot TCA and in lipid solvents (14). I failed to detect any radioactivity by the same assay procedure I used for the secretory studies.

To determine if the pilocarpine-induced apparent increase in secretion by the lung might in fact have been due to a higher protein specific activity of blood proteins, I measured the influence of pilocarpine on the protein specific activity of blood. To do this I pulse-labeled six rabbits with [14C]leucine. Half of these rabbits received pilocarpine, as described above, and the other three received only saline. I withdrew venous blood from the rabbits at 0, 60, and 120 min after the pulse-labeling and assayed it for its protein specific radioactivity (24). The protein specific activity of blood from control rabbits at these times was 8±1, 14±3, and 18±5 dpm/mg protein (mean±SEM); the values for pilocarpine treated rabbits are 7±2, 15±4, and 17±3 dpm/mg protein. Finally, in an attempt to examine the influence of pilocarpine on the surface-active fraction, I injected rabbits with radioactive leucine, as described above, but did not follow with an injection of non-
radioactive leucine. The animals \( (n = 3) \) were sacrificed after 1 h and the surface-active fraction isolated. This was divided into samples of known protein radioactivity, which were then mixed with lung homogenates and lung lavage returns from three nonradioactive pilocarpine-treated rabbits and three rabbits untreated with pilocarpine. The surface-active fraction was then isolated from these lavage returns, homogenates and assayed for protein radioactivity. The recovery from the lavage returns were 92\pm3\% (mean\pmSEM) and 90\pm4\% for control and pilocarpine-injected rabbits, and 85\pm5\% and 87\pm7\% for the homogenates of control and pilocarpine-treated rabbits, respectively.

Time course studies on radioactive protein secretion in rabbits, rats, and mice. These experiments involved a pulse-labeling procedure but not agents to stimulate or inhibit secretion. Rabbits were given a pulse of radioactive leucine and their lungs were lavaged as described above. Rats were given 0.1 ml of \( l-[U-{ }^{14}C] \) leucine (17.5 \times 10^{-6} \mumol; sp act 280 \mu Ci/\mu mol) i.p.; 4 min later they received 0.2 ml of \( l-[U-{ }^{14}C] \) leucine (3.2 \mu mol) i.p. Mice were given 0.05 ml of \( l-[U-{ }^{14}C] \) leucine (8.9 \times 10^{-6} \mumol, sp act \mu Ci/\mu mol) i.p., followed 4 min later by 0.1 ml of \( l-[U-{ }^{14}C] \) leucine (1.6 \mu mol) i.p. Since I expressed the protein radioactivity in the lavage surface-active fraction as a percent of the protein radioactivity in the lavage plus the tissue surface-active fraction, these different routes of administering the radioactive substrate would not influence the results.

The rabbit lungs were lavaged as described. The rat lungs were lavaged five times with 5 ml of cold saline containing 10^{-3} M tris buffer. Mouse lungs were lavaged four times with 0.5 ml of this lavage solution for each lavage. After each lavage the trachea was unclamped and the lung emptied by its own recoil and gentle kneading. The lungs and lavage returns were then treated as described for rabbits to isolate the surface-active fractions and to assay their protein radioactivity. In this part of the study the animal weights (mean\pmSD) were: rabbits 2.7\pm0.2 kg, rats 0.305\pm0.04 kg, mice 0.025\pm0.002 kg.

**Lung lavage for nonradioactive protein.** For these experiments we used mice, rats, rabbits, cats, and dogs. In the mice, rats, and rabbits, lung lavage was performed as described. In dogs and cats, the lungs were inflated with enough lavage fluid to smoothly inflate all areas. This volume of solution was then used for four additional lavages with fresh solution each time. In those experiments with the dogs and cats where I wished to determine the percent of protein in the total surface-active fraction (lavage plus tissue) in the lavage returns, I lavaged a lobe and homogenized and isolated the tissue surface-active fraction from that lobe. To determine the percent distribution of nonradioactive protein between the lavage and tissue surface-active fractions, I divided the amount of protein in the lavage surface-active fraction by the amount of protein in the lavage and tissue surface-active fraction and multiplied the quotient by 100 to convert it to a percentage.

