INFLUENCE OF MOLECULAR CHARGE UPON THE ENDOCYTOSIS AND INTRACELLULAR FATE OF PEROXIDASE ACTIVITY IN CULTURED ARTERIAL ENDOTHELIUM

PETER F. DAVIES*, HELMUT G. RENNKE AND RAMZI S. COTRAN

From the Vascular Pathophysiology Laboratory, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, U.S.A.

SUMMARY

The molecular charge of the macromolecule, horseradish peroxidase (HRPase, 40 000 mol. wt), was modified to yield highly anionic (pI < 3.68) and cationic (pI = 9.5-10.5) derivatives. The effects upon the interactions between HRPase and arterial endothelium were then studied in vitro. The net rate of uptake of HRPase into endocytic vesicles and vacuoles of confluent endothelium was influenced by its molecular charge, there being less internalization of the anionic HRPase than of the native (pI = 7.9-8.2) and cationic derivatives. The molecular diameter was not significantly different between the cationic (A_r = 28.8 Å), anionic (A_r = 31.2 Å) and native (A_r = 29.6 Å) HRPase. The rate of uptake of [U-14C]sucrose, a tracer of bulk fluid endocytosis, was unaffected by the presence of the differently charged HRPase, indicating that the volume of vesicles formed per cell per hour remained constant. The intracellular fate of HRPase of different charge was investigated biochemically and morphologically. The rate of loss of internalized HRPase activity in the endothelial cells approximated first-order kinetics. The rate of disappearance of intracellular HRPase activity was much greater for cationic (t_1/2 = 8 h) and native (t_1/2 = 18 h) than for anionic HRPase (t_1/2 = 80-100 h). By electron microscopy, all 3 forms of HRPase were restricted to intracellular membrane-bounded vesicles and vacuoles consistent with a vesicle-lysosomal pathway. Studies with purified lysosomal cathepsin D indicated that the differences in the intracellular half-lives of HRPase may be attributable in small part to decreased and increased rates of lysosomal proteolysis of anionic and cationic HRPase, respectively, in comparison with native HRPase. Pre-labelling of endothelial secondary lysosomes by inhibitors of phagosome-lysosome fusion (dextran sulphate, polyglutamate) lengthened the intracellular half-life of native HRPase, while introduction of cationic ferritin to cells pulsed with anionic HRPase greatly decreased its half-life. Thus an influence of molecular charge upon endosome-lysosome fusion cannot be excluded. The studies indicate that the net charge carried by exogenous HRPase influences both its internalization in endocytic vesicles and its subsequent intracellular fate, which in turn may be modified by the introduction of other differently charged macromolecules. These results are discussed in relation to macromolecular transport by vascular endothelium in vivo.

* Correspondence to: Dr P. F. Davies, Pathology Research MRB-6, Brigham and Women’s Hospital, 75 Francis Street, Boston, MA 02115, U.S.A.
INTRODUCTION

Vascular endothelial cells contain many endocytic vesicles that are involved in the uptake of macromolecules from the extracellular environment (French, 1966; Florey & Sheppard, 1970; Schwartz & Benditt, 1972; Hütten, Boutet & More, 1973a, b; Stein & Stein, 1976; Vlodavsky, Fielding, Fielding & Gospodarowicz, 1978; Simionescu & Simionescu, 1978). The fate of such macromolecule-laden vesicles in vivo is unclear, but a number of studies suggest that there is some involvement in trans-endothelial transport in both capillary and arterial endothelium (Palade, 1960; French, 1966; Schwartz & Benditt, 1972; Hütten et al., 1973b; Simionescu, Simionescu & Palade, 1973). In vitro, however, many endocytic vesicles fuse with primary lysomes with exposure of their contents to degradative enzymes, and presumably a similar pathway exists for some of the vesicles observed in vivo. It appears likely therefore that the interactions of plasma macromolecules with the endothelial surface, their endocytosis and subsequent intracellular fate, are of importance with respect both to endothelial cell metabolism and to potential macromolecular transport.

