Anionized and Cationized Hemeundecapeptides as Probes for Cell Surface Charge and Permeability Studies: Differentiated Labeling of Endothelial Plasmalemmal Vesicles

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ABSTRACT To obtain small membrane markers easily accessible to the charged groups of the cell surface, we prepared, from hemeundecapeptide (HUP), three derivatives that maintain the peroxidatic activity: (*a*) the anionized hemeundecapeptide, M_r 1,963, estimated diameter 1.68 nm, pI 3.5, for the detection of basic groups; and both (*b*) a cationized hemeundecapeptide containing predominantly tertiary amino groups, M_r 2,215, estimated diameter 1.75 nm, pI 9.0, and (*c*) a cationized hemeundecapeptide containing only primary amino groups, M_r 2,271, estimated diameter 1.75 nm, pI 10.6, for labeling acidic residues. The markers were perfused in situ in mice to label the luminal surface of fenestrated endothelium of pancreatic capillaries. Specimens were processed through the cytochemical reaction for peroxidatic activity and examined by electron microscopy. The anionized HUP and HUP (pI 4.85) marked the plasmalemma proper, the coated pits, and the membrane and diaphragms of plasmalemmal vesicles and transendothelial channels. The cationized HUP containing predominantly tertiary amino groups (pI 9.0) decorated all cell surface components with the exception of plasmalemmal vesicles and channels; the latter were, however, labeled by the cationized HUP containing only primary groups (pI 10.6), which suggests that these structures contain on their luminal surface very weak acidic residues of high pK_a values. The fact that the membrane of plasmalemmal vesicles can discriminate against permeant cationic macromolecules only up to a pI of ~9.0 indicates that in the electrostatic restriction there is a charge limit. In the case of fenestrated capillary endothelium, the upper charge limit seems to be a pI of ~9.0. In these vessels, the charge discrimination is effective for molecules as small as 2 nm.

Several markers have been used to gain information about the cell surface charge. They have the limitation of either requiring prefixation (e.g., by colloidal iron), or concomitant fixation (e.g., by ruthenium red), or of being large enough (e.g., cationized ferritin) to raise the problem of their accessibility to the cell surface moieties. In order to circumvent these limitations, we prepared from hemeundecapeptide (HUP)[1] (microperoxidase M-11) two groups of derivatives that maintain their peroxidatic activity: anionized hemeundecapeptide (aHUP) for the detection of basic residues, and two cationized hemeundecapeptides (cHUP) for the detection of acidic groups. These compounds have the following advantages: (*a*) they can be applied to fresh tissues in vivo, in situ, and in vitro; (*b*) owing to their small size (about 2 nm in diameter), their access to the groups of cell surface is assumed to be easier than that of most of the markers so far employed in such studies; and (*c*) they can be used as probes both for detecting the cell surface charges and for exploring the charge effect in permeability studies.

We report the results obtained by using these synthesized markers for the detection of the anionic and cationic sites of the luminal surface of fenestrated capillary endothelium in mouse pancreas. Among these markers, the native hemeundecapeptide was previously used only as a fluid phase tracer in capillary permeability (5, 20, 21).

[1] *Abbreviations used in this paper:* aHUP, anionized hemeundecapeptide; cHUP, cationized hemeundecapeptide; $cHUP_p$, cationized hemeundecapeptide (pI 10.6) containing primary amino groups only; $cHUP_t$, cationized hemeundecapeptide (pI 9.0) containing predominantly tertiary amino groups; HUP, hemeundecapeptide (microperoxidase-11).

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MATERIALS AND METHODS

Reagents

Microperoxidase (M-11), thereafter referred to as hemeundecapeptide (HUP), 3,3′-diaminobenzidine tetrahydrochloride, and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Cytochrome c, *n,N*-dimethyl-trimethylenediamine (DMTA), hexamethylenediamine (HMD), and acetic anhydride were obtained from E. Merck (Darmstadt, Federal Republic of Germany). Agarose IEF, Pharmalyte pH 3-10, and Sephadex G25 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of HUP Derivatives

ANIONIZED HUP: This derivative was prepared by covalent blocking of the free amino groups of the HUP with acetic anhydride (6): $HUP(NH_2)_2 + 2(CH_3CO)_2O \rightarrow HUP(NHCOCH_3)_2$.

The following protocol was used: 20 mg HUP was dissolved in 1.5 ml distilled water to which 1.5 ml of saturated solution of sodium acetate was added. The solution was cooled at 0°C, and 20 μl acetic anhydride (4 × 5 μl) was added. After 60 min, the mixture was transferred to a stoppered vial and left overnight at 4°C. The aHUP was further purified by gel filtration and concentrated as indicated for cHUP.

