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Effects of Polyethyleneimine on Endocytosis and Lysosome Stability

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ABSTRACT. Polyethyleneimine (PEI) is shown to destabilize isolated rat liver lysosomes, as indicated by a decrease in the latency of their acid *N*-acetyl-β-glucosaminidase. PEI also inhibited the generation of radiolabeled digestion products from ^{125}I-labeled bovine serum albumin endocytosed by rat visceral yolk sac *in vitro*. However, PEI did not greatly inhibit the endocytic uptake of a nondigestible fluid-phase substrate, fluorescein isothiocyanate (FITC)-dextran. It is hypothesized that PEI inhibits the adsorptive endocytosis of ^{125}I-labeled bovine serum albumin, and thus its subsequent intralysosomal digestion, by competing with and displacing the labeled protein from its binding sites on the visceral yolk sac cell surface. This hypothesis suggests a plausible explanation for the ability of PEI to act as an efficient vector for gene and oligonucleotide transfer into mammalian cells. PEI present in the culture medium is carried into cells by adsorptive endocytosis. Concentrated thus on the endosome membrane, it permeabilizes this membrane and so affords DNA conjugated to the PEI an otherwise unavailable mode of access into the cytoplasm. BIOCHEM PHARMACOL **56**;1:41–46, 1998.

KEY WORDS. endocytosis; lysosome; polyethyleneimine

PEI† has been reported to act as an efficient vector for gene and oligonucleotide transfer into mammalian cells [1]. Its mechanism of action is unknown, but presumably involves the creation of pores in the plasma membrane or the endosome membrane. DNA is too large and too polar a molecule to cross membranes by passive diffusion, so that an increased rate of diffusion is an implausible explanation. Furthermore, there is no evidence that PEI could insert into a biomembrane to create a channel through which DNA could pass.

The PEI molecule contains primary, secondary, and tertiary aliphatic amine groups, in the ratio 1:2:1 [2]. The protonation pattern of PEI [2] indicates clearly the influence of multiple nitrogen atoms in close proximity. Whereas typical aliphatic amines have discrete pK_a values well above 7, PEI acquires protons incrementally over a wide pH range. At pH 7.5, only 11% of the nitrogen atoms in a PEI solution of concentration 0.01 resM (see Materials and Methods for a definition of resM) are protonated; this figure rises steadily to 67% as the pH is decreased to 4.0 [2]. Thus, PEI behaves as a "proton sponge" [1] over the pH range that a macromolecule will encounter in its passage from the extracellular space to the lysosome compartment.

The buffering capacity of PEI within the physiological pH range has been proposed [1] as contributing to the ability of the polymer to deliver DNA to cells. Thus, PEI could buffer the endosome/lysosome and prevent lysosomal degradation of DNA. It was also proposed, perhaps less plausibly, that a consequence of this inhibition of lysosomal degradation of DNA would be "lysosomal swelling and rupture that provide an escape mechanism for the PEI/DNA particles."

In this paper, we report experiments designed to evaluate the effects of PEI on the stability of the lysosomal membrane, on fluid-phase endocytosis, and on the endocytosis and intracellular degradation of a digestible macromolecule. Effects on lysosome stability were studied using lysosomes from rat liver. Endocytic uptake and subsequent intralysosomal digestion were measured in the 17.5-day rat visceral yolk sac cultured *in vitro*. The aim of the work has been to shed light on the mechanism by which PEI delivers DNA in living cells.‡

MATERIALS AND METHODS

Materials

All chemicals were from the Sigma Chemical Co. or the Aldrich Chemical Co. FITC-dextran (average molecular weight 38.9 K; Sigma product FD-40S) was dissolved in

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† *Abbreviations:* FITC, fluorescein isothiocyanate; and PEI, polyethyleneimine.

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water at 80 mg/mL and dialyzed for 48 hr against at least five changes of water.