In those experiments where I wished to compare the amount of protein in the lavage surface-active fraction to the species alveolar surface area, I lavaged the entire dog or cat lung but did not make any measurements of the tissue surface-active fraction. The lavage returns were collected and the surface-active fraction was isolated from the lavage returns as described above. In this section of the study, protein was measured directly on the surface-active fraction without the addition of carrier albumin and without the acid precipitation steps. I used crystallized albumin (Mann Research Laboratories Inc., New York) as the standard for the assay (27).

**Oxygen consumption.** To obtain lungs to measure oxygen consumption \((O_{2})\) the rabbits were anesthetized with Nembutal (pentobarbital, Abbott Laboratories, North Chicago, Ill.) (30 mg/kg i.v.) and then sacrificed as described above. To measure lung \( O_{2} \), the lungs were removed from the thorax, rinsed externally in cold Krebs-Ringer phosphate medium (KRP) 1 and sliced with a McClymont tissue slicer (Brinkmann Instruments, Inc., Westbury, N. Y.), set to make slices 1.0 mm thick. The slices were weighed and about 200 mg of tissue was placed in Warburg flasks containing 2.5 ml KRP medium. The flask center well contained 0.2 ml of 10\% KOH and a 2-cm square paper strip. The flasks were attached to a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.) at 40OC, and shaken at a frequency of 120/min. The sidearms were then closed and the flasks allowed to equilibrate for 20 min. Measurements of oxygen consumption were then made every 10 min for 60 min. At the conclusion of the incubation the tissue was chilled and 2.5 ml of cold 20\% TCA were added. The lung was then homogenized and assayed for DNA as previously described (28). \( O_{2} \) is expressed as microliters \( O_{2} \) consumed per hour per milligram DNA.

**Statistical calculations.** Comparisons of two samples were made by an unpaired t-test analysis. Regression lines were calculated by the least squares method (29).

**RESULTS**

**Influence of experimental conditions on the recovery of protein and fluid from lung lavage returns.** In all species studied in preliminary experiments (rabbit, rat, mouse, cat, and dog), slightly more than 90\% of the protein in the surface-active fraction recovered after 10 separate lavages is found in the first three lavage returns. I therefore used five lavages in all experiments in all species studied. The various experimental conditions used in the rabbit experiments did not influence the volume of fluid recovered after five separate lavages. Thus in saline \((n = 6)\), pilocarpine \((n = 4)\), pilocarpine plus atropine \((n = 3)\), colchicine plus pilocarpine \((n = 4)\), and iodoacetate plus pilocarpine \((n = 6)\), after five separate lavages of 50 ml each, the volume recovered was 90.6\%\pm1.4\% (mean\pmSEM), 89.1\%\pm4.8\%, 83.4\%\pm5.7\%, 93.5\%\pm1.5\%, and 81.9\%\pm4.9\%, respectively. In experiments where five separate lavages were performed the volume recovered in different species, as a percent of the volume injected, was mouse \((n = 4)\) 52\%\pm2\% (mean\pmSEM), rat \((n = 18)\) 82.3\%\pm1.1\%, rabbit \((n = 38)\) 88.7\%\pm1.0\%, cat \((n = 6)\) 95.3\%\pm2.9\%, and dog \((n = 6)\) 89.0\%\pm3.6\%. There is very little difference in the volume of lavage fluid recovered from different species except for the mouse. I think the low recovery from the mouse may have been caused by the difficulty experienced in working with the small mouse trachea.

**Stimulation and inhibition of protein secretion.** Pilocarpine produced a 2.5-fold increase in the percentage

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1*Abbreviations used in this paper: DPL, dipalmityl lecithin; KRP, Krebs-Ringer phosphate buffer; TCA, trichloroacetic acid.*

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Species influence on secretion and on the amount of protein in the lavage surface-active fraction. The appearance of radioactive protein in the lavage surface-active fraction varies between species. At each time studied I found a greater percent of the radioactive protein in the surface-active fraction in the lavage material in the mouse than the rat; the rat has a greater percent in the lavage surface-active fraction than the rabbit (Fig. 3).