CATIONIZED HUP: Synthesis of cHUP was based on the activation of the carboxyl groups of HUP with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, and the subsequent reaction of activated carboxyl with either (a) *N,N*-dimethyltrimethylenediamine to obtain a derivative that contains predominantly tertiary amino groups ($cHUP_t$), or (b) to produce a derivative with primary amino groups only ($cHUP_p$) (reference 3).[2]

To obtain these two cationic components the following procedure was used: in a 5 ml beaker placed on a magnetic stirrer, 20 mg of HUP was added to 2 ml of aqueous solution of *N,N*-dimethyltrimethylenediamine (or hexamethylenediamine) adjusted to pH 4.0 with 10 N HCl. After HUP solubilization, 80 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide was added and the pH was maintained at 4.0 with 0.2 N HCl with continuous stirring until no detectable changes in pH were recorded (~3 h). The solution was then transferred to a stoppered vial, left for 48 h at 22°C, and passed through a Sephadex G25 fine column (90 × 1.5 cm), with distilled water as eluent. cHUP were concentrated by lyophilization (Multi-dry; FTS System Inc., Stone Ridge, NY).

Characterization of HUP Derivatives

MOLECULAR CHARGE: The isoelectric point of these HUP derivatives was determined by isoelectric focusing. Slabs (9 × 10 cm) were prepared from a gel containing 1% agarose IEF, 12% sorbitol, and 2% Pharmalyte pH 3-10. The pH was measured on the gel slab at 5-mm intervals by use of a stibium electrode. The pH gradient so obtained was virtually linear over the range of 3.4 to 9.6. The HUP bands were localized via the peroxidatic reaction by use of 3,3′-diaminobenzidine as H_2 donor (10).

OPTIMUM PH FOR PEROXIDATIC REACTION: The optimum for peroxidatic reaction was determined at different pH and various hemepeptide concentrations with 3,3′-diaminobenzidine as hydrogen donor (10). The HUP concentration was determined by spectrophotometry with a molar extinction coefficient of $E_{417\,nm} = 1.2 \times 10^5$ (1/mol × cm) (reference 15).

MOLECULAR DIAMETER: The diameter values of HUP and its derivatives were estimated under the assumption that each molecule is a sphere with $V = (1/6)\,\pi \cdot d^3 = M_r/\rho N$ so that $d = (6M_r/\pi\rho N)^{1/3}$, where V is the volume of the HUP molecule, d its diameter, M_r the molecular weight, ρ the density of HUP molecule (assumed to be 1.3 g/cm³ [5]), and N Avogadro's number.

ABSORPTION SPECTRUM: The absorption spectrum of each HUP derivative was recorded by comparison with the absorption spectrum of HUP in 0.15 NaCl at pH 7.0.

[2] (*a*) $HUP(COOH)_4 + \frac{4H_2N(CH_2)_3N(CH_3)_2}{EDC} HUP(CONH[CH_2]_3N[CH_3]_2)_4$ (cationized HUP derivative with predominantly tertiary amino groups). (*b*) $HUP(COOH)_4 + \frac{4H_2N(CH_2)_6NH_2}{EDC} HUP(CONH[CH_2]_6NH_2)_4$ (cationized HUP derivative with free primary amino groups only). EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.

Experimental Procedure

The HUP derivatives synthesized were tested as membrane markers: $cHUP_t$ and $cHUP_p$ for the detection of anionic sites, and HUP and aHUP for the localization of cationic groups on the luminal cell surface of the fenestrated capillaries in the mouse pancreas.

ANIMALS: Experiments were carried out on 30 male RAP mice, 20–25 g. Before use, animals were kept for a few days under similar standard housing and feeding conditions.

EXPERIMENTAL PROTOCOL: After light anesthesia with ethyl ether and laparotomy, the vena cava caudalis and the abdominal aorta were catheterized and the vasculature was washed free of blood by retrograde perfusion with Dulbecco's phosphate-buffered saline (PBS), pH 7.2, warmed to 37°C. At this temperature the isoelectric points of the probe molecules remained unchanged. With the vena cava as the outlet, the perfusion was performed in an open circuit at a flow rate of 3 ml/min for 6 min before the marker was added to the perfusate. Hemepeptides were injected at a concentration of 2 mg/ml and maintained within the vasculature for 2 min. The unbound tracer was flushed with PBS (3 ml/min for 4 min), and the vasculature was fixed by perfusion for 10 min with a mixture of 5% formaldehyde and 3% glutaraldehyde in 0.1 M HCl-Na cacodylate buffer, pH 7.2–7.4. Six mice were perfused with each probe; from each pancreas 6–10 specimens were randomly collected; from each of them 60–90 sections were cut, and in each section 18–25 vascular profiles were examined.

These hemepeptide derivatives have also been used to study the charge effect on capillary permeability (manuscript in preparation).

Controls

Controls consisted of pancreas specimens collected from six mice which, without being perfused with markers, were processed histochemically for peroxidatic reaction.

