Ascorbate uptake by isolated rat alveolar macrophages and type II cells

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CASTRANOVA, V., J. R. WRIGHT, H. D. COLBY, AND P. R. MILES. *Ascorbate uptake by isolated rat alveolar macrophages and type II cells.* J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 54(1): 208–214, 1983.—Studies were conducted to measure intracellular ascorbate content and to characterize ascorbate uptake in three fractions of isolated rat pneumocytes (i.e., alveolar macrophages, alveolar type II epithelial cells, and another fraction of small pneumocytes that contains neither macrophages nor type II cells). When cells are incubated in medium containing 0.1 mM ascorbate (i.e., the concentration normally found in plasma), intracellular ascorbate concentrations are 3.2 mM in alveolar macrophages and type II cells and 0.9 mM in other lung cells; ascorbate influx is 1.5 nmol·10^7 cells^{-1}·h^{-1} for alveolar macrophages, 0.24 nmol·10^7 cells^{-1}·h^{-1} for type II cells, and very slow in other pneumocytes. Ascorbate influx displays saturation kinetics in both alveolar macrophages ($K_{1/2}$ = 2 mM; V_{max} = 32.2 nmol·10^7 cells^{-1}·h^{-1}) and type II cells ($K_{1/2}$ = 5 mM; V_{max} = 14.2 nmol·10^7 cells^{-1}·h^{-1}). After correction for differences in the membrane surface areas of these two types of lung cells, the rates for maximum ascorbate influx (V_{max}) are similar in alveolar macrophages and type II cells. In addition, ascorbate uptake by alveolar macrophages and type II cells is dependent on metabolic activity and extracellular sodium. In contrast, ascorbate uptake in other lung cells does not exhibit saturation kinetics and is not dependent on metabolism or sodium. Thus alveolar macrophages and type II cells possess an energy-dependent cotransport system for ascorbate and sodium influx. The high ascorbate content and the existence of a specialized transport mechanism for ascorbate uptake may explain the relative resistance of alveolar macrophages and type II cells to oxidant injury.

ascorbate transport; pneumocytes

EXPOSURE TO OXIDANTS, such as hyperbaric oxygen, nitrogen dioxide, and ozone, results in injury to pulmonary tissue, impairment of gas exchange, and eventually death (12, 22). Lipid peroxidation has been implicated as a possible cause of oxidant injury in lung tissue (11, 24). It has been shown that ascorbate, an antioxidant, inhibits pulmonary lipid peroxidation in vitro (28, 29) and reduces lung damage (17) and mortality (21), resulting from in vivo exposures to oxidants. Therefore ascorbate may play an important role in protecting pulmonary tissue from oxidant injury.

Evidence indicates that alveolar type II epithelial cells are relatively resistant to oxidant damage (12). Indeed, after oxidant exposure, type II cells proliferate, spread along the alveolar epithelium, and transform into type I cells (1, 13). In this manner, type II cells are thought to repair oxidant-induced damage to the alveolar epithelium by replacing injured type I cells. Alveolar macrophages also exhibit resistance to oxidant injury. After exposure to oxidant gases, alveolar macrophages increase in number and remain viable (do not become leaky) (19). Whether the relative resistance of type II cells and alveolar macrophages to oxidant injury is related to ascorbate is as yet unknown.

We have shown previously that a mixed population of lung cells has the ability to accumulate ascorbate via a specialized transport pathway (30). However, the transport characteristics for ascorbate in individual types of pneumocytes have yet to be determined. The objectives of this investigation were *1*) to measure the intracellular concentration of ascorbate in three types of lung cells, i.e., alveolar macrophages, type II cells, and other lung cells (a mixture of small cells that does not include type II cells or alveolar macrophages), and *2*) to study the characteristics of ascorbate uptake in these three types of pneumocytes. A preliminary report of these results has appeared previously (10).