A 50% (w/v) aqueous solution of PEI (average molecular weight 50 K; Sigma product P 3143) was diluted with water to 0.9% (9 mg/mL). After adjusting the pH to 7.0, the solution was dialyzed for 48 hr against at least three changes of water, readjusted to pH 7.0, and diluted to a nominal final concentration of 4.5 mg/mL. Because of the polydispersity of synthetic macromolecules such as PEI, it is common to express the concentrations of aqueous solutions in terms of the monomer residue molar concentration (resM). The monomer residue in PEI is $-CH_2-CH_2-NH-$, formula weight 43, so that a PEI solution of 4.5 mg/mL is approximately 0.1 resM. This stock solution was used to prepare further dilutions of PEI, as detailed below.

[^{125}I]Iodination of Bovine Serum Albumin

Two washed Iodo-beads® and 90 μL of 50 mM of sodium phosphate buffer (pH 7.0) were placed in a 1.5-mL Eppendorf tube. Then Na ^{125}I solution (2–3 μL; 200–300 μCi) was added, and the mixture was kept at room temperature for 5 min. Next bovine serum albumin (fraction V; Sigma product A9647; 2 mg in 1 mL water) was added, and the iodination reaction was allowed to proceed at room temperature for 15 min. The solution was then separated from the beads, and free [^{125}I]iodide was removed by adding a small KI crystal and dialyzing for 48 hr against at least five changes of 1% (w/v) KI in phosphate-buffered saline, pH 7.4. The product was stored at −20° in 100-μL portions and thawed just before use.

Lysosome Stability

A lysosome-rich subcellular fraction was prepared from the liver of a rat deprived of food overnight. The liver was pushed through a sieve, and the pulp was homogenized in 10 mL of 250 mM of ice-cold sucrose per gram, followed by centrifugation at 4° and 1100 *g* for 10 min. The pellet was discarded, and 20 mL of the supernate was centrifuged at 4° and 22,500 *g* for 10 min. The resulting pellet was gently resuspended in 1 mL of 250 mM of ice-cold sucrose.

To determine the ability of PEI to destabilize lysosomes, test solutions were prepared containing 250 mM of sucrose and various concentrations of dialyzed PEI, and adjusted with KOH or HCl to pH 5.0, 6.0, 7.0, or 8.0. To 3.8 mL of test solution at 25° was added 200 μL of the resuspended lysosome-rich pellet. This diluted suspension was maintained at 25°, and 50- or 100-μL samples were taken at 0, 30, and 60 min.

The integrity of the lysosomes in these samples was measured by determining the free activity of *N*-acetyl-β-glucosaminidase at 25°. The assay mixture contained the substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (2.5 mM) and sucrose (250 mM) in 200 mM of sodium citrate–HCl buffer, pH 5.0. Incubation was for 2 or 5 min and was terminated by the addition of 5 vol. of sodium carbonate (1 M). Liberated 4-methylumbelliferone was measured by determining the fluorescence of a 400-μL sample in the well-plate attachment of a Perkin Elmer luminescence spectrometer (model LS 50B) with excitation wavelength 360 nm and emission wavelength 448 nm. In controls, substrate was added after the sodium carbonate. The total *N*-acetyl-β-glucosaminidase activity was measured in simultaneous parallel assays containing 0.2% Triton X-100. Free and total activities were expressed in arbitrary fluorescence units, and the free:total ratio was expressed as a percentage.

Endocytic Uptake and Intralysosomal Digestion

Visceral yolk sacs from 17.5-day pregnant rats were incubated (1 or 2 per flask) with agitation at 37° in Erlenmeyer flasks containing 4.4 mL medium 199 (with $NaHCO_3$) and 0.5 mL of heat-inactivated calf serum, in an atmosphere of O_2:CO_2 (95:5), as originally described by Williams *et al.* [3, 4]. After a 15-min equilibration period, 100 μL of dialyzed FITC-dextran and/or 20–50 μL of dialyzed ^{125}I-labeled albumin solution was added. In some experiments, dialyzed PEI was also added at this time. At timed intervals yolk sacs were removed, dissected free from any remaining fragments of amnion, and washed in three changes of ice-cold PBS. Each yolk sac was dissolved in 0.5 M of NaOH (2.0 mL) by incubation with agitation for 1 hr at 37°. Duplicate 50-μL portions of the digest were used to measure protein content [5], with bovine serum albumin (Sigma Fraction V) as the reference protein.