Tissue Processing for Electron Microscopy

Pancreas specimens fixed in situ were immersed for 90 min in same aldehyde solution as above, then incubated for 60 min at 22°C in 0.15% 3,3′-diaminobenzidine, 0.02% H_2O_2 in 0.05 M Tris-HCl buffer (9, 21) at pH 8.0 for cHUP, and at pH 9.0 for HUP and aHUP. Specimens were postfixed for 90 min at 4°C in 1% OsO_4 in 0.1 M HCl-Na cacodylate buffer, pH 7.2–7.4, stained en bloc for 30 min in an aqueous solution of 0.5% uranyl acetate, dehydrated in ethanol, and embedded in Epon-812. Thin sections cut with a diamond knife on OmU_3C Reichert ultramicrotome (C. Reichert A. G., Wien, Austria) or an Ultracut (American Optical Scientific Instruments, Warner-Lambert Co., Buffalo, NY) were stained with lead citrate and examined with Philips 201C and Philips 400 HM electron microscopes operated at 80 kV (Philips Electronic Instruments, Inc., Mahwah, NJ).

RESULTS

Physicochemical Properties of HUP Markers

The structural formulae of the tracers used are illustrated in Fig. 1, and some physicochemical properties are listed in Table I.

The isoelectric point of the HUP markers was 3.5 for aHUP, 4.85 for HUP, 9.0 for $cHUP_t$, and 10.6 for $cHUP_p$ (Table I and Fig. 2).

The optimum pH for peroxidatic activity was different for each derivative: 9.4 for aHUP, 9.0 for HUP, and 8.1 for both $cHUP_t$ and $cHUP_p$ (Fig. 3).

If a spherical configuration of the HUP molecule was assumed, the estimated diameters were: 1.68 nm for aHUP, 1.66 nm and HUP, and 1.75 nm for both $cHUP_t$ and $cHUP_p$. Because of the nature of this assumption (see Materials and Methods), these calculated values obviously provide only an approximation of the actual dimensions of these molecules.

Absorption spectra were quite similar for HUP and its derivatives, with a maximum absorption at 417 nm (Fig. 4).

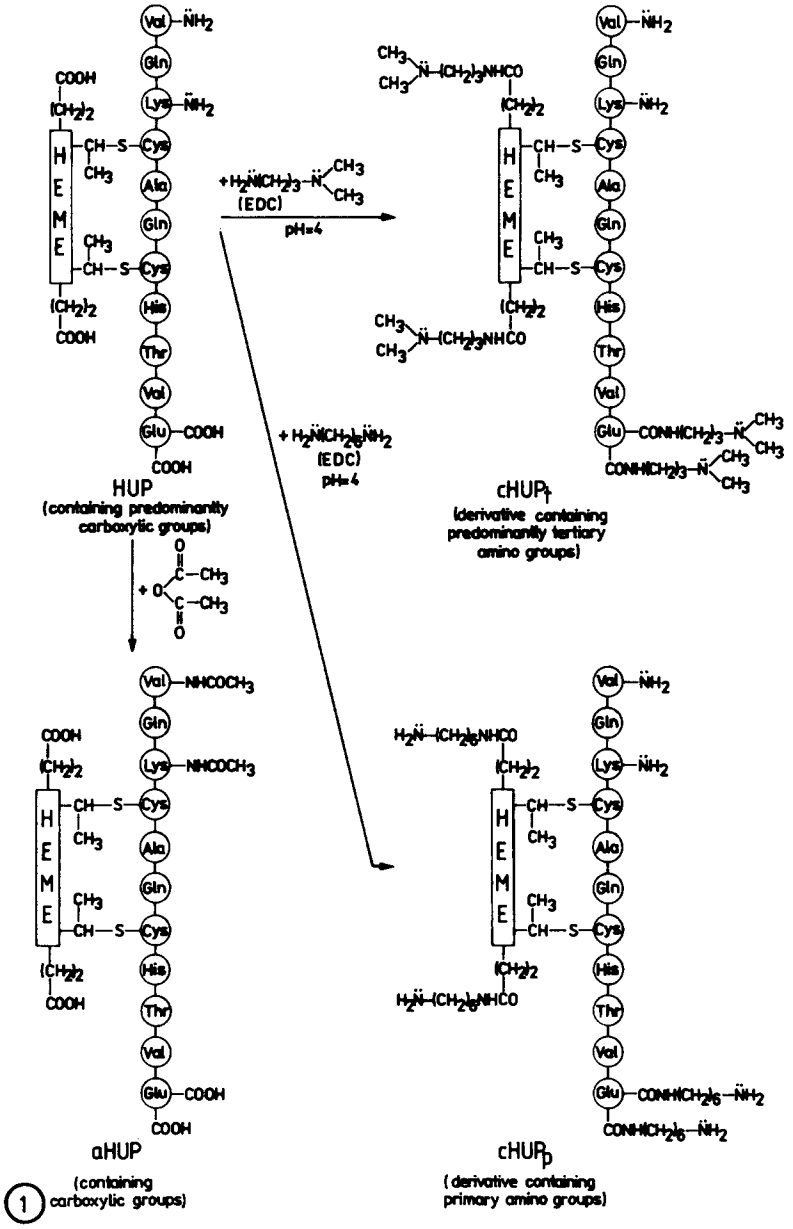

FIGURE 1 Structural formulae of the HUP, aHUP, cHUP$_t$, and cHUP$_p$.

