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Research Article

Solute Absorption from the Airways of the Isolated Rat Lung. III. Absorption of Several Peptidase-Resistant, Synthetic Polypeptides: Poly-(2-Hydroxyethyl)-Aspartamides

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A series of samples of the synthetic polypeptide poly-α,β-[*N*(2-hydroxyethyl)-DL-aspartamide] (PHEA), containing covalently bound fluorophore, ethylcarbonyl-6-aminofluorescein, and exhibiting different molecular weight distributions with weight average molecular weights ranging from approximately 4 to 43 kD, was prepared and characterized. Aqueous solutions of the polymers were administered to the airways of isolated perfused rat lung preparations, and transfer to the perfusate was measured. Polymers administered directly to the perfusate were not degraded during the experiment. Polymer transfer rates were dependent upon starting molecular weight distribution, larger molecules being absorbed more slowly. In the case of a polymer with a median molecular weight of 7.2 kD, the absorbed species appeared to be smaller molecules than those which were originally administered. This was not the case for a 3.98-kD polymer; absorbed material had a gel permeation chromatography elution volume equivalent to that of the administered material. Absorption for the 3.98-kD polymer was found to be dose dependent. Approximately 70% absorption of a 0.2-mg dose occurred in 100 min. Much larger polymers (up to 11.65 kD) were also absorbed at finite rates. Results are discussed in the context of macromolecular delivery to the systemic circulation via the lung.

KEY WORDS: aerosols; lungs; polypeptides; proteins; polymers; pulmonary absorption.

INTRODUCTION

This paper is the third in a series of articles describing the use of the isolated rat lung to survey pulmonary xenobiotic disposition (1,2). Inhalation is a nonenteral alternative route for new generation proteins and peptides (3). Insulin is known to be active when administered by aerosol (4), and inhalation therapy is generally well received by patients. Presently, however, there is little information on the absorption of proteins and peptides via the lung, even though extracellular peptidase activity is much lower in the lung than in the gastrointestinal tract, and the surface area available for absorption is large (5). What information is available is difficult to interpret because of poor dosimetry (6,7) and the fact that structural changes in proteins simultaneously vary the molecule's physical chemistry and absorption rate in addition to its susceptibility to lung peptidases. This paper is the first of several in which the disposition of a series of synthetic, peptidase-resistant poly-aspartamides in the isolated rat lung is described. These polymers have been proposed as plasma expanders and drug carriers (8–10). They were chosen to explore the effects of molecular variation upon residence kinetics and absorption from the airways, in the absence of significant metabolism.

N-Substituted poly-α,β-DL-aspartamides have been used previously as model polymers in studies of macromolecular disposition (11) and to determine the effects of chemical structure of macromolecules on their cellular uptake (12,13). The structure of the products (Scheme I and Table I) may be varied in terms of molecular weight distribution, net electric charge, and hydrophobicity by varying the base with which polysuccinimide (II, Scheme I) is reacted (11,12). Furthermore, these polymers can carry groups of special interest; if *R* is fluorescent (*R* = ethylcarbonyl-6-aminofluorescein; Table I), partial substitution in III (Table I) produces a labeled, easily assayed, polymer with a covalently bound, stable fluorophore with spectral qualities and quantum yield similar to fluorescein (14).

Many of these molecules are apparently nonimmunogenic (9) and they are not readily metabolized by peptidases (10), due to the use of a racemic mixture of aspartic acid as a synthetic precursor and the α,β linkages produced during ring opening (Scheme I). Therefore, these molecules are ideal candidates to elucidate the importance of molecular structure in pulmonary absorption and/or retention at the absorption site. Specifically, this paper describes the syn-