METHODS

Isolation of lung cells. Male Sprague-Dawley rats (200–300 kg) were anesthetized with pentobarbital sodium (65 mg/kg body wt). Alveolar macrophages were obtained by pulmonary lavage with 80 ml of ice-cold Ca^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5.5 mM glucose; pH = 7.4) as described previously (8). Type II cells and other pneumocytes were isolated by elastase digestion and purified by centrifugal elutriation (18). Briefly, the lungs and heart were removed en bloc. The lungs were perfused with 0.9% NaCl to remove blood cells. Free alveolar macrophages were removed by pulmonary lavage with phosphate-buffered medium. Lungs were then filled with elastase solution [40 U/ml elastase (type I) and 0.006% deoxyribonuclease (DNase, Sigma Chemical, St. Louis, MO) in phosphate-buffered medium] and incubated at 37°C for 30 min to free lung cells. Following enzymatic digestion, lungs were minced with a tissue chopper (slice thickness of 0.5 mm), and digestion was arrested by incubating the suspension at 37°C for 10 min in 20 ml of inhibitor solution (25% fetal calf serum and 0.006% DNase in phosphate-buffered medium). The cell suspension was strained through nylon mesh, and

the isolated lung cells were loaded into an elutriator (model J-21 centrifuge equipped with a model JE-6 rotor, Beckman Instrument Company, Fullerton, CA) at a flow rate of 10 ml/min and a rotor speed of 2,000 rpm. The other lung cell fraction was collected by infusing 200 ml of phosphate-buffered medium containing 0.5% bovine serum albumin (BSA) through the elutriator at this flow rate and centrifuge speed. Type II cells were then recovered at a flow rate of 18.9 ml/min and a rotor speed of 2,000 rpm. The three cell fractions were concentrated by centrifugation at 1,000 *g* for 5 min at 2°C. The supernatant was removed by aspiration and cells were resuspended in phosphate-buffered medium containing 1 mM $CaCl_2$ and 0.5% BSA.

The number of cells in each fraction was determined with an electronic cell counter (model ZB, Coulter Instrument, Hialeah, FL). Cellular identification was by light, fluorescent, and electron microscopy as described previously (18). The macrophage fraction contained 95% alveolar macrophages. The type II cell fraction was more than 85% pure. Minor contaminants in the type II cell fraction were mainly polymorphonuclear leukocytes, although some alveolar macrophages and unidentified cells were also present. The other lung cell fraction contained small pneumocytes, such as lymphocytes, fibroblasts, ciliated cells, and other unidentified cells. This fraction did not contain red blood cells, granulocytes, or alveolar macrophages but did contain 33 ± 2% type II cells. In this investigation the ascorbate content and fluxes for the other lung cell fraction were corrected for the contribution of these type II cells as follows

value for other lung cells = (raw data)

$$- (0.33 \times \text{measured value for type II cells})$$

Therefore the data presented here for the other lung cell fraction represent values for pneumocytes other than alveolar macrophages and type II cells.

Viability of cellular fractions was tested by measuring oxygen consumption with a Gilson K-IC oxygraph equipped with a Clark electrode (Gilson Medical Electronics, Middleton, WI). Oxygen consumption of the cellular fractions are approximately 215 nmol $O_2 \cdot 10^6$ $cells^{-1} \cdot h^{-1}$ for type II cells, 130 for other lung cells, and 120 for alveolar macrophages. In addition, cellular fractions appear viable because of their ability to accumulate ascorbate (Table 1).

Measurement of intracellular ascorbate. The intracellular concentration of ascorbate in alveolar macrophages, type II cells, and other pneumocytes was measured according to the method of Zannoni et al. (31). Each of the three lung fractions were preincubated for 30 min at 37°C in phosphate-buffered medium containing 1 mM $CaCl_2$, 0.5% BSA, 0.006% DNase, and 0.1 mM ascorbate [the normal plasma level in rats (28)]. The cells were separated from the medium by centrifugation at 1,000 *g* for 5 min, washed three times in ascorbate-free medium to remove external ascorbate, and resuspended in ascorbate-free, phosphate-buffered medium at 10^7 cells/ml. One milliliter of this cell suspension was added to 4 ml of 5% trichloroacetic acid, and the samples were allowed to stand at 2°C for 10 min. The protein was then removed by centrifugation at 15,000 *g* for 15 min. One milliliter of the supernatant was mixed with 0.1 ml of each of the following: 85% orthophosphoric acid, 8% dipyridyl in ethanol, and 3% $FeCl_3$. This mixture was allowed to stand at 22°C for 30 min. Ascorbate levels were measured by reading the optical densities of the samples against standards at 525 nm with a Gilford spectrophotometer (model 300-N, Gilford Instrument, Oberlin, OH).