Studies of the interactions of vascular endothelium with various ligands (Skutelsky, Rudich & Danon, 1975; Skutelsky & Danon, 1976; Stein, Chajek & Stein, 1976; Pelikan, Gimbrone & Cotran, 1979), hormones (Buonassisi & Venter, 1976; Gimbrone & Alexander, 1977), and lipoproteins (Stein & Stein, 1973, 1976; Vlodavsky, Fielding, Fielding & Gospodarowicz, 1978; Reckless, Weinstein & Steinberg, 1978) have shown that certain blood-borne macromolecules associate with the endothelial cell surface and are taken up by endocytosis. In endothelial and other cultured cells, uptake of macromolecules such as low-density lipoproteins, growth factors and model tracers by high-affinity low-capacity endocytosis, as well as by low-affinity high-capacity adsorptive and bulk-fluid endocytosis has been demonstrated (Brown & Goldstein, 1975; Williams, Kidston, Beck & Lloyd, 1975; Carpenter & Cohen, 1976; Leake & Bowyer, 1977; Davies & Ross, 1978, 1980; Vlodavsky et al., 1978; Davies, Selden & Schwartz, 1980). In such circumstances, the charges carried by macromolecules may greatly influence their uptake by endocytosis.

Using cationic, electron-dense probes, Skutelsky & Danon (1976) and Skutelsky et al. (1976) demonstrated evenly distributed negative charges over the surface of vascular endothelium in situ. Pelikan et al. (1979) reported a similar distribution in cultured human endothelium and suggested that this might influence endothelial endocytosis. The net negative charge at physiological pH is attributable to sulphated mucopolysaccharides associated with intrinsic proteins of the plasma membrane (Buonassisi & Root, 1975). Shen & Ryser (1978) have demonstrated a remarkable increase in the uptake of both albumin and HRPase by L929 fibroblasts following conjugation of the proteins with cationic poly-L-lysine. Morphological studies in the glomerulus by Rennke, Cotran & Venkatachalam (1975) have indicated a charge-dependent selectivity in renal filtration of plasma molecules and a morphometric study by Simionescu & Simionescu (1978) implicates molecular charge as a major factor modulating endocytosis in isolated myocardial endothelial cells.

In this paper we have used horseradish peroxidase (HRPase) as a model macro-
molecule to quantify the influences of molecular charge upon endocytosis by cultured arterial endothelial cells. The results demonstrate a charge-related effect upon the uptake of HRPase into endocytic vesicles. Subsequent to endocytosis, the rates of degradation of HRPase activity in the cells were greatly influenced by molecular charge. The studies are consistent with the existence of charge-selectivity in the uptake, degradation and, potentially, the transport of plasma macromolecules by the vascular endothelium.

MATERIALS AND METHODS

Cell culture

Bovine arterial endothelium was obtained by gentle application of a sterile cotton swab to the luminal surface of calf aorta following a 10-min exposure to crude collagenase (Clostridium histolyticum, type 1, 1 mg/ml, Worthington Biochemicals, Freehold, N.J.) at 37 °C. The swab, with attached cells, was rotated in a 25 cm² Falcon flask containing tissue culture medium to release the sheets of endothelial cells. The detached cells became adherent to the flask’s surface as sheets and islands of endothelium. A confluent monolayer was rapidly established in tissue culture medium containing 5% bovine plasma-derived serum, prepared as described by Vogel et al. (1958). The plasma-derived serum inhibited the growth of occasional contaminating smooth-muscle cells without affecting the proliferation of endothelial cells. Serial passage of endothelial cells (split ratio, 1:3) eliminated, by dilution, any contaminating smooth-muscle cells. Beyond the 3rd passage, cells were fed every other day with tissue culture medium containing 10% calf serum (M.A. Bioproducts, Bethesda, MD). For the studies described here, endothelial cells in passages 11-12 were used, usually 2 days after attaining confluence. The basal medium was Dulbecco’s modified Eagle’s medium supplemented with 2 μmol/ml glutamine and 100 units each of penicillin and streptomycin per ml.

Purification of HRPase

Horseradish peroxidase was purchased from Sigma Chemical Co., St Louis, MO (type II R.Z. 1.6). The main isoenzymes were purified as reported previously (Rennke & Venkatachalam, 1979), by gel filtration on an acrylamide/agarose column (Ultrigel AcA 44, LKB Produkter, AB, Sweden) followed by ion-exchange chromatography on a diethylaminoethyl cellulose column (DE52, Whatman, Inc., Clifton, N.Y.) equilibrated with 0.02 M-Tris-HCl buffer, pH 8.5.

Modification reactions

The polyanionic derivative (HRPₐ) was prepared by acylation with succinic anhydride (Eastman Kodak Company, Rochester, N.Y.) according to the method of Klotz (1967). For this purpose 840 mg of succinic anhydride was added in small increments to 420 mg of purified HRPase dissolved in 80 ml of distilled water. The pH was maintained at around 8.5 throughout the reaction (2 h). The final solution was kept overnight at 40 °C, dialysed extensively against distilled water, concentrated and lyophilized.