TABLE I

Some Physicochemical Characteristics of the Hemeundepeptide Markers Used

Physicochemical properties	aHUP	HUP	cHUP$_t$	cHUP$_p$
Molecular weight (M_r)	1,963	1,879	2,215	2,271
Number of free primary amino groups	0	2	2	6
Number of tertiary amino groups	0	0	4	0
Number of free carboxyl groups	4	4	0	0
Optimum pH for peroxidatic reaction (DAB as H_2 donor)	9.4	9.0	8.1	8.1
Isoelectric point	3.5	4.85	9.0	10.6
Molecular diameter* (*nm*)	1.68	1.66	1.75	1.75

DAB, 3,3'-diaminobenzidine.
* Estimated as indicated in Materials and Methods.

Binding of HUP Markers to the Endothelial Cell Surface

In the fenestrated endothelium of pancreatic capillaries, the luminal cell surface was heterogeneously decorated by the markers used. The decoration pattern was examined on the plasmalemma proper, the coated pits, the membrane and diaphragms of plasmalemmal vesicles open on the luminal front, the membrane and diaphragms of transendothelial channels, and the luminal aspect of the fenestral diaphragms.

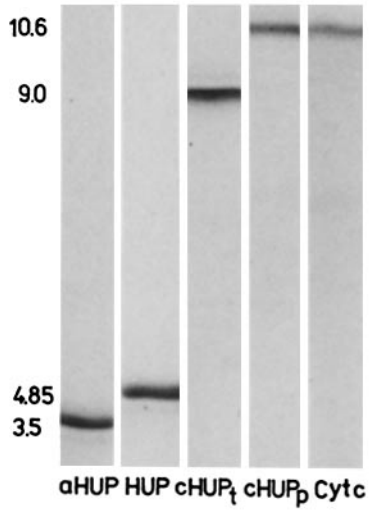

FIGURE 2 Gel electrofocusing of HUP, aHUP, $cHUP_t$, and $cHUP_p$, and the cytochrome c (Cytc). The pH gradient was prepared with 2% ampholyte in the range of 3 to 10.

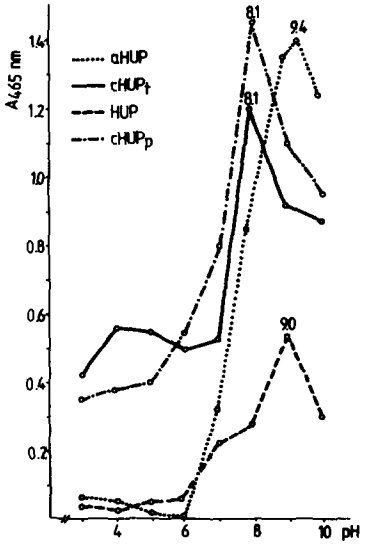

FIGURE 3 Effect of pH on the peroxidatic activity of HUP, aHUP, $cHUP_t$, and $cHUP_p$. Citric acid-Na_2HPO_4 buffer (0.1 M) was used for pH 3 to 6, and Tris-HCl buffer (0.1 M) was used for pH >7.0.

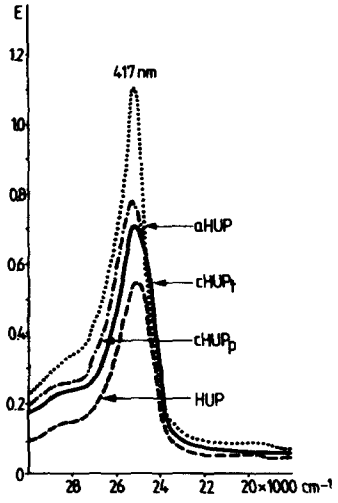

FIGURE 4 Absorption spectra of the hemeundecapeptide (HUP) and its anionic (aHUP) and cationic ($cHUP_t$ and $cHUP_p$) derivatives.

Occasionally, in the case of cHUP, additional information was obtained by tracer labeling of the endothelial basal lamina (basement membrane).

As a general feature, the anionic compounds such as HUP and aHUP gave a comparable decoration pattern, which involved all the surface domains mentioned above. Among the cationic derivatives, $cHUP_t$ labeled the endothelial cell surface, with the exception of the membrane and diaphragms of plasmalemmal vesicles. In contrast, $cHUP_p$ also marked the plasmalemmal vesicles and their associated diaphragms. Examined in more detail, the decoration pattern had some characteristics for each marker used.