The FITC-dextran content of duplicate 10- or 50-μL portions of yolk-sac digests and culture media was assayed [6] by dilution with 500 mM of sodium phosphate buffer, pH 7.4, and determining the fluorescence of a 400-μL sample in the well-plate attachment of a Perkin Elmer luminescence spectrometer (model LS 50B) with excitation wavelength 495 nm and emission wavelength 520 nm.

The radioactivity in duplicate 250-μL portions of yolk-sac digests and culture media was measured. Acid-soluble ^{125}I in each culture medium was also measured, by adding 1.0 mL of 15% trichloroacetic acid to a 250-μL portion, incubating for 1 hr at 4°, and centrifugation at 4600 *g* for 10 min. Duplicate 250-μL samples of the supernate were counted. The radioactivity measurements for any given experiment were all made on the same day, using a Beckman LS 7500 gamma-spectrometer.

To permit quantitative comparisons between the measured values for different substrates, substrate accumulation by tissues and the generation of digestion products were calculated and expressed as clearances. Clearance is defined here as the volume (μL) of culture medium whose substrate content has been accumulated or processed by the tissue. Clearances are normalized further by expression as microliters per milligram of tissue protein.

RESULTS

Table 1 shows the *N*-acetylglucosaminidase free activity of rat liver lysosomes incubated at 25° in 250 mM of

TABLE 1. Free activity of *N*-acetyl-β-D-glucosaminidase in rat liver lysosomes preincubated in 250 mM of sucrose containing PEI*

Concentration of PEI (resM†)	pH	Free activity (% of total activity) 0 min	30 min	60 min
0.002	5.0	18 ± 5	58 ± 5	83 ± 25
	6.0	12 ± 1	48 ± 11	63 ± 14
	7.0	11 ± 0	49 ± 9	69 ± 16
	8.0	9 ± 2	65 ± 16	70 ± 4
0.001	5.0	25 ± 2	41 ± 3	53 ± 9
	6.0	18 ± 1	39 ± 4	50 ± 5
	7.0	18 ± 4	44 ± 7	49 ± 6
	8.0	14 ± 4	35 ± 10	40 ± 2
0.0005	5.0	20 ± 2	34 ± 2	37 ± 5
	6.0	13 ± 6	26 ± 6	32 ± 4
	7.0	15 ± 1	35 ± 5	37 ± 6
	8.0	10 ± 1	22 ± 1	24 ± 5
0.0002	5.0	13 ± 1	18 ± 2	20 ± 2
	6.0	6 ± 6	13 ± 5	21 ± 2
	7.0	8 ± 3	16 ± 2	18 ± 1
	8.0	6 ± 1	12 ± 2	13 ± 4
0.0001	5.0	10 ± 2	14 ± 2	14 ± 2
	6.0	8 ± 4	12 ± 3	13 ± 4
	7.0	9 ± 3	15 ± 6	14 ± 2
	8.0	6 ± 1	12 ± 2	12 ± 1

*A lysosome-rich fraction from rat liver was diluted into the indicated solution, at 4°, and then incubated at 25°. Samples were removed for assay of *N*-acetyl-β-D-glucosaminidase at the indicated times. Enzyme activities were expressed in arbitrary fluorescence units, and the free activity was calculated as a percentage of the total activity measured in the presence of Triton X-100 (0.2%). Each value shown is the mean ± SD for three experiments.