The polycationic derivative (HRPₜ) was prepared by substituting carboxyl groups on the protein molecules with hexanediamine (Hoare & Koshland, 1967; Rennke & Venkatachalam, 1979). The reaction was carried out in a solution containing 1 mg/ml of the purified HRPase, 0.08 M-hexanediamine (Eastman Kodak Company, Rochester, N.Y.) and 0.02 M-1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Calbiochem, San Diego, CA). This solution was maintained at pH 5.0 for 4 h at room temperature, kept overnight at 4 °C, dialysed against distilled water, concentrated and lyophilized. A stock solution of each HRPase (50 mg/ml Balanced Salt Solution, BSS) was prepared.
Isoelectric points

The isoelectric points of the tracers were established by isoelectric focusing on a slab containing 5% acrylamide, 0.18% bisacrylamide, 2% ampholyte (pH 3.5–10) (Ampholine, LKB Produkter, AB, Sweden) and 10% glycerol. The proteins were loaded on 4-μl wells contained in the gel and focused for 6 h on a model M150 slab isoelectrofocusing apparatus (MRA Corporation, Clearwater, FL). The pH was measured on the gel at 5-mm intervals by means of a surface microelectrode (Ingold Electrodes, Inc., Lexington, MA) connected to a Radiometer PHM 62 pH meter. The gel was then stained with Coomassie Blue (Righetti & Drysdale, 1974).

Chromatographic procedures

The molecular sizes of the purified native enzyme and the anionic and cationic derivatives dissolved either in buffer or in tissue culture medium were determined by gel filtration on a 60 cm × 2.2 cm column of acrylamide/agarose mixture (Ultrogel AcA 44). The column was equilibrated with a solution containing 0.15 M-NaCl, 0.05 M-phosphate buffer, pH 7.4, 0.001 M-EDTA to prevent bacterial growth. Samples of 1.5–2 ml were applied to the column under constant pressure, and fractions of about 1.3 ml were collected. The enzyme concentration in the fractions was determined at 403 nm on a Beckman DBGT spectrophotometer (Beckman Instruments, Inc., Irvine, CA) and biochemically by the diaminobenzidine assay (Herzog & Fahimi, 1973). Fractions obtained from loadings containing tissue culture medium or the proteins of known molecular radius that served as standards were read at 280 nm on the spectrophotometer. The void volume was determined repeatedly by the elution of blue dextran. Distribution coefficients ($K_V$) were calculated for all proteins. The unknown molecular radii of the tracers were then obtained from the standard curve, in which the molecular radii of the proteins that served as standards were plotted against their respective distribution coefficients on semilogarithmic graph paper (Laurent & Killander, 1964). All chromatographic procedures were carried out on the same column under identical elution conditions.

Quantitative endocytosis

HRPase was added to tissue culture medium to a final concentration of 1 mg HRPase/ml unless otherwise indicated in the figures. Confluent endothelial cell cultures were incubated with HRPase at 37 °C. Following incubation, the monolayer was washed 5 times with BSS containing 0.2% bovine albumin (BSS/BSA), rinsed with BSS and then exposed to trypsin (0.05 mg/ml). The detached cells were centrifuged in the cold (200 g, 7 min), the supernatant was removed and the cells were dissolved in 1 ml Triton X-100 (0.01% aqueous soln). Intra-cellular HRPase activity was assayed spectrophotometrically by the method of Steinman & Cohn (1972) using hydrogen peroxide and σ-dianisidine substrate at pH 5.0. The rate of increase in optical density with time was measured and recorded with a Gilford 250 spectrophotometer with flatbed recorder. Maximum sensitivity was 0.05 ng HRPase. A range of standards for each HRPase was prepared by dilution of carefully weighed, HRPase solutions. There was no detectable endogenous peroxidase activity in cultured endothelium. The preparation of cationized HRPase reduced its enzymic activity by 28%, whereas anionic HRPase retained 92% of the activity of the native enzyme (see Table 1). These differences, inherent in the standard curves, were taken into account in all determinations of HRPase internalization rates. There was no detectable HRPase activity in cells that were incubated with HRPase at 4 °C to inhibit endocytosis, washed and assayed by the above procedures.

In pulse-chase experiments to determine the half-life of intracellular HRPase, cells were incubated with the enzyme for 1–3 h, washed 5 times with BSS/BSA as above, then reincubated with HRPase-free tissue culture medium for a long chase period (up to 96 h). Duplicate dishes of cells were removed periodically for assay of intracellular HRPase activity after solution in Triton.