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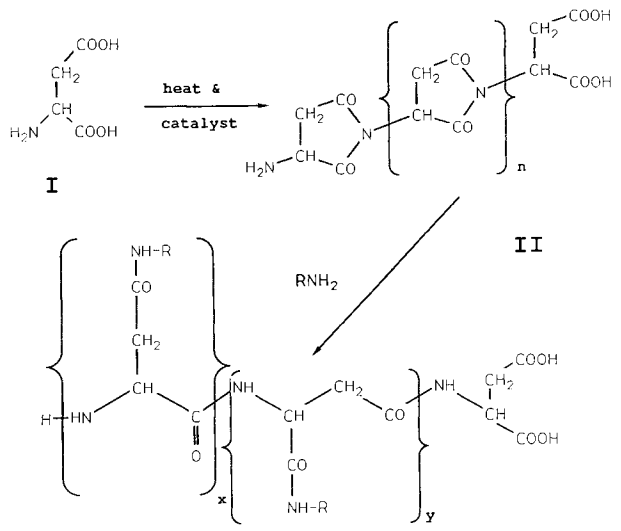


Scheme I. Synthetic route to *N*-substituted poly-α,β-DL-aspartamides (III, Table I) and related copolymers. Poly-DL-succinimide (II) can be synthesized with different degrees of polymerization from *L* or D, L-aspartic acid (I). Fractional precipitation of II can be employed prior to reaction with bases (Table I) to produce the desired MWD of the starting precursor. The chemical structure of resulting products can be modified via a consecutive reaction with different bases (R-NH_2). The MWD of product polymers can be further narrowed by gel permeation chromatography.

thesis and characterization of poly-(2-hydroxyethyl)-aspartamide (PHEA) labeled with 2-aminoethylcarbonyl-6-aminofluorescein IIIF, Table I) and the molecular weight dependency of its absorption in the lung.

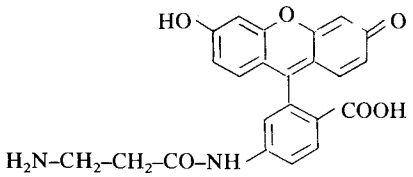
MATERIALS AND METHODS

Preparation of IIIF. Polysuccinimide (II) was prepared and fractionated by a fractional precipitation method (15) and reacted with 2-aminoethylcarbonyl-6-aminofluorescein (F; Ref. 14). Typically, to produce a label density approximately 0.5% F by weight (Table II), 3 g II was reacted with 162 mg F for 4 days at 60°C in 30 ml dimethylformamide (DMF) containing 0.4 mmol triethylamine. IIF was precipitated with methanol, unreacted F was removed by washing, and the product was dried under vacuum (80°C, 48 hr). The product was redissolved in 28 ml of DMF, then cooled to 0°C, and excess ethanolamine (2 mol equivalents with respect to succinimide units) was added. This mixture was stirred at ambient temperature for 24 hr, neutralized with acetic acid, dialyzed against distilled water, and concentrated under vacuum prior to gel permeation chromatography (GPC) on Sephadex G-25F (50 × 400-mm column, Pharmacia LKB, Piscataway, NJ) using water as the mobile phase. Total IIIF was isolated from the collected fractions by freeze drying. A typical yield (from 3 g of II) was about 3.1 g IIIF. Samples with a narrow molecular weight distribution (MWD, Table II) were prepared by dissolving IIIF in pH 7.8 phosphate-buffered saline (0.05 *M* phosphate, 0.15 *M* NaCl) followed by GPC on Sephacryl S-200 (Pharmacia, 40 × 700-mm column) using the same buffer as the mobile phase. Individual fractions from the broad polymer peak were concentrated to less than 20 ml and desalted by GPC on Sephadex G-25F (50 × 400-mm column) in water. After freeze-drying, lyophilized polymers were stored in the dark over silica gel prior to use. Two sets of polymers were prepared (Table II) with small degrees of polydispersity ($[M_w/M_n] < 1.6$ in all cases). Their absorption was studied in the isolated perfused rat lung. Polymer series A and B contained approximately 0.5 and 1% fluorescein equivalents respectively; series B was prepared with increased fluorophore content to improve assay sensitivity when performing GPC on perfusate samples.