†Residue molar concentration.

sucrose containing PEI. Within the pH range 5–8, lysosomes were stable for at least 60 min when incubated in sucrose alone (data not shown, but see [7]) or in 250 mM of sucrose containing 0.0001 resM of PEI. However, 0.002 resM of PEI caused more than 50% of the lysosomal enzyme to become accessible to substrate within 30 min, and similar effects were seen with 0.001 resM after 60 min. No pH-dependence of the effects of PEI at these concentrations was apparent. At 0.0005 resM, lysosome stability was decreased substantially; this effect was greater at pH values of 7 and below, and was already maximal by 30 min. PEI at 0.0002 resM had minimal effects.

Figure 1 shows the results of a typical experiment in which visceral yolk sacs were incubated in the presence of ^{125}I-labeled albumin. Little radioactivity accumulated in the tissue, but there was a steady release of acid-soluble radioactivity into the medium. This pattern has been shown [4] to indicate the steady endocytic uptake of the labeled protein, followed by its transfer to the lysosomes, where proteolysis leads to the generation of [^{125}I]iodotyrosine and its release into the culture medium. The rate of production of acid-soluble radioactivity can be equated with the rate of endocytic uptake of the labeled protein, and expressed as a clearance value [3, 4].

When these experiments were undertaken with PEI (0.003 resM) present in the culture medium, the rate of production of acid-soluble radioactivity was decreased sharply (Fig. 2). There was, however, no concomitant accumulation of radioactivity in the tissue itself. Table 2 summarizes the results of these experiments.

Since these results suggested that PEI inhibits endocytosis of ^{125}I-labeled albumin by the 17.5-day rat visceral yolk sac, the uptake of the fluid-phase marker FITC-dextran was also measured. Figure 3 shows the pattern of uptake and the effect of two concentrations of PEI. Even at 0.003 resM, PEI had little effect.

DISCUSSION

In the experiments described above, we have determined the effects of PEI on lysosomes and on the endocytosis and subsequent lysosomal digestion of ^{125}I-labeled albumin.

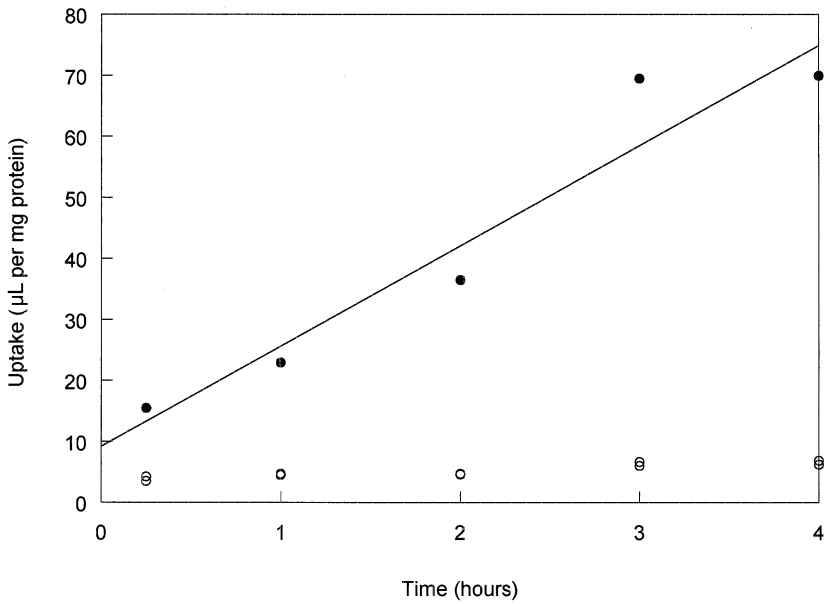

FIG. 1. Uptake and digestion of ^{125}I-labeled bovine serum albumin by the rat visceral yolk sac cultured *in vitro*. The total radioactivity present in the yolk sac tissue (○) and the acid-soluble radioactivity found in the culture medium (●) are shown. This is a typical experiment, representative of three experiments.