I found a linear relation between the log of the nonradioactive protein in the lavage surface-active fraction and the log of the species alveolar area obtained from the literature (Fig. 4a) (33). The smaller species have more protein in the lavage surface-active fraction per square meter of alveolar surface area than the larger species and the amount of protein in the lavage surface-active fraction per square meter of alveolar surface area is related in a direct linear manner with the species respiratory rate (Fig. 4b). The log of the protein in the lavage surface-active fraction varies inversely with the log of the species alveolar diameter (Fig. 4c). The values for respiratory rate (34) and alveolar diameter (35) were obtained from the literature. Of the protein in the total surface-active fraction (lavage plus tissue), there is a direct linear relationship between the percent in the lavage surface-active fraction and the species respiratory rate (Fig. 5a); there is an inverse relation with the species alveolar diameter (Fig. 5b).

Table I

Influence of Stimulants and Inhibitors of Secretion on Lung Oxygen Consumption

<table>
<thead>
<tr>
<th>Agent</th>
<th>Oxygen consumption (μl O₂·h⁻¹·mg DNA⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (9)</td>
<td>150.4±4.1</td>
</tr>
<tr>
<td>Pilocarpine (4)</td>
<td>141.2±6.0</td>
</tr>
<tr>
<td>Pilocarpine and atropine (4)</td>
<td>142.3±5.6</td>
</tr>
<tr>
<td>Colchicine (4)</td>
<td>153.5±11.3</td>
</tr>
</tbody>
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All rabbits were given these agents, as described in Fig. 1. All measurements of QO₂ were made in triplicate. The numbers in parentheses indicate the number of animals used. Mean ±SEM are given. None of the differences between groups are statistically significant.
DISCUSSION

Studies on a wide variety of tissues have defined the characteristics of cell systems that secrete protein (36). In general these exhibit the following features: (a) they respond to certain chemicals or hormones, such as various cholinergic agents, with an increase in secretion; (b) energy, usually derived from oxidative metabolism, is essential for secretion, as evidenced by a decrease in the secretory response to a stimulant when metabolism is inhibited; (c) the secreted molecules are derived directly from intracellular storage granules. The present work and other recently reported biochemical and ultrastructural studies provide evidence that the intact lung meets these criteria. I have shown that the administration of pilocarpine, a cholinergic agent, results in an increase in the relative amount of radioactive protein in the surface-active fraction of the alveolar compartment and that this response can be blocked by atropine. Pilocarpine and atropine have a similar effect on phospholipid secretion (37, 38). In addition, blocking this pilocarpine effect with iodoacetate, which inhibits lung oxygen consumption, indicates that the stimulatory effect of pilocarpine is influenced by the level of the lung’s oxidative metabolism.

Evidence that the secreted proteins and phospholipids are derived directly from intracellular storage granules is less direct. Goldenberg, Buckingham, and Sommers concluded from nonquantitative morphological observations, that pilocarpine stimulates secretion of lamellar body contents into the alveolar lumen (39). This conclusion received support from recent stereological studies that report that after pilocarpine administration there is a decrease in the volume density of lamellar bodies per granular pneumocyte profile; atropine blocks this response (37). These morphological studies and the evidence that lamellar bodies are rich in phospholipid and contain protein (40) support the concept that the secreted phospholipids and proteins are derived, at least in part, from the intracellular storage granules that the lamellar bodies are considered to represent.

Microtubules and microfilaments are thought to play a role in the secretion of proteins from various tissues (30–32). This concept is based on the finding that agents such as colchicine and vinblastine, which interrupt the structural integrity of microtubules, also inhibit the secretion of various proteins. The response associated with the administration of colchicine suggests that protein secretion in the surface-active fraction is dependent on the integrity of microtubules.

The present study and those reported by others (37–39) indicate that a neurohumoral mechanism is involved in the secretion of surface-active material, but we have little insight into what factors might influence this secretory process. However, physiological studies sug-

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**Figure 2** Influence of iodoacetate on lung QO₂ and pilocarpine induced secretion. a. These studies were performed as in Fig. 1 except that some rabbits received iodoacetate before the administration of pilocarpine but after the pulse-labeling procedure. The animals were sacrificed 2 h after administration of [¹⁴C]leucine. In the iodoacetate-treated animals each point is the mean of two animals. The other value is the mean±SEM of four rabbits. b. Rabbits were given the indicated amount of iodoacetate or an equal volume of saline i.p. They were then sacrificed immediately (●—●) or after 2 h (○—○) and the QO₂ of lung slices was measured. Mean±SEM are given.
gest that respiratory frequency may be a determinant of the rate at which surface-active material is utilized on the alveolar surface (41). Since replacement should keep pace with utilization, respiratory rate might also be a determinant of secretion. In addition, current concepts of alveolar surface forces imply that the smaller the airspace, the lower its surface tension must be if air-space stability is to be maintained (3). These considerations suggest that since different species have widely different respiratory rates and alveolar diameters, there might be interspecies differences in the lungs' secretory activity.