TABLE 1. *Intracellular concentrations of ascorbate in isolated rat lung cells*

Cell Fraction	Cell Water, $\mu l/10^7$ cells	Ascorbate Content	
		nmol/10^7 cells	mM
Type II cells	2.26 ± 0.14	7.27 ± 1.19	3.21 ± 0.52
Alveolar macrophages	7.50 ± 0.35	24.09 ± 3.12	3.21 ± 0.41
Other lung cells*	1.23 ± 0.12	1.05 ± 0.08	0.85 ± 0.06

Isolated lung cells were incubated at 37° C in phosphate-buffered medium containing of 0.1 mM ascorbate, 1 mM $CaCl_2$, 0.5% BSA, and 0.006% DNase for 30 min prior to determination of intracellular ascorbate and water levels. Values are means ± SE for 6 determinations. * Data for other lung cell fraction have been corrected for 33% contamination by alveolar type II cells.

Intracellular ascorbate concentrations were expressed as nanomoles of ascorbate per liter of intracellular water. Intracellular water content was determined according to a method described previously (7, 18). Briefly, fractions of isolated lung cells were centrifuged through cushions of dibutyl phthalate. Intracellular water content was calculated from wet-to-dry weight measurements after correcting for trapped extracellular space which was measured with tritiated inulin (New England Nuclear, Boston, MA).

Measurement of ascorbate uptake. Ascorbate uptake by alveolar macrophages, type II cells, and other pneumocytes was measured by a method described previously (28). Briefly, each of the three cell fractions (2×10^7 cells/ml) were preincubated for 30 min at 37°C in phosphate-buffered medium containing 1 mM $CaCl_2$, 0.5% BSA, and 0.006% DNase. In some experiments, cells were preincubated in low-sodium medium (10 mM sodium and 145 mM tetraethylammonium) or in the presence of metabolic inhibitors. After preincubation, 0.25 μCi of [1-^{14}C]ascorbic acid (New England Nuclear) plus various concentrations of unlabeled ascorbate were added to each milliliter of suspension to initiate uptake. After various periods of incubation at 37°C, ascorbate transport was monitored by taking 0.5-ml samples from the appropriate cell suspensions. The samples were centrifuged for 5 s at 12,800 *g* using a model 5412 Eppendorf microcentrifuge (model 5412, Brinkmann Instrument, Westbury, NY). The supernatants were removed, and the cell pellets were washed three times by alternate resuspension and centrifugation with ice-cold phosphate-buffered medium. After the final wash, the cell pellets were resuspended in 100 μl of Protosol (New England Nuclear) and incubated for 1 h at 55°C to digest the protein. The digested samples were then added to 10 ml of Aquasol (New England Nuclear). Radioactivity was measured using a TriCarb liquid scintillation spectrometer with an automatic quence correction (model 3380,

Packard Instrument, Downers Grove, IL). Ascorbate uptake was calculated from disintegrations per minute and the specific activity and was expressed as nanomoles of ascorbate taken up per 10^7 cells.

As with the previous study of ascorbate uptake in mixed lung cells (28), 0.5 mM thiourea was added to all cell suspensions to prevent the oxidation of ascorbate to dehydroascorbate. Wright et al. (28) have employed thin-layer chromatography to show that the [1-^{14}C]ascorbate accumulated by lung cells is not converted to dehydroascorbate. They have also reported that less than 1% of the cell-associated [1-^{14}C]ascorbate was found in the trichloroacetic acid precipitate, i.e., there is little radioactivity bound to cellular protein. Therefore the ascorbate uptake described in the present study seems to be true influx.