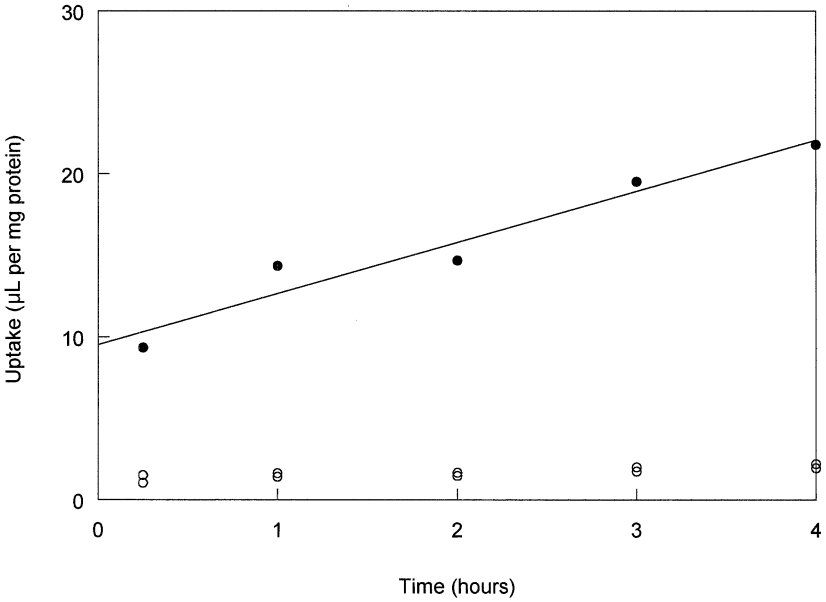

FIG. 2. Uptake and digestion of ^{125}I-labeled bovine serum albumin by the rat visceral yolk sac cultured *in vitro* in the presence of 0.003 resM (monomer residue molar concentration) of PEI. The total radioactivity present in the yolk sac tissue (○) and the acid-soluble radioactivity found in the culture medium (●) are shown. This is a typical experiment, representative of four experiments.

The methods chosen have been used extensively in the past for similar studies, so that a wealth of background literature is available. Investigations using the liver lysosome method were reviewed in 1992 [8] and those using the visceral yolk sac method in 1990 [9].

We first showed that concentrations of PEI greater than 0.0002 resM have the ability to damage the membrane of lysosomes, such that their *N*-acetylglucosaminidase becomes accessible to a normally non-permeant substrate. Contrary to expectation, the effect was not affected much by pH, within the range 5–8, indicating that it is not determined by the degree of protonation of PEI. PEI in similar concentrations has been shown recently to increase the permeability of the outer membrane of Gram-negative bacteria [10].

In previous experiments using the visceral yolk sac system, it had been shown that digestible macromolecules containing ^{125}I-labeled phenolic residues are endocytosed and delivered to the lysosomes, where enzyme-catalyzed digestion takes place, with release of low molecular weight radioactivity into the culture medium (see [9] for references). If a lysosomal protease inhibitor is present, the production of [^{125}I]iodotyrosine is decreased, with a concomitant accumulation of the undigested macromolecule in the cells [11, 12]. We argued that, since PEI disrupts the lysosome membrane, it is likely to have the same effect on the endosome membrane and alter the fate of an endocytosed macromolecule that in the absence of PEI is delivered to the lysosomes and digested. If PEI allowed endocytosed substrate to escape into the cytoplasm, the results might resemble those observed in the presence of a lysosomal protease inhibitor: the production of [^{125}I]iodotyrosine would decrease and undigested macromolecule would accumulate in the cells.

TABLE 2. Effects of PEI on the uptake and digestion of ^{125}I-labeled bovine serum albumin by 17.5-day rat visceral yolk sacs cultured *in vitro*

Concentration of PEI (resM*)	Rate of generation of acid-soluble radioactivity† (µL/mg/hr)
0	16.7 ± 3.4 (3)
0.0015	4.2 ± 1.6 (3)
0.003	2.2 ± 0.9 (4)

*Residue molar concentration.