For studies of fluid pinocytosis rates, [U-14C]sucrose (New England Nuclear, Boston, MA) was added to the culture medium as a fluid tracer (10 μCi/ml, 2 × 10^-8 M) and assayed as
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described fully elsewhere (Davies & Ross, 1978; Davies et al. 1980). There was no measurable interaction between HRPase and [14C]sucrose when they were present in the same medium.

In all experiments, aliquots of each Triton lysate were removed for assay of cellular protein by the Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951).

Lactate dehydrogenase (LDHase; EC 1.1.1.27) released from cells was measured as an index of toxicity of HRPase by the Worthington assay procedure (Worthington Biochemicals, Freehold, N.J.) using rabbit skeletal muscle LDHase (type XI, Sigma Chemical Co.) as standard (Stambaugh & Post, 1966).

Table 1. Characteristic of anionic and cationic horseradish peroxidases

<table>
<thead>
<tr>
<th></th>
<th>pI range</th>
<th>( A_\text{d} (\text{Å}) )</th>
<th>% activity of HRPase ( \text{E}^{1\text{mol}}_\text{at 403 nm} )</th>
</tr>
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<tbody>
<tr>
<td>Anionic HRPase (HRPA)</td>
<td>&lt; 3.68</td>
<td>31.2</td>
<td>92.1 ( \times 10^4 )</td>
</tr>
<tr>
<td>Native HRPase (HRPN)</td>
<td>6.9 (trace)</td>
<td>29.6</td>
<td>100</td>
</tr>
<tr>
<td>Cationic HRPase (HRP(\text{c} ))</td>
<td>7.94-8.17 (&gt; 92%)</td>
<td>9.54 ( \times 10^4 )</td>
<td></td>
</tr>
</tbody>
</table>

* Comparison on the basis of per unit weight of enzyme protein.

Cytochemical localization of HRPase

Cultures were exposed to HRPase (1 mg/ml) for 1 h at 37 °C and 4 °C. The cells were washed 5 times with BSS/BSA, rinsed twice with BSS then fixed with 3 % glutaraldehyde in 0.1 M-cacodylate buffer with 0.05 % CaCl\(_2\). Peroxidase activity was localized in the cells by a modification of the technique of Graham & Karnovsky (1966). The cells were preincubated for 30 min in 50 mg % diaminobenzidine in 0.05 M-Tris-HCl at pH 7.6. \( \text{H}_2\text{O}_2 \) was then added to the diaminobenzidine to a final concentration of 0.01 % for 30 min. After rinsing with cacodylate buffer, cells were postfixed in 1 % OsO\(_4\), dehydrated and embedded in Epon 812 directly in the Petri dish. After polymerization the cells were remounted on Epon blanks and cut transversely. Thin sections, stained lightly with lead citrate, were examined in a Philips 201 electron microscope.

Inactivation of HRPase activity by purified lysosomal cathepsin D

Differently charged HRPases were incubated at 25 °C with the following purified lysosomal enzymes: cathepsins C and D (bovine spleen), β-glucosidase (yeast), and neuraminidase (Clostridium perfringens), all obtained from Sigma Chemical Co., St Louis, MO. The incubation mixture (20 µl) contained the lysosomal enzyme sample (20 µl), HRPase (20–500 ng) in 3.4 mM-tartrate buffer, pH 4.0 (Barrett, 1967). Aliquots (100 µl) were withdrawn at various times up to 12 h and assayed for HRPase enzymic activity in phosphate buffer (0.2 M) at pH 5.0. The lysosomal enzymes alone did not affect the substrate mixture for the HRPase enzyme assay. We were only able to detect significant decrease of HRPase activity with preparations of cathepsin D (0.27 mU/ml). Heat-inactivated (100 °C, 5 min) cathepsin D was used as a control. The specific inhibitor of cathepsin D, pepstatin (Barrett & Dingle, 1972) obtained from the Sigma Chemical Co. also directly inhibited HRPase activity and so was not useful in this assay system.

RESULTS

Modification of molecular charge

The modification reactions resulted in derivatives of distinctly different isoelectric points (Table 1). The purified HRPase had a major component (> 92 % activity) with an isoelectric point of 7.94–8.17; a minor isoenzyme with isoelectric point of 6.9 was also identified. The succinyl-derivative (HRPA) was identified as a narrow
Fig. 1. Elution profiles of anionic (HRPₐ, upper) native (HRPₙ, middle) and cationic (HRP⁺, lower) HRPases in buffer (left panels) and in tissue culture medium (right panels) chromatographed on a 60 cm x 2-2 cm column of Ultrogel® AcA 44. Total volume, \( V_T = 214 \) ml; void volume, \( V_0 = 85.9 \) ml; elution volume, \( V_E \); distribution coefficient, \( K_w = (V_E-V_0)/(V_T-V_0) \).
band close to the edge of the gel slab; its isoelectric point was below 3.68, the first pH reading of the gel. The hexanediamine-substituted derivative had an isoelectric point of 9.5–10.5.