RESULTS

The intracellular ascorbate and water contents for alveolar macrophages, type II cells, and other pneumocytes are given in Table 1. The ascorbate concentration in each cellular fraction is much higher than that present in the medium (0.1 mM). Therefore these lung cell fractions have the ability to accumulate ascorbate. These data agree with results obtained previously for a mixed population of pneumocytes (28). However, data from the present investigation indicate that alveolar macrophages and type II cells accumulate ascorbate to a much greater extent than does the other fraction of pneumocytes. Therefore it is possible that macrophages and type II cells possess an ascorbate transport system which differs from that of other lung cells.

The time courses for ascorbate uptake by these three lung cell fractions are shown in Fig. 1. In these experiments, the extracellular ascorbate concentration was 0.1 mM, i.e., the normal plasma level of ascorbate. The time course of ascorbate uptake in each cell type displays two components. The initial minor component of uptake is rapid and short in duration (<15 min), whereas the major component of uptake is linear from 15 to 120 min. The rate of ascorbate uptake during this major component of transport is 1.5 nmol·10^7 cells^{-1}·h^{-1} for alveolar macrophages, 0.24 nmol·10^7 cells^{-1}·h^{-1} for type II cells, and very slow in the other fraction of lung cells. Note that lung cells which exhibit the slowest rate of ascorbate uptake also have the lowest ascorbate content.

Since the three fractions of pneumocytes contain different amounts of ascorbate and exhibit different rates of transport, it was of interest to further characterize the transport system of these lung cells. The dependence of ascorbate influx on the external ascorbate concentration is shown in Fig. 2. In all three cell fractions, ascorbate uptake increases as the extracellular ascorbate concentration is increased. However, type II cells and alveolar macrophages exhibit saturation kinetics, whereas the other fraction of pneumocytes exhibits a linear concentration dependence. The data were analyzed using double-reciprocal plots to estimate V_{max} (i.e., the maximum rate of uptake) and $K_{1/2}$ (i.e., the external ascorbate concentration that results in one-half of the maximal