Polymer Molecular Weight Distributions. The MWD of IIIF fractions (Table II) was determined by GPC using a previously calibrated, mixed-bed column (Sephacryl S-200:Sephadex G-25SF; 3:1, 12 × 300 mm, Pharmacia LKB) and a mobile phase of pH 7.4 phosphate-buffered saline (0.05 *M* phosphate, 0.15 *M* NaCl). The column was calibrated with three overlapping fractions of PHEA with known MWD using the curve summation method (16). The linear portion of the calibration curve relating logMW and the elution volume, V_e, covered the range 1.5 through 45 kD and could be expressed by the equation $\log MW = 6.50 - 0.19V_e$, where MW is in daltons. Elution of IIIF was monitored spectrophotometrically at 490 nm (Model 1840 UV detector, Isco Inc., Lincoln, NE).

Polymer Stability and Fluorophore Content. Polymers held in aqueous solution at pH 7.4 and 21°C for 10 days showed no changes in their elution profiles on GPC; no hydrolysis of the polymer or the fluorophore–polymer bonds occurred during this time period. Fluorophore content was quantified relative to fluorescein itself assuming that the

Table I. Bases for Reaction with II (Scheme I) to Produce Poly-Hydroxyethyl-Aspartamide (III)[a]

Base	R-NH_2	Polymer type
Ethanolamine	$H_2N-CH_2-CH_2-OH$	III
2-Aminoethylcarbonyl 6-Aminofluorescein		Fluorescent label, F (used with polymer III, produces IIIF)

[a] In practice, II is partly labeled with fluorophore, F, prior to ethanolamine, to produce IIIF (Table II)

Table II. Characteristics of the Various Fluorophore-Labeled Poly-Hydroxyethyl-Aspartamides (IIIF)

Polymer	[F][a]	MWD			
		Median[b]	M_n[c]	M_w[c]	Pd[d]
Series A					
AIIIF43250	0.58	43,250	—	—	—
AIIIF11650	0.47	11,650	10,200	12,200	1.20
AIIIF8230	0.60	8,230	6,860	8,800	1.28
AIIIF5175	0.56	5,175	3,900	5,700	1.46
Series B					
BIIIF7200	1.21	7,200	4,700	7,430	1.58
BIIIF3980	1.07	3,980	3,310	4,680	1.41

[a] Fluorescein concentration [Eq. (1)].
[b] Molecular weight from elution volume corresponding to maximum peak height.
[c] Number (M_n)- and weight (M_w)-averaged molecular weight.
[d] Polydispersity, Pd = M_w/M_n.

quantum yield due to both fluorophores was identical; in practice they are very similar (14). Using excitation and emission wavelengths of 486 and 516 nm, respectively, fluorescent intensities of solutions containing known weights of polymer and disodium fluorescein were compared (Aminco-Bowman Ratio II Spectrofluorimeter, American Instrument Company, Silver Spring, MD). The apparent fluorescein concentration as a percentage by weight, [F], in the polymers was calculated from

$$[F] = 100 \times \frac{[I_p \times \text{fluorescein concentration}]}{[I_f \times \text{polymer concentration}]} \quad (1)$$

where I_p and I_f are fluorescent intensities from polymer and fluorescein solutions, respectively.

Pulmonary Absorption in the Isolated Lung. Isolated rat lungs were prepared and housed as described previously (1,2,17). To determine the effects of MWD on polymer absorption into the circulating perfusate (Krebs/Henseleit buffer plus 4%, w/v, bovine serum albumin), approximately 2 or 4 mg of IIIF with different MWDs, dissolved in 100 μl distilled water, was administered intratracheally using a "cartridge" and forced inflation technique described in detail elsewhere (17). After dosing, samples were removed from the perfusate and assayed for total fluorescence relative to standard fluorescein solutions. In one dose ranging study different concentrations were prepared and dosed to deliver 100-μl aqueous solutions containing 0.2, 0.5, 2.0, or 7.5 mg of polymer. In some cases involving polymers of lower molecular weight, GPC was performed on polymer in perfusate solutions before and after absorption to determine changes in MWD induced by the lung and absorption process. This used a calibrated Sephadex G50F 10 × 500-mm column with a pH 9.2 aqueous mobile phase (0.1 *M* Na_2HPO_4, 0.15 *M* NaCl) at a flow rate of 0.5 ml/min (Model 740 controller and pump, Model M420E fluorescence detector, Waters-Millipore, Milford, MA). Detection employed a 450-nm bandpass excitation filter and 490-nm longpass emission filter. Elution volumes were determined before and after absorption as an indicator of the MWD of the samples.