The calculated molecular radii for the 3 tracer enzymes are given in Table 1. The elution profiles of the tracers in buffer and in the presence of tissue culture medium after incubation with endothelial cells are shown in Fig. 1. All 3 enzymes eluted as a single peak except for HRPc in which a small amount of enzymic activity (less than 2% of the total enzyme) appeared in earlier fractions with a $K_v$ of 0.2720 and a calculated $A_e$ of 37.89 Å. Fractions that contained proteins of the tissue culture medium were consistently devoid of enzymic activity.

![Graph showing uptake of differently charged HRPase into endocytic vesicles](image)

**Fig. 2.** Uptake of anionic (●), native (■) and cationic (△) HRPases in confluent cultured arterial endothelial cells at 37 °C. Cells were incubated with 1 mg HRPase/ml medium. Intracellular HRPase activity was determined in duplicate cultures following washing, dispersal of cells with trypsin and solution in Triton X-100.

**Uptake of differently charged HRPase into endocytic vesicles**

The influence of molecular charge upon the net uptake of HRPase by endothelial cells at 37 °C is shown in Fig. 2. The rate of net uptake of the cationic form of the protein was greater than that of the native enzyme. The anionic HRPase, however, was taken up at a significantly lower rate than native and cationic enzymes. These differences were not attributable to any effect of the differently charged HRPases upon cell viability as determined by direct observation of the cultures and Trypan Blue exclusion. Lactate dehydrogenase activity was determined to test for toxic effects of different HRPases (Table 2). LDHase released into the culture media in the presence of the different HRPases was 2%, 0% and 6% of dish total (cells + medium) for anionic, native and cationic HRPases, respectively.

The possibility that differences in HRPase uptake could be explained because of interaction of the differently charged HRPase with components of the tissue culture medium, or with substances produced by the cell monolayer, was investigated. HRPase activity remained unaffected by the presence or absence of tissue culture medium (Fig. 1), and the presence or absence of cells at 4 °C and 37 °C over a wide range of
exposure times (not shown). Gel filtration of HRPase-containing medium resulted in an elution pattern that was identical to that obtained from the purified HRPase molecules; there was no alteration in molecular size or molecular charge and the

Table 2. Viability of endothelial cell cultures following incubation with differently charged HRPase as determined by cellular lactate dehydrogenase (LDHase; EC 1.1.1.27) release

<table>
<thead>
<tr>
<th>Lactate dehydrogenase activity (μmol NADH oxidized/min per ml culture medium or cell lysate)</th>
<th>Medium</th>
<th>Cells</th>
<th>% LDHase activity retained by cells</th>
</tr>
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<tbody>
<tr>
<td>HRP&lt;sub&gt;a&lt;/sub&gt;</td>
<td>0.30</td>
<td>14.13</td>
<td>98</td>
</tr>
<tr>
<td>HRP&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Not detectable</td>
<td>11.99</td>
<td>100</td>
</tr>
<tr>
<td>HRP&lt;sub&gt;c&lt;/sub&gt;</td>
<td>15.25</td>
<td>15.25</td>
<td>94</td>
</tr>
</tbody>
</table>

Viability of endothelial cultures following incubation with differently charged HRPase. Confluent cultures were incubated at 37 °C for 1 h with anionic (HRP<sub>a</sub>), native (HRP<sub>n</sub>) or cationic (HRP<sub>c</sub>) HRPase (1 mg/ml). The media were removed and 100 µl of each were assayed for LDHase activity. The cells were rinsed with BSS, freeze-thawed 5 times and the cell lysates were suspended in 1 ml Dulbecco's modified Eagle's medium. Aliquots (100 µl) were assayed for LDHase activity.

Fig. 3. Time course of fluid endocytosis by confluent cultured endothelium in the presence of anionic (○), native (■) and cationic (△) HRPases (1 mg/ml). [14C]-glucose was added to the culture medium as a tracer of fluid endocytosis. After incubation, cells were processed as described for Fig. 2, and the Triton lysate was assayed for 14C radioactivity by liquid scintillation counting. Enzymic specific activities of the HRPase were identical to those of the purified molecules. We concluded that there was no association of HRPase with substances in the tissue culture medium and no activation or degradation of HRPase activity by substances produced by the endothelial cells. Therefore, the observed differences in uptake rates appear attributable to HRPase-cell interactions.
It was possible that the molecular charge of HRPase might alter the rate of formation of endocytic vesicles or, alternatively, might alter the volume of newly formed endocytic vesicles. In either case a decrease in the rate of fluid-phase endocytosis would result. In order to exclude these possibilities, the rate of bulk fluid endocytosis was determined independently using [14C]sucrose (Davies et al. 1980) in the presence of differently charged HRPase (Fig. 3). There were no significant differences in rates of fluid endocytosis in the presence of any of the HRPases.