†Acid-soluble radioactivity is expressed as a clearance value: the volume of culture medium whose content of ^{125}I-labeled albumin is converted into the acid-soluble form, per mg yolk-sac protein, per hour. Each value is the mean ± SD for the number of experiments shown in parentheses.

The results we obtained, using ^{125}I-labeled albumin as substrate, did not conform to this expected pattern. Although they indicated that PEI profoundly inhibits the generation of [^{125}I]iodotyrosine, there was no accumulation of substrate in the tissue. This outcome is not compatible with explanations that envision PEI diverting endocytosed ^{125}I-labeled albumin from undergoing lysosomal proteolysis, either by causing endosome rupture, with the release of the labeled protein into the cytoplasm, or by inhibiting lysosomal proteinases. Rather, they indicate that PEI is inhibiting the endocytosis of ^{125}I-labeled albumin, which is known to occur by adsorptive endocytosis [4]. However, a further experiment showed that there is only slight inhibition of fluid-phase endocytosis at concentrations of PEI that profoundly inhibit the uptake of ^{125}I-labeled albumin.

These data demonstrate that PEI inhibits the adsorptive endocytosis of a protein without concomitantly inhibiting fluid-phase endocytosis. Although other explanations are possible, such as an effect of PEI on receptor trafficking in the endosome, the simplest and most plausible is that PEI competes with and displaces ^{125}I-labeled albumin from the plasma membrane binding sites that are responsible for adsorptive endocytosis of the labeled protein. There is