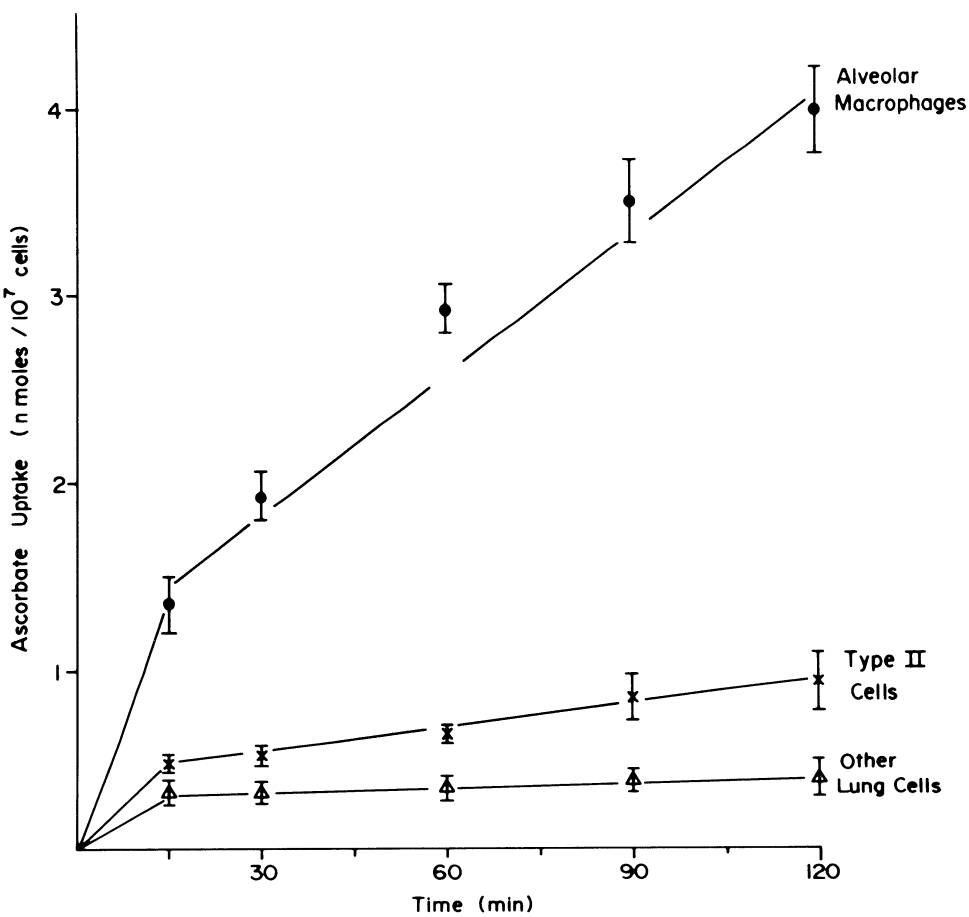
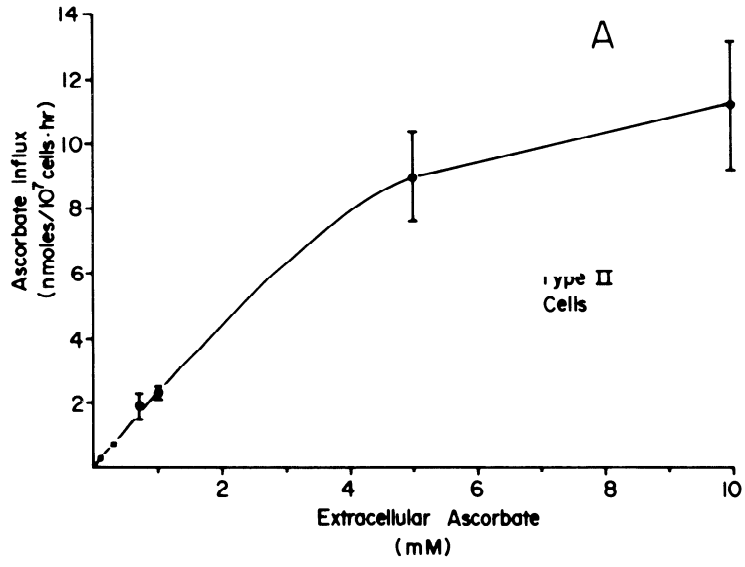
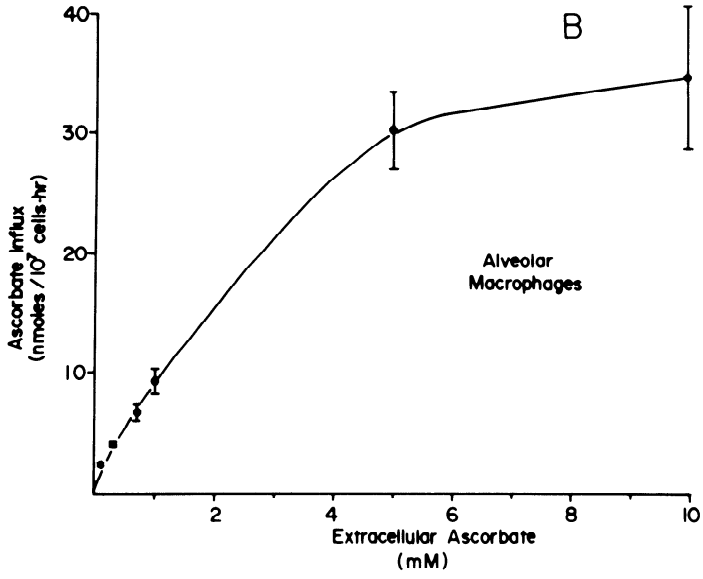


FIG. 1. Time course of ascorbate uptake by alveolar macrophages (●), type II cells (×), and other lung cells (△). Cells were incubated at 37°C for 30 min in phosphate-buffered medium containing 1 mM $CaCl_2$, 0.5% BSA, and 0.006% DNase before addition of 0.1 mM [1-^{14}C]ascorbate. At various times samples were taken and centrifuged, and the supernatant removed. Cell pellets were washed 3 times, digested, and prepared for scintillation counting. Ascorbate uptake was calculated from dpm and specific activity (see METHODS for further details). Values are means ± SE for 4 experiments. Data for other lung cells are raw data that have not been corrected for contamination by type II cells.