Incubation of endothelium with increasing concentrations of HRPase (0.5-2.0 mg/ml) for 1 h at 37 °C resulted in linear uptake of the 3 tracers (Fig. 4) with no indication of saturability as would occur with receptor-mediated adsorptive endocytosis (O'Brien, 1979). These data indicate that the 3 forms of HRPase behave primarily as tracers of fluid endocytosis and weak adsorptive endocytosis, a conclusion that was also supported by the morphological studies reported below.

**Half-life of intracellular HRPase activity**

The half-lives ($t_1$) of HRPase enzymic activities subsequent to internalization by cultured endothelium at 4 °C and 37 °C are shown for differently charged HRPase in Fig. 5. At 4 °C (inset, Fig. 5) there was little loss of activity, consistent with inhibition of both lysosomal enzymic activity and exocytosis of intact HRPase. At 37 °C, loss of
HRPase activity followed first-order kinetics for the 3 HRPase tracers. The $t_1$ for anionic HRPase was considerably longer than for the native and cationic forms; 96, 18 and 8 h, respectively. When these results are taken into account, it is apparent

![Graph showing kinetics of loss of intracellular HRPase activity from confluent cultured arterial endothelium at 37°C and at 4°C (inset).](image)

that the uptake rates shown in Fig. 2 underestimate the true rates of uptake by a variable margin. Thus the underestimate is greatest for HRP_C and smallest for HRP_A, where, because of very slow intracellular degradation, its uptake was almost cumulative. Table 3 is a summary of the $t_1$ values for HRPase from 5 experiments. Thus the main findings from these pulse-chase experiments was the charge-related order of the half-life of intracellular HRPase activity: HRP_A \( \gg \) HRP_N \( > \) HRP_C.

**Table 3. Half-life intracellular enzyme activities for endocytosed anionic, native and cationic HRPase in confluent cultured endothelial cells**

<table>
<thead>
<tr>
<th></th>
<th>Mean $t_1$ (h)</th>
<th>Mean $K_1$</th>
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<tr>
<td>HRP_A</td>
<td>95.8 ± 7.0</td>
<td>8.7 ( \times ) 10^{-4} h^{-1}</td>
</tr>
<tr>
<td>HRP_N</td>
<td>18.0 ± 1.3</td>
<td>3.5 ( \times ) 10^{-4} h^{-1}</td>
</tr>
<tr>
<td>HRP_C</td>
<td>8.2 ± 0.8</td>
<td>6.9 ( \times ) 10^{-3} h^{-1}</td>
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</table>

Summary table of intracellular half-lives of differently charged HRPase activities in confluent cultures of arterial endothelium.
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Cytochemical localization of HRPase

All 3 forms of HRPase were detected only in membrane-bounded cytoplasmic vesicles (Fig. 6). To maximize the sensitivity of detection of small amounts of HRPase, counterstaining was minimized or omitted during preparation for transmission electron microscopy. There was no evidence of intracellular, extravesicular HRPase activity. The cell surface, cytoplasm and other organelles were devoid of reaction product. HRPase was not detected in association with the cell surface even for the cationic protein, and this was consistent with the biochemical data. HRPase was not present in all vesicles, an observation that is most easily explained by the fact that a considerable proportion of apparent 'vesicles' are in fact invaginations of the cell surface. They appear as vesicles only because the plane of section excludes their continuity with the cell surface. In the peripheral regions of cultured vascular endothelium up to 70% of vesicles are continuous with the cell surface as determined by ruthenium red staining (Davies & Kuczera, unpublished). Therefore, the absence of HRPase from a proportion of vesicles (Fig. 6) indicates that the washing procedure effectively eluted HRPase from all sites that were continuous with the cell surface.