I found that the radioactive protein of the surface-active fraction appeared in the alveolar compartment more rapidly in mice (respiratory rate about 160/min), than in rats (respiratory rate about 85/min), than in rabbits (respiratory rate about 35/min). These findings are consistent with the hypothesis that respiratory rate is a determinant of the secretory activity, but to be conclusive one needs to show that changes in respiratory rate are followed by changes in the rate of secretion. I also found that species with faster respiratory rates and smaller alveoli have more protein per square meter of alveolar surface area in the lavage surface-active fraction than species with lower respiratory rates.

These findings suggest that there might be two in-series replacement mechanisms for the ventilatory-induced depletion of alveolar film surface-active material. The first mechanism, a physical effect, would relate to a greater rate of adsorption of surface-active material into the lining film from the hypophase of the duplex lining layer. This might relate to a greater amount of protein in the hypophase which, in vitro, seems to accelerate adsorption of surface-active material (42), or to an excess of both lipid and protein. Breath-by-breath replacement of surface-active material in the surface film, more rapidly needed in species with high respiratory rates, would occur from this source. The second replacement mechanism, also related to respiratory rate, would be secretion of surface-active material into the hypophase. Because the secretion of molecules from storage granules is a bulk affair, I view this second mechanism, as incapable of the fine control that breath-by-breath adjustments would require.

The means by which respiratory rate might be coupled to neurohumoral-mediated secretion is unclear. This could be achieved by the presence in the alveolar wall of nerve endings sensitive to changes in pressure or distention. This potential mechanism has some basis in recent anatomical findings. Hung, Hertweck, Hardy, and Loosli found two types of nerve endings in close association with alveolar epithelial cells (43). One type, located in the alveolar epithelial cells, has anatomical features similar to sensory fibers in other organs (44, 45) and similar to the "type J" receptors considered to respond to changes in pulmonary interstitial pressure (46). The other type of nerve ending in the alveolus is found in close association with the granular pneumocyte, and, because of its anatomical similarity to nerves in endocrine organs, may have a motor function (47). Protein secretion may be one of this nerve ending's motor functions.

The findings in the present studies can be compared with certain aspects of two relevant studies. First, Young and Tierney made quantitative determinations on the amount of dipalmitoyl lecithin (DPL) in lung tissue and lavage fluid in rats (19). They found the DPL in the lavage fluid was 12.1% of the total DPL recovered from the lung tissue plus the lavage fluid. I found that the protein in the lavage surface-active fraction was 12.4% of the total protein recovered from the surface-active fraction of lung tissue plus lavage fluid. It would be of interest to know if this close relationship between the distribution of DPL and protein exists in other species and if the distribution of DPL varies be-
between species with respiratory rate and alveolar diameter as does the distribution of protein in the surface-active fraction. Secondly, although I have not surveyed the synthesis of protein in the surface-active lung fractions of different species, since secretory rates are different, I expect rates of synthesis would also differ. This concept is supported from recent work by Goldner and Brumley (48). They found interspecies variation in the incorporation of "P into lung phosphatidyl choline. There was a highly significant correlation of incorporation with alveolar surface area, alveolar diameter, and, by inference, respiratory rate.

Figure 4 Protein in the lavage surface-active fraction in different species. The animals were sacrificed without prior manipulation and their lungs lavaged. Each point represents the mean value for the species; figures in parentheses indicate the number of animals studied. The values for alveolar surface area, respiratory rate, and alveolar diameter were culled from the literature.

Figure 5 Distribution of protein between the lavage and tissue surface-active fraction. Each point represents the mean value for the species; figures in parentheses indicate the number of animals studied.
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REFERENCES


D. Massaro