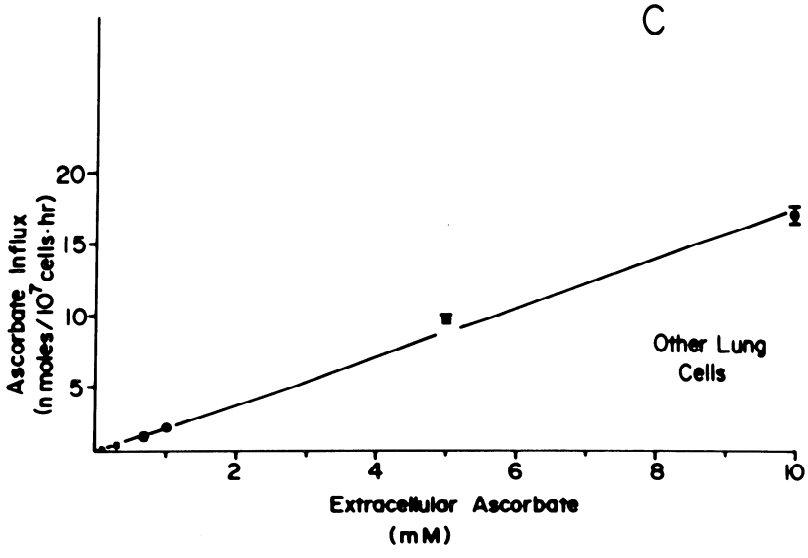


FIG. 2. Ascorbate influx as a function of extracellular ascorbate concentration in type II cells (*A*), alveolar macrophages (*B*), and other lung cells (*C*). Cells were incubated in phosphate-buffered medium containing 1 mM $CaCl_2$, 0.5% BSA, and 0.006% DNase at 37°C for 30 min prior to addition of various concentrations of [1-^{14}C]ascorbate. Uptake was measured 1 h after addition of ascorbate (see METHODS for further details). Values are means ± SE for 3 experiments. Data for other lung cells have been corrected for 33% contamination by alveolar type II cells.

influx). These data are given in Table 2. Alveolar macrophages exhibit a V_{max} of 32.2 nmol·10^7 cells^{-1}·h^{-1} and a $K_{1/2}$ of 2.2 mM; V_{max} for type II cells is 14.2 nmol·10^7 cells^{-1}·h^{-1} and $K_{1/2}$ is 4.9 mM. The high ascorbate flux for alveolar macrophages may be due to the large surface area of these cells. Relative surface areas were obtained by assuming a spherical shape and using values for cellular volume obtained from the cell water data in Table 1 (7, 18). From this analysis, the surface area of alveolar macrophages is 2.2 times that of type II cells. If one uses this value to normalize for surface area, V_{max} values for ascorbate influx in alveolar macrophages and type II cells are similar. However, macrophages possess a ruffled surface. Thus the permeability of type II cells to ascorbate may in fact be greater than alveolar macrophages.

The effects of extracellular sodium and metabolic inhibitors on ascorbate influx in alveolar macrophages, type II cells, and other pneumocytes are given in Table 3. Ascorbate influx in alveolar macrophages and type II cells is dependent on the presence of external sodium; i.e., ascorbate influx decreases as extracellular sodium is removed. Such sodium dependence is not characteristic of ascorbate transport in the other fraction of pneumocytes. Ascorbate influx in alveolar macrophages and type II cells is also linked to metabolism; i.e., ascorbate transport is decreased by metabolic inhibitors. It is of interest that the glycolytic inhibitor, iodoacetate, is a more effective inhibitor of ascorbate uptake in alveolar macrophages, whereas cyanide, an inhibitor of the mitochondrial electron transport chain, is more effective with type II cells. This difference in potency may be explained by metabolic differences in these cells (15). In contrast, neither metabolic inhibitor affects ascorbate influx in the other fraction of pneumocytes. These data suggest that alveolar macrophages and type II cells possess a cotransport system for the influx of sodium and ascorbate and that the accumulation of ascorbate in these cells is linked to metabolism as an energy source. The mechanism by which other lung cells accumulate ascorbate is unclear. However, it does not seem to depend directly on sodium or metabolism.