BINDING OF ANIONIZED HUP (pI 3.5): The reaction product appeared as a rather uniform layer 15–20 nm thick closely bound to the plasma membrane, the coated pits, the membrane of plasmalemmal vesicles open on the luminal front, and the transendothelial channels. The labeling was usually heavier on the diaphragms associated with these vesicles and channels (Fig. 5*b*). The decoration of fenestral diaphragms was either comparable or less intense than that of the adjacent plasmalemma (Fig. 5*a*). The marker labeled the infundibula leading to the intercellular junctions but did not penetrate beyond the latter.

BINDING OF HUP (pI 4.85): The native HUP produced a labeling pattern similar to that described for aHUP (not illustrated).

BINDING OF CATIONIZED HUP_t (pI 9.0): The cytochemical reaction product of this cationic derivative appeared usually as a less homogeneous layer; it was in general thicker (~20–40 nm) than that produced by the HUP and aHUP, and had the occasional tendency to form small aggregates ~20 to 35 nm wide and located at a certain distance (~10–20 nm) from the outer electron opaque leaflet of the plasmalemma membrane. 2 min after its perfusion in situ, $cHUP_t$ labeled plasmalemma proper and the junctional infundibula, and it usually displayed a higher density on coated pits and coated vesicles. The large majority of fenestral diaphragms were marked on their luminal aspect by variable amounts of reaction product (Fig. 6*b*). In some locations, the marker passed through fenestral diaphragms (Fig. 7) and labeled the underlying endothelial basal lamina. On the latter, the decoration appeared as small (~15–20 nm diameter) aggregates with a relatively uniform distribution (~75–95 nm apart) predominantly present on the lamina rara externa (not illustrated). In contrast to the structures mentioned above, plasmalemmal vesicles open on the luminal front, transendothelial channels, and their associated diaphragms remained unlabeled (Fig. 6, *c–f*). Occasionally, on plasmalemmal vesicles incompletely opened on the cell surface, a small amount of reaction product could be seen in the area of vesicle stomata, probably bound to their infundibulum.

BINDING OF CATIONIZED HUP_p (pI 10.6): The reaction product of this marker labeled almost continuously the plasmalemma proper with a higher concentration on coated pits and coated vesicles (Fig. 7, *a–d*). As with the previous markers, the decoration of fenestral diaphragms was variable, being in general comparable or slightly less intense than on the adjacent plasmalemma (Fig. 7*a*). Characteristically, however, and unlike $cHUP_t$ (pI 9.0), this cationic derivative of high pI (10.6) consistently had access to and labeled also the membranes of plasmalemmal vesicles, transendothelial channels, and their diaphragms (Fig. 7 *b–d*). A similar binding pattern was obtained with cytochrome c, pI 10.6 (not illustrated; manuscript in preparation).

DISCUSSION

Procedures used for HUP anionization and cationization do not significantly change the molecular weight, molecular diameter, and the absorption spectrum, but for each compound