Loss of HRPase enzymic activity in the presence of purified cathepsin D

Straus (1958) proposed that intracellular HRPase activity declined as a result of degradation by lysosomal enzymes following fusion of incoming HRPase-laden pinosomes with primary lysosomes. We investigated the possibility that differences in $t_1$ values for HRPase of modified charge may reflect altered affinity of acid proteases for the HRPase substrate. HRPase activity was measured at various times during incubation with cathepsin D at pH 4.0. There was a decrease in activity with time of incubation for all 3 forms of HRPase (Fig. 7). The rate of loss of enzymic activity was of the order: cationic > native > anionic. Although the order of susceptibility to cathepsin D action is consistent with the $t_1$ values determined in endothelial cells, the magnitude of the differences is not, there being a twelvefold difference between $t_1$ values for intracellular cationic and anionic HRPase. Lineweaver–Burk analysis of the data (Fig. 8) demonstrated that the $K_m$ values for all 3 HRPases were very similar (range $1.36-1.44 \times 10^{-8}$ mol l$^{-1}$), but with different $V_{max}$ values.

Influences of inhibitors of phagosome–lysosome fusion

Recently Hart, Young and co-workers (Hart & Young, 1975, 1978, 1979; Geisow, Beaven, Hart & Young, 1980) have described the inhibition of phagosome–lysosome fusion in cultured macrophages by various anionic macromolecules. The site of action of such inhibition was localized to the secondary lysosome (Geisow et al. 1980). It was possible that the effects of molecular charge upon the intracellular HRPase half-life that we observed might be attributable to modified endosome–lysosome fusion. Pulse–chase experiments with HRPase were therefore conducted in the presence of the anionic macromolecules, dextran sulphate and polyglutamic acid. Fig. 9 demonstrates that the normal short half-life of cationic HRPase in these cells was
lengthened significantly in the presence of the anions. Conversely, cationized ferritin prepared as reported previously (Rennke et al. 1975) significantly shortened the intracellular half-life of anionic HRPase (Fig. 10). These influences were entirely intracellular, there being no HRPase associated with the cell membrane at the time of exposure to the ferritin, dextran sulphate or polyglutamate.

**DISCUSSION**

Our studies demonstrate that molecular charge may confer selectivity upon the uptake and intracellular fate of macromolecules by the endothelium. In tissue culture, molecular charge significantly influences the net uptake of HRPase into arterial endothelium, presumably because of charge-related interactions at the cell surface. Subsequent to internalization, there is resistance of negatively charged HRPase to intracellular inactivation and, conversely, enhanced inactivation of the positively charged enzyme.
Fig. 9. Altered intracellular fate of native HRPase activity in the presence of dextran sulphate (DS) or polyglutamic acid (PGA). Cells were pulse-labelled with native HRPase, thoroughly washed and then exposed to culture medium, which in some dishes contained DS (▼, 10 mg/ml) or PGA (□, 100 mg/ml). Cellular HRPase activity was determined at various time points during the chase period.

Fig. 10. Accelerated intracellular disappearance of anionic HRPase activity in the presence of cationized ferritin. Endothelial cells were pulse-labelled with anionic HRPase, washed and then exposed to fresh culture medium. Cationized ferritin (cf) was added (1 mg/ml) 20 h (▼) and 48 h (■) later. Cellular HRPase activity was determined at various time points during the chase period.

Concerning endocytosis of charged HRPase

HRPases of negative, neutral or positive net charge behave as tracers of bulk fluid uptake or weak adsorptive endocytosis. There are, however, significant differences in the net uptake of differently charged HRPases into endocytic vesicles. The effect could not be explained by any measurable toxic effects upon these cells, or by interaction of HRPase with components of the tissue culture medium. The HRPases were taken up exclusively in vesicles with no evidence of HRPase reaction product in the cytosol. The uptake of [14C]sucrose by fluid endocytosis was the same whether anionic, native or cationic enzyme was present in the culture medium. These data suggest that at a constant rate of formation of endocytic vesicle volume, the differences in uptake of HRPases of differing pI values reside in charge effects at the cell surface.

Anionic sites have been shown to be uniformly distributed over the surface of vascular endothelial cells in culture (Pelikan et al. 1979) and in vivo (Skutelsky & Danon, 1976). Ruthenium red staining (Luft, 1971) reveals a 'glycocalyx' on the cell surface. Its composition includes highly negative heparan sulphate (Buonassisi & Root, 1975). It appears reasonable to expect differential uptake of positively and
negatively charged macromolecules under conditions in which a high affinity receptor is absent.