TABLE 2. *Concentration dependence of ascorbate influx in isolated rat lung cells*

Cell Fraction	Concentration Dependence	V_{max}, nmol·10^7 cells^{-1}·h^{-1}	$K_{1/2}$, mM	R
Type II cells	Saturation	14.2	4.9	1.00
Alveolar macrophages	Saturation	32.2	2.2	0.97
Other lung cells*	Linear			

Data from Fig. 2 were plotted using double-reciprocal plots. Data were fitted with straight lines by linear regression to obtain V_{max} (maximal rate of uptake), and $K_{1/2}$ (half-maximal concentration). R is the correlation coefficient for these lines. * Data for other lung cell fraction have been corrected for 33% contamination by alveolar type II cells.

TABLE 3. *Effects of extracellular sodium and metabolic inhibitors on ascorbate influx*

Treatment (Concn)	% Inhibition		
	Type II cells	Alveolar macrophages	Other lung cells*
Control (155 mM Na^+)	0	0	0
Low extracellular Na^+ (10 mM Na^+)	52.2 ± 10.1%	34.0 ± 4.6%	3.3 ± 3.3%
Potassium cyanide (1 mM)	47.0 ± 9.5%	18.8 ± 4.5%	0.7 ± 0.7%
Iodoacetic acid (1 mM)	14.2 ± 7.1%	62.3 ± 9.0%	1.0 ± 1.0%

Isolated lung cells were incubated at 37°C in normal or low-sodium media (i.e., phosphate-buffered media containing 1 mM $CaCl_2$, 0.5% BSA, and 0.006% DNase), in the absence or presence of inhibitors for 30 min prior to addition of 0.1 mM external [1-^{14}C]ascorbate. In low-sodium medium, tetraethylammonium was used as a sodium substitute. Uptake was measured 1 h after addition of ascorbate. Values are means ± SE for 5 experiments. * Data for other lung cells have been corrected for 33% contamination by alveolar type II cells.

DISCUSSION

In this investigation we have studied the characteristics of ascorbate transport in three fractions of isolated rate pneumocytes, i.e., alveolar macrophages, type II cells, and other lung cells. This study is an extension of previous work in which we characterized ascorbate transport in a heterogenous population of lung cells (28). Each cell fraction displays the ability to accumulate ascorbate. However, the intracellular ascorbate concentration of alveolar macrophages and type II cells is greater than in the other fraction of pneumocytes. The rate of ascorbate uptake in alveolar macrophages and type II cells is also greater than that in the other fraction of pneumocytes. In alveolar macrophages and type II cells, ascorbate influx follows saturation kinetics and is dependent on external sodium and metabolic energy. Therefore those lung cells which accumulate large amounts of ascorbate and which exhibit relatively high rates of uptake seem to possess a specialized carrier mechanism for the influx of ascorbate against a concentration gradient. The data suggest that this mechanism may involve the cotransport of sodium and ascorbate where influx of ascorbate against a concentration barrier is linked to the influx of sodium down an electrochemical gradient. In contrast, the other fraction of lung cells accumulates less ascorbate at a slower rate and does not exhibit saturation kinetics, sodium dependence, or energy dependence. Therefore the transport system for ascorbate in these cells differs from that for alveolar macrophages and type II cells. As yet, the nature of the transport mechanism in other lung cells is unknown.