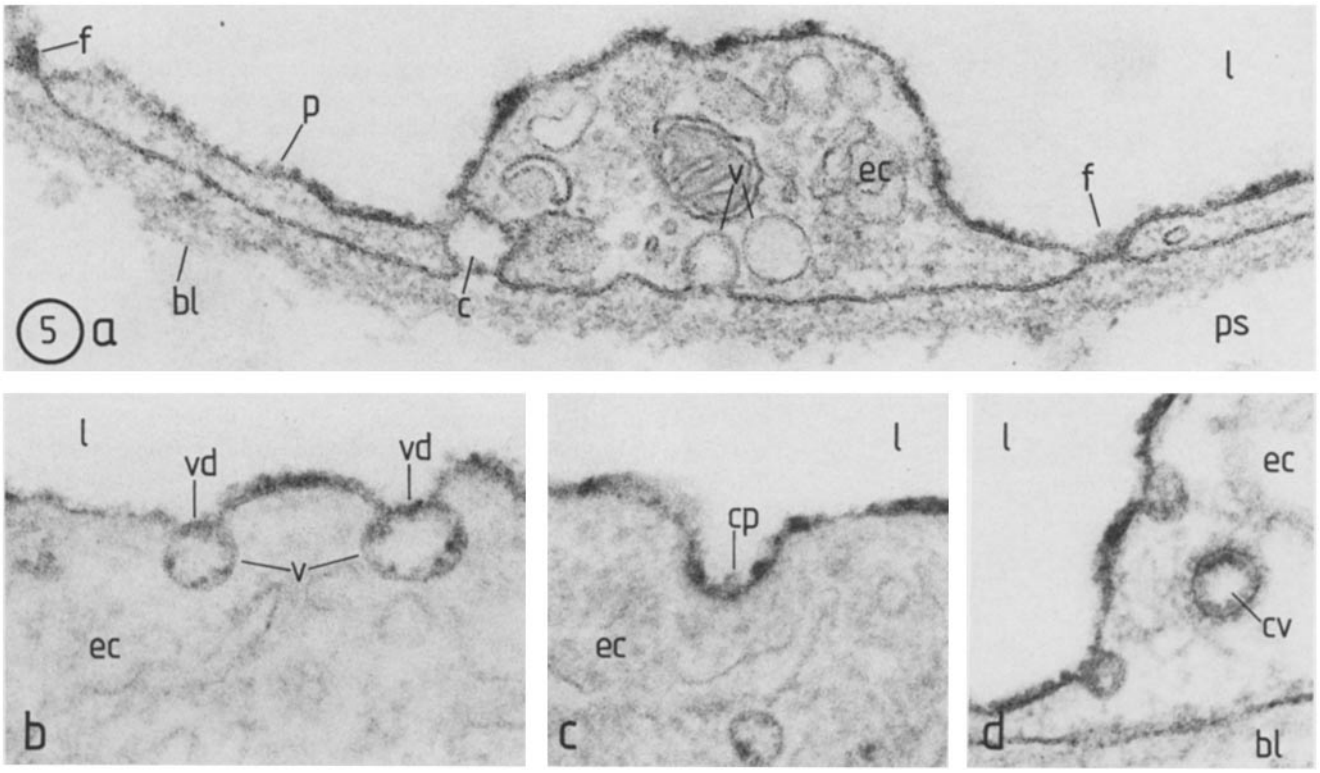

FIGURE 5 Blood capillaries of mouse pancreas: binding of aHUP (pI 3.5) to the luminal endothelial surface (similar labeling was obtained with HUP, pI 4.85). (*a*) Reaction product marks the plasmalemma proper (*p*) and the fenestral diaphragm (*f*), as well as the membrane and diaphragm of transendothelial channels (*c*). (*b*) The marker also labels rather uniformly the membrane of plasmalemmal vesicles (*v*) open on the luminal front and their associated diaphragms (*vd*). (*c*) Peroxidatic reaction product usually labels markedly the coated pits (*cp*). (*d*) aHUP labeling of a coated vesicle (*cv*). *bl*, Basal lamina; *ec*, endothelial cell; *l*, lumen. (*a*) × 88,000; (*b*) × 108,000; (*c*) × 85,000.

the pH optimum for peroxidatic activity is different (Figs. 3 and 4 and Table I). The isoelectric points of these derivatives cover a relatively broad spectrum; this makes them useful probes for detecting cell surface acidic and basic residues of various charge density and strength. These tracers can also be used as small charged probes for permeability studies.

Previous work with cationized ferritin, pI 8.4, and alcian blue (17–19, 20, 22), as well as with HUP, colloidal gold-conjugated albumin (1, 8), or hydrazinated ferritin (V. Muresan and M. Constantinescu, Institute of Cellular Biology, Bucharest, manuscript submitted for publication), have established that cell surface of capillary endothelium contains differentiated microdomains generated by the preferential distribution of anionic sites and some glycoconjugates. In our present experiments, the results obtained with the cHUP of pI 9.0 were similar to those previously reported for cationized ferritin of pI 8.4 (17–19, 20, 22), i.e., both ligands decorated plasmalemma, coated pits, and fenestral diaphragms but failed to label the membrane and the diaphragm of plasmalemmal vesicles and vesicle-derived channels. In contrast, the cHUP of a high positive charge (pI 10.6), in addition to marking the cell surface domains decorated by cationized ferritin and $cHUP_t$, also marks the diaphragm and the membrane of plasmalemmal vesicles and channels. The same labeling pattern was observed after perfusion with cytochrome *c*. The avid binding of cytochrome *c* to the membrane of plasmalemmal vesicles may explain at least in part why this probe crosses the capillary wall more rapidly than expected on the basis of size alone (2). Our findings suggest that the membrane of plasmalemmal vesicles is not completely devoid of exposed acidic groups, but these may be represented by very weak anionic residues. Likely candidates for the latter are the phenolic hydroxylic groups (pK_a, 10.07) of thyrosine residues, the free sulfhydryl groups (pK_a, 8.33) of cysteine, the carboxyl groups of glutamic acid (pK_a, 4.25), and the carboxyl groups of glycosaminoglycans (pK_a, 3.21–3.93) (4, 12, 16). These observations further support the idea that the vesicle membrane is chemically different from plasma membrane, and that during their fusion, the two membranes do not intermix (20).

When anionic ferritin (M_r 480,000, molecular diameter ~11 nm, pI 4.5) was used to detect the cell surface basic residues, no labeling was observed on the luminal front of fenestrated endothelium (22). When we used other anionic markers such as aHUP and HUP with a pI very close (3.5 and 4.85, respectively) to that of the anionic ferritin but much smaller than the latter, almost the entire cell surface was uniformly labeled (except for fenestral diaphragms on which the decoration was rather poor and inhomogeneous). The difference in binding capacity between anionic ferritin and aHUP can be explained either in terms of accessibility or by the density of cationic sites required for binding a large vs. a small tracer. The intense aHUP labeling of vesicle membrane and diaphragm indicates that these structures contain many basic groups. As expected, these residues appear to be at lower density, a ubiquitous component of the endothelial plasma membrane.