Jacques (1972) reported that adsorption to, or repulsion from, the plasma membrane is a potential mechanism for selectivity of internalization by endocytosis. Our results agree in principle with those of Shen & Ryser (1978) who demonstrated large increases in cellular accumulation of HRPase conjugated to poly-L-lysine. The smaller charge-related increase in net uptake of cationic HRPase that we observed in endothelium is most likely attributable to a more limited enhancement of molecular surface charge density by hexanediamine substitution than Shen & Ryser (1978) obtained by conjugation with polylysine. Lloyd and coworkers (Pratten, Duncan & Lloyd, 1978) have also demonstrated enhanced uptake of cationic tracers into cultured cells solely on the basis of increased adsorption to the plasma membrane. Similar mechanisms may account for the disproportionate uptake of plasma proteins by the liver parenchyma. Removal of sialic acid from caeruloplasmin, which greatly increases its positive charge, resulted in much greater uptake than when sialic acid was an integral part of the molecule (Gregoriades, Morell, Sternlieb & Scheinberg, 1970).

Effects of molecular charge upon intracellular degradation of HRPase activity

Gordon (1975) discussed on theoretical grounds the importance of regulation of vesicular transport of macromolecules to their subsequent catabolism in lysosomes, endocytosis being an obvious limiting step in the delivery of exogenous proteins to the lysosomes. In the endothelium, selectivity of uptake based on molecular charge will be a controlling step for transcellular transport of proteins in addition to determining their delivery to lysosomes. Endothelial cells in vivo and in vitro contain lysosomes, and some of the metabolic requirements of endothelial cells are met by lysosomal degradation of endocytosed proteins; the effects of molecular charge upon this process are therefore of interest.

When endothelial cells were pulse-labelled with HRPase of different charge, the kinetics of disappearance of intracellular HRPase activity proceeded as a first-order reaction in each case, but with large, consistent differences in intracellular half-lives. Anionic HRPase activity was degraded at a very slow rate (t1/2, 96 h), while cationic HRPase activity was lost about twice as fast (t1/2, 8 h) as the native form (t1/2, 18 h). Inactivation of intracellular HRPase activity could occur by several mechanisms. There may be fusion of HRPase-laden vesicles with primary lysosomes and the subsequent proteolysis of HRPase as described by Straus (1958) and supported by the studies of Steinman and coworkers (Steinman, Silver & Cohn, 1974). Alternatively, there is indirect evidence from our use of phagosome–lysosome fusion inhibitors that the loading of vesicles with HRPase of different charge may influence the fusion of the vesicles with lysosomes.

A proven pathway for HRPase degradation is via lysosomal proteolysis. In contrast to the physiological pH (7.2) existing at the cell surface when HRPase is endocytosed, the intralysosomal pH has been estimated to be 5.0 (Mego, 1971). At this pH, the anionic HRPase is still anionic but less so, while the native and cationic forms are more cationic than at neutral pH. If it is assumed that this pathway predominates,
our results suggest that HRPase inactivation is selective for molecular charge; in particular, that anionic HRPase is resistant to lysosomal inactivation. Some supporting evidence was obtained by test-tube experiments in which lysosomal cathepsin D, the enzyme responsible for initial proteolysis in secondary lysosomes (Gordon, 1975), degraded HRPase activity at pH 4.0 in the order: $\text{HRP}_C > \text{HRP}_N > \text{HRP}_A$. There is evidence that lysosomal enzymes of rat kidney and liver are cationic (Koenig, 1969) and that, in vitro, a variety of polyanions reversibly inhibit their activity through electrostatic interaction (Bernfeld, 1963). Our results with cathepsin D are consistent with such a mechanism of inhibition. The differences in intracellular half-life of the charged HRPases, however, were much greater than the differences in loss of activity in the presence of cathepsin D, particularly for anionic HRPase. While this might be due to the absence of a true lysosomal environment in the test-tube, it is also possible that different rates of vesicle–lysosome fusion may influence loss of intracellular HRPase activity, analogous to the inhibition of phagosome–lysosome fusion by polyanions in cultured macrophages as described by Hart & Young (1975). This hypothesis can be investigated more directly using morphometric techniques at the macromolecular level in the intact cell. Whichever mechanism operates, it will be of interest to determine if charge selectivity is expressed for the lysosomal proteolysis of other differently labelled, exogenous proteins and in other cells.

It is possible that, in vivo, some endothelial vesicles are targeted for the lysosomal system while others are engaged in transendothelial transport of macromolecules. It is also likely that the function of endocytic vesicles varies in endothelia from different segments of the vascular tree. Although we did not attempt in the present study to measure precise intracellular pathways of endocytic vesicles in arterial endothelium, the experiments indicate that the charge carried on a macromolecule contained within an incoming endocytic vesicle can influence its intracellular fate. Whether or not this phenomenon reflects altered vesicle pathways within the endothelium remains to be investigated.

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REFERENCES


Vascular endothelial endocytosis


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