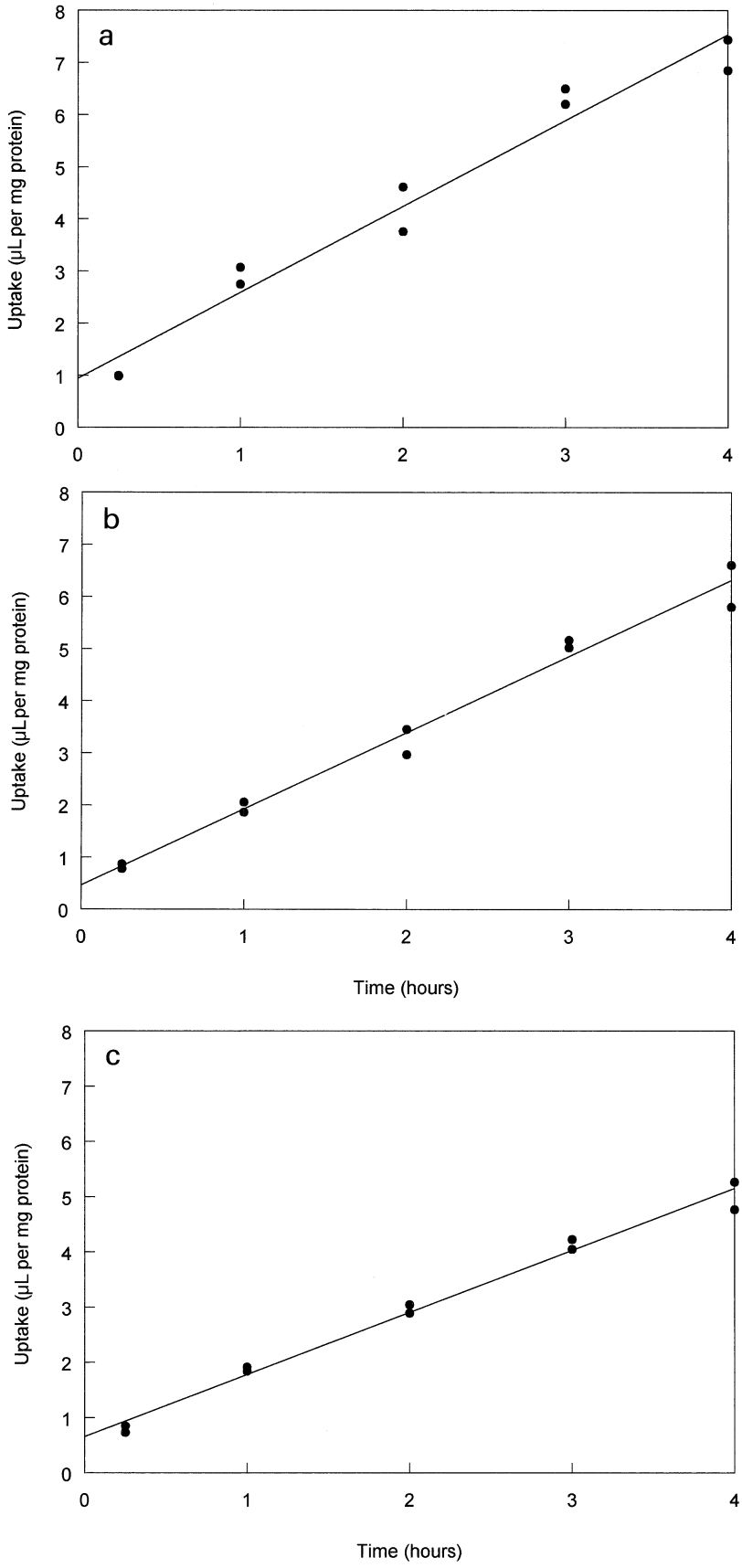

FIG. 3. Uptake of FITC-dextran by the rat visceral yolk sac cultured *in vitro* in the presence of PEI. The graphs show the FITC-dextran present in the tissue after cultures (a) in the absence of PEI; (b) in the presence of 0.0015 resM (monomer residue molar concentration) of PEI; and (c) in the presence of 0.003 resM of PEI. Three or four experiments were conducted at each concentration.

evidence that other polycations, for example poly-L-ornithine and poly-L-lysine [13, 14], enter the visceral yolk sac by adsorptive endocytosis, and it has been shown that cationic domains are important for the adsorptive endocytosis of certain proteins [15] by this tissue. In future work, we hope to ascertain whether PEI is a substrate for endocytosis in the visceral yolk sac and, if so, whether its rate of uptake is indicative of adsorptive or of fluid-phase endocytosis.

Taken together, our results suggest a mechanism for the ability of PEI to promote gene transfection. PEI present in low concentration in the culture medium is carried into cells by adsorptive endocytosis. Concentrated thus on the endosome membrane, it permeabilizes this membrane, and so affords DNA conjugated to the PEI an otherwise unavailable mode of access into the cytoplasm. The mechanism by which PEI modifies endosomal membrane structure is unknown, but may resemble that proposed recently for cationic liposomes [16].

We comment finally on the concentrations at which the effects of PEI on lysosomal integrity occur. These are significantly greater than those required for gene transfection. Transfection efficiency has been reported [1] to be maximal at approximately 10 equivalents of PEI nitrogen per DNA phosphate. Thus 2 μg of DNA (6 nmol of phosphate) and 60 res.nmol of PEI (60 nmol of nitrogen) were added to cells in a final volume of 1.1 mL, achieving a final PEI concentration of approximately 55 res.nmol/mL, or 5.5×10^{-5} resM. In our experiments, isolated lysosomes were unaffected by PEI at twice this concentration, and did not show significant damage until ten times (5×10^{-4} resM). The explanation for this discrepancy could lie in a different susceptibility of lysosomes and endosomes, or in a greater destabilizing effect of PEI when it is complexed to DNA. However, the explanation is more probably to be found in the orientation of the lysosomes in our experiment. Isolated lysosomes present their cytoplasmic face to the incubation medium, whereas endocytosed PEI will encounter the inside surface of endosomes, and this is the surface that bears the binding sites. Thus, PEI will be concentrated on this internal surface following adsorptive endocytosis, but probably not by the cytoplasmic face of isolated lysosomes.

In summary, we report the first direct evidence that PEI can disrupt lysosomes and modify adsorptive but not fluid-phase endocytosis. A hypothesis is formulated that explains the effects observed and the ability of PEI to promote gene transfection. Further experimental work will be required to test the various elements of this hypothesis.

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