RESULTS

Pulmonary Absorption in the Isolated Lung. Table III shows data for polymer transfer from airways to perfusate. Figures 1a and b show transfer of Series A and Series B polymers, respectively. Nominal administered doses of ≈2 mg were employed in all cases with the exception of AIIIF11650, which was dosed at 4 mg to aid in its detection in perfusate. Mean administered doses are shown in Table III. Polymer AIIIF43250 showed negligible transfer, indicating its retention in the airways over the 3-hr lifetime of this preparation. Other polymers showed increasing transfer rates with decreasing molecular weight. In no cases was transfer complete in 2–3 hr but continuous transfer occurred at a finite rate for all MWDs with the exception of the 43.25-kD material.

Evidence for the existence of a molecular sieving effect which favors the absorption of smaller polymer molecules is shown in Fig. 2. In initial experiments conducted with Series A polymers, there was insufficient assay sensitivity to perform successful analytical GPC on perfusate samples. Experiments with the smallest polymer, BIIIF3980, indicated that the molecular size of the transferred material was indistinguishable from that which was administered to the airways (elution volume after gel permeation chromatography. V_e, was unchanged before and after absorption). This was not the case with BIIIF7200. Comparison of the open circles and the dashed profile in Fig. 2 indicates that the median molecular weight of the transferred polymer, in this case, was smaller than that which was administered.

Dose Dependency. BIIIF3980 was synthesized in sufficient quantity to perform a dose ranging study (0.2–7.5 mg; $n \geq 4$). Data in Fig. 3 and Table III show that nonproportional increases occurred in percentage transfer as the dose of this polymer was decreased. While experiments at the lowest doses suffered from increased experimental scatter, the transfer profiles at the 0.22-mg mean dose (Table III) were apparently first order (2,18).

DISCUSSION

When polymers were injected into the circulating per-

Table III. Percentage Transfer of the Administered Dose to the Perfusate 100 min After Dosing

Polymer	Mean dose (mg)[a]	% transfer[b]
Series A		
AIIIF5175	2.27	29.6 ± 13.9
AIIIF8230	1.97	12.9 ± 5.2
AIIIF11650	4.82	4.0 ± 1.4
Series B		
BIIIF3980	0.22	68.4 ± 11.3
	0.55	49.0 ± 5.0
	2.16	16.8 ± 3.0
	7.79	10.2 ± 3.9
BIIIF7200	2.18	11.9 ± 3.0

[a] The mean administered dose ($n \geq 4$ in all cases).
[b] Percentage transfered to the perfusate 100 min after dosing ± standard deviation.