The ability of mammalian cells to accumulate ascorbate has been reported using various tissues. Willis and Kratzing (26, 27) have reported ascorbate uptake in lung slices and perfused lungs. As with alveolar macrophages and type II alveolar epithelial cells, ascorbate uptake exhibits saturation, sodium dependence, and energy dependence in the retina, small intestine, adrenal cortex, and adrenal medulla (4, 14, 16, 23). In contrast, leukocytes accumulate ascorbate by a system that exhibits a linear concentration dependence and is not directly linked to an energy source (3, 5, 6). Such transport is similar to that reported here for the other lung cell fraction.

The time course of ascorbate uptake displays two components, i.e., a minor rapid component (0–15 min) and a major component (15–120 min) (Fig. 1). A similar two-component time course has been reported for the

transport of cations in dog red blood cells (9). The nature of this minor component of uptake is unknown. However, it is probably not extracellular radioactivity trapped in the cell pellet during centrifugation, since experiments indicate that little radioactivity appears in the cell pellet immediately after addition of [1-^{14}C]ascorbate (data not shown). By definition, uptake is directly dependent on cellular surface area. Relative surface areas, calculated assuming spherical shape and using values for cellular volume obtained from cell water data (Table 1), are approximately 2.2 for alveolar macrophages, 1.0 for type II cells, and 0.7 for other lung cells. Thus, when the minor components of uptake in the three cell fractions are normalized for surface area, the rate constants for influx are similar for each cell type, even though the characteristics of ascorbate transport are very different in these three cell types. Ascorbate influx during the major component of uptake is greatest for alveolar macrophages and lowest for the other lung cell fraction. These differences in influx are not explained by differences in surface area. However, the difference in the rate of ascorbate uptake between alveolar macrophages and type II cells at 0.1 mM external ascorbate (Fig. 1) seems to be due to different $K_{1/2}$ values for transport in these cells (Table 2). Indeed, after normalization for surface area the V_{max} values for alveolar macrophage and type II cells are similar. In fact, the ascorbate permeability of type II cells may actually exceed that for alveolar macrophages, since we may have underestimated the surface area of alveolar macrophages due to their ruffled membrane.

We have reported in a previous paper (28) that the concentration dependence for ascorbate influx in a heterogenous population of lung cells consists of two components, i.e., a saturable and a linear component. We have suggested that these data may have resulted from the use of a mixed population of cells, some of which possess a linear concentration dependence while others exhibit saturation kinetics. Data from the present study support this suggestion, since alveolar macrophages and type II cells display saturation kinetics, whereas other pneumocytes exhibit a linear concentration dependence.

The studies with metabolic inhibitors indicate that ascorbate influx in type II cells is more sensitive to inhibition by cyanide (an inhibitor of the electron transport chain) and less sensitive to iodoacetic acid (an inhibitor of glycolysis) than is the ascorbate transport system of alveolar macrophages. These data could be explained by metabolic differences between these two cell types. Indeed, Fisher et al. (15) have reported that energy generation in type II cells is more dependent on mitochondrial electron transport and less dependent on glycolysis than in alveolar macrophages.

In this paper, we have proposed that type II cells and alveolar macrophages possess an energy-dependent cotransport system for the uptake of sodium and ascorbate. This specialized carrier mechanism results in the rapid uptake and accumulation of ascorbate by these two types of lung cells. Other lungs cells, which do not possess this specialized transport system, are less able to accumulate ascorbate. Since ascorbate is an antioxidant in the lung (29, 30), it is possible that these differences in ascorbate transport may be related to the relative resistance of alveolar macrophages and type II cells to oxidant injury. We have shown that in all cell types ascorbant uptake can be increased by increasing the external ascorbate concentration above the normal plasma level of 0.1 mM. These data suggest that high dietary levels of ascorbate may be useful in protecting the lung from oxidant injury. Indeed, other investigators have reported that lung ascorbate levels decline after exposure to oxone or hyperbaric oxygen (3, 20, 25). Thus these results also imply a role for ascorbate as an antioxidant in the lung. In addition, ascorbate has been shown to reduce pulmonary edema and mortality following exposure to ozone or hyperbaric oxygen (17, 21). However, Arad et al. (2) have not been able to demonstrate protection of the lung using ascorbate. Therefore the role of ascorbate in the protection of the lung from oxidant injury requires further study.

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