Because it contains both types of oppositely charged residues (four carboxyl groups and two amino groups), HUP can theoretically bind concomitantly to both cationic and anionic

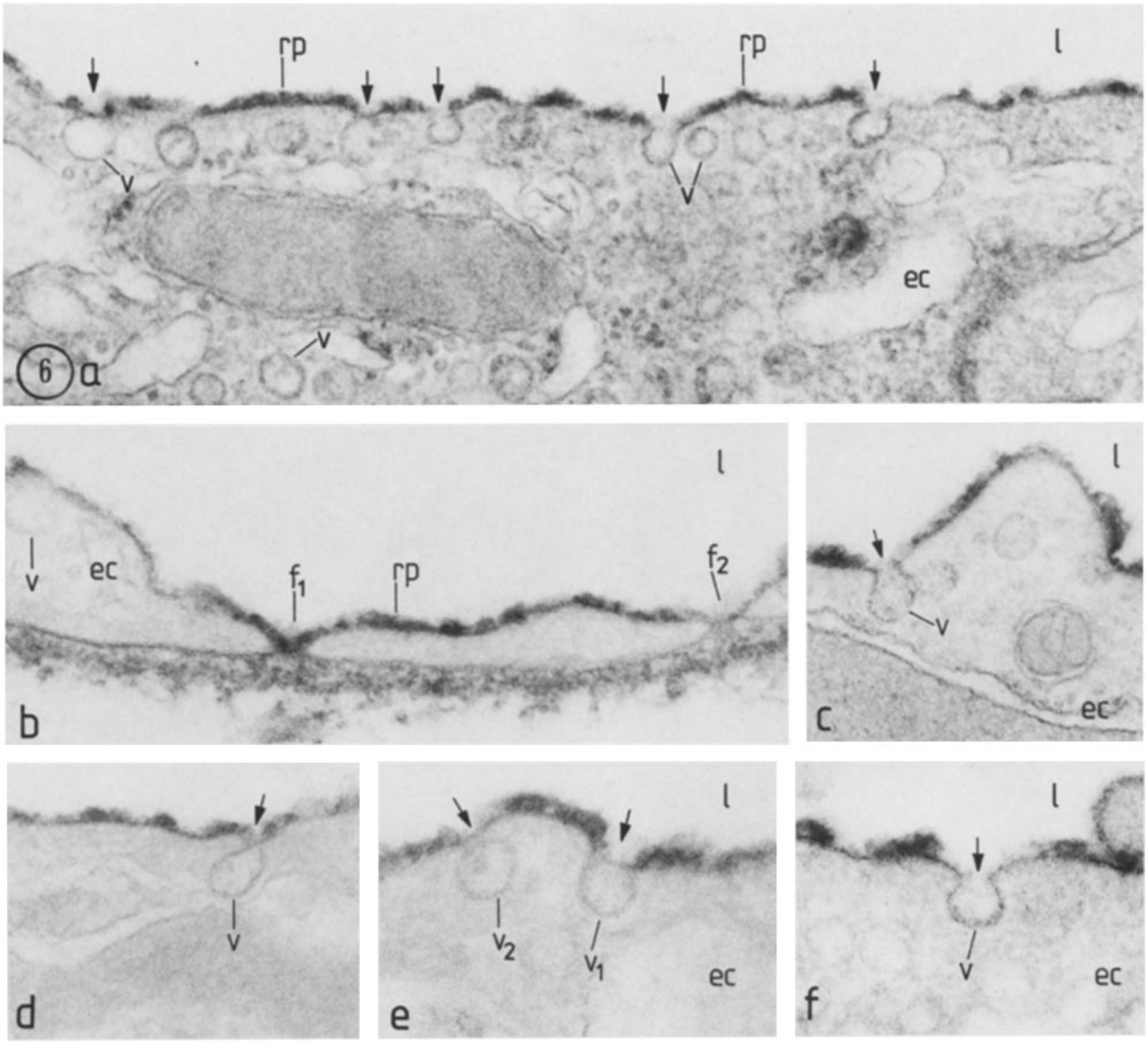

FIGURE 6 Blood capillaries of mouse pancreas: binding pattern of the cHUP, of pI 9.0 to the luminal endothelial surface. (*a*) Peroxidase reaction product (*rp*) labels rather homogeneously the plasmalemma proper but fails to decorate markedly the membrane and the diaphragm of plasmalemmal vesicles (*v*) open on the cell surface (arrows). (*b*) The marker decorates variably the fenestral diaphragms—see differences between f_1 and f_2. (*c–f*) Gallery of plasmalemmal vesicles, with various degree of opening on the luminal surface, which are not labeled by the reaction product. In e, the vesicle v_1 displays a conspicuous stomal diaphragm unlabeled by the marker (arrow); vesicle v_2 contains a moderately contrasted round particle, probably a low density lipoprotein. ec, Endothelial cell; *l*, lumen. (*a*) × 70,500; (*b*) × 111,000; (*c*) × 82,000; (*d*) 108,000. (e and *f*) × 117,000.