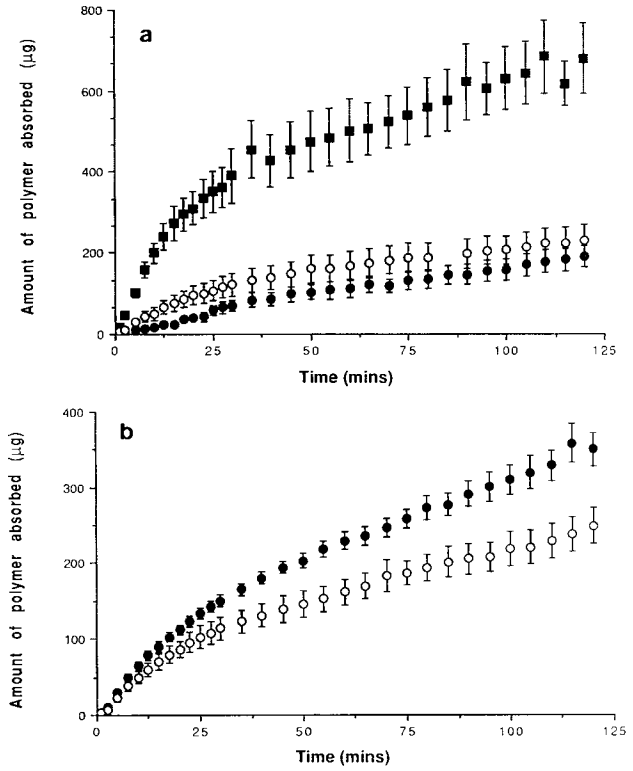


Fig. 1. Pulmonary absorption of fluorophore-labeled poly-2-hydroxyethyl-aspartamide: (a) AIIIF5175 (■; n = 5), AIIIF8230 (○; n = 5), and AIIIF11650 (●; n = 5); (b) BIIIF3980 (●; n = 5) and BIIIF7200 (○; n = 5). Mean administered doses are shown in Table III; BIIIF3980 was administered at a mean dose of 2.16 mg. Error bars are standard deviations.

fusate of the isolated lung preparation, there was no observable decrease in concentration over the 180-min lifetime of the preparation. Thus, binding and/or metabolism was not important on the systemic side of this preparation after absorption had occurred. Nevertheless, the difference in elution volumes after absorption for BIIIF7200 (Fig. 2) may have been due to selective absorption of the smaller molecules in the airways and/or partial metabolism of BIIIF7200 during the absorption process. Metabolism is unlikely, given this compound's molecular polydispersity and BIIIF3980's identical elution volume before and after absorption (larger

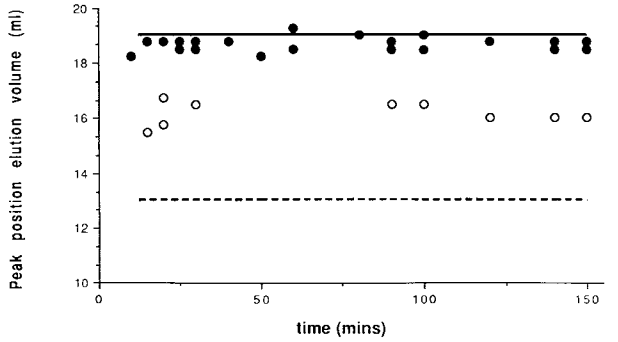


Fig. 2. Peak position elution volumes for BIIIF3980 (●) and BIIIF7200 (○) after pulmonary absorption and for control samples (——, 3980; ---, 7200) before administration to the airways.

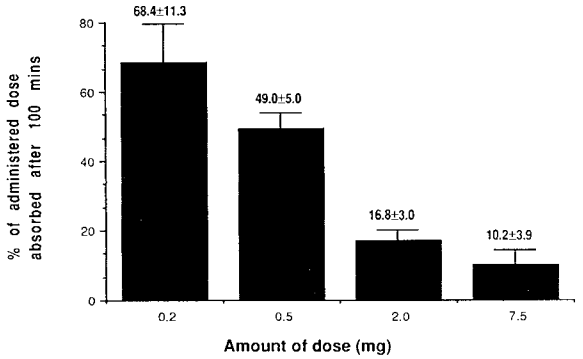


Fig. 3. The mean percentage of BIIIF3980 absorbed across the pulmonary barrier 100 min after dosing as a function of the nominal dose administered to the lung. Error bars are standard deviations ($n \geq 4$). Analysis of variance showed that differences existed in the absorption of the polymer as a function of dose (F = 100.1 with 3 and 18 degrees of freedom).

values for V_e in the perfusate indicate lower molecular weight material). The appearance of the absorption profiles in Fig. 1 clearly indicates a biphasic type of absorption, with an initial rapid phase followed by a transfer process which appears roughly zero order. It is likely that the small molecules from each distribution will be absorbed faster than the large molecules. While this may partly explain the shape of these profiles, there are complicating factors which remain to be studied; the absence of a progressive change in the peak elution volume with time (Fig. 2) would appear to contradict this explanation. On the other hand, polymer chromatography peaks on GPC were broad and small and were less reliable at early time points when transfer was minimal. The implication is that a possible molecular weight cutoff point exists for these polymers somewhere between 4 and 7 kD, below which any molecular weight can transfer without sieving being observed. Future publications on additional polymers will employ improvements in GPC techniques and address this subject in greater detail.