sites. Since the decoration pattern obtained with this heme-peptide is similar to that of aHUP, it is very likely that during its electrostatic adsorption on the cell surface, HUP behaves predominantly as an anionic marker. Since HUP has been used as tracer for capillary permeability (5, 21, 24), its ingestion by plasmalemmal vesicles should be considered a combined process of adsorptive (electrostatic) and fluid-phase uptake. The lack of strong anionic sites on vesicles and channels has been inferred to be a possible explanation for the reduced transport of cationic macromolecules, and enhanced exchange of anionic molecules in intestinal capillaries (11, 14). Because in our present experiments none of the tracers were detected in the intercellular junctions of capillary endothelia examined, it is unlikely that either the negative or the positive charges on such solutes per se contributes to exclusion and restriction to diffusion in intercellular junctions (2). The latter may be tight enough to exclude molecules of the size of hemeundecapeptides (~ 2 nm) (21).

A salient finding of the present experiments is that plasmalemmal vesicles appear to be able to discriminate against cationic molecules only up to a pI of ~9.0. At higher isoelectric points (e.g., cHUP of pI 10.6 and cytochrome *c* of pI 10.6), this electrostatic restriction ceases. This probably applies also to a larger tracer such as the cationized ferritin of pI > 9.0 used at a very high concentration by some investigators (13, 23). Our observations suggest that in the electrostatic restriction to permeant macromolecules there is a charge limit; in the case of fenestrated capillary endothelium of mouse pancreas, the upper charge limit appears to be ~pI 9.0. The lower and upper charge limits may vary from one molec-

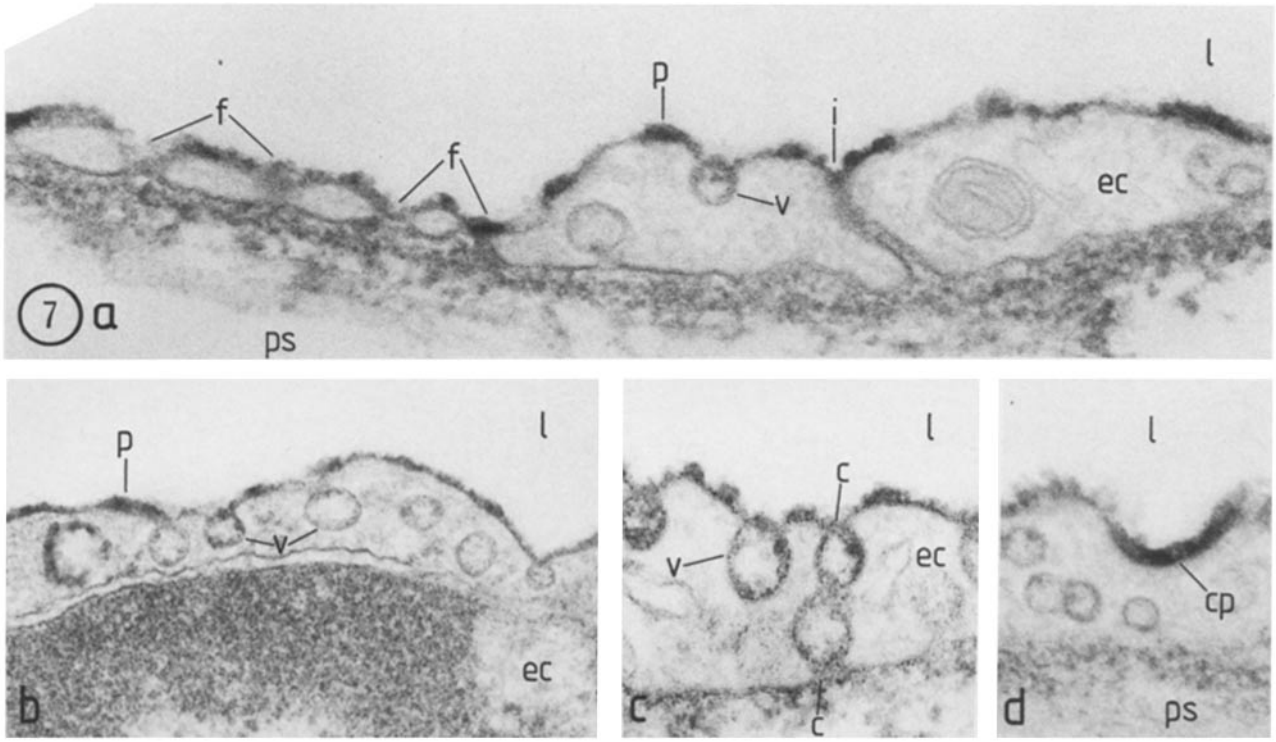

FIGURE 7 Blood capillaries