Dose Dependency. When the data for BIIIF3980 at the 0.22-mg dose (Fig. 3) were modeled by a first-order approximation (2), the half-life for absorption was ≈14.5 min. This value is surprisingly close to that for fluorescein [dianion molecular weight = 0.33 kD, transfer half life = 12.7 ± 1.22 min (1,2)] in the same model. The results in Fig. 3 indicate the existence of a saturable absorption mechanism which may or may not be influenced by the presence of the fluorophore. Like fluorescein, the fluorophore carries two negative charges at physiologic pH (6). This dose dependency is presently being studied with mixtures of fluorophore-labeled and unlabeled III with different MWDs, while varying doses and solution concentrations at constant doses of the fluorescent analyte, IIIF.

The dependence of absorption rates upon molecular weight has been reported for widely different molecules in various model systems (3). Saturable mechanisms for pulmonary absorption have been recognized for other negatively charged compounds such as phenol red and cromolyn sodium (19–21). In all instances the doses were much higher than would normally be used for delivery by aerosol (2), and thus, for reasons of experimental facility, some studies may

predispose the system to apparent saturation kinetics and low values for absorption. Hydrophilic compounds appear to cross the lung at a rate determined typically by the reciprocal of the square root of their molecular weight (22). Junctional passive diffusion (23,24) can explain many results in the literature, if the rate-limiting barrier is the epithelium (3) with pore radii in the 6- to 10-Å range (24). There are some indications that the rate of transfer may be independent of molecular weights up to ≈6 kD (24). Our data (Fig. 2) are roughly consistent with this finding. On the other hand, it is likely that absorption through the lung is even more complex than these statements suggest. The influence of formulation upon solvent flux in the lung is poorly understood and water is absorbed extremely rapidly from the alveoli (26). Whether solutes can be carried along with such a mass transfer from the airways is presently unknown. If vesicular transport takes place (endocytosis) it is likely that the significance of this mechanism is highly dependent on molecular structure and size. Concentration dependence may still occur due to either energy limitations or limits on the number of available vesicles or both (27,28). The importance of water-filled membrane pores is also a function of the molecules being studied. Furthermore, the precise nature of the pulmonary membranes should also be taken into account. The predominance of negative charge in the basement membranes and interstitia of the lung (29) is almost certain to influence the speed of passage of charged molecules through electrostatic interactions. Overall, absorption of xenobiotics is a complex function of their size, concentration, charge, and molecular conformation and their interactions with the lung.

Although we have shown that the pulmonary absorption of polypeptides is molecular weight dependent, there is every reason to believe that systemic protein and peptide delivery by aerosol is not only feasible, but desirable. Provided that doses are not overly large, dosimetry can be made quite reproducible in human subjects without lung disease (30). Most contemporary drugs are inhaled by human patients in very small doses in comparison to the doses administered to the rat lung. In the absence of metabolic complications, about 70% absorption of a 0.2-mg dose of BIIIF3980 occurred in less than 2 hr and pulmonary absorption kinetics in most mammalian species proceeds at similar rates (3). Even larger polymers are absorbed at finite rates, and provided that these are delivered to the peripheral regions of the lung where clearance processes are slow (31,32), extended durations of residence may be expected, with absorption occurring over a 10- to 12-hr time frame. Consequently, by systematically studying the absorption of poly-hydroxyethyl-aspartamide and its copolymers, it is possible to characterize the disposition of synthetic polypeptides in the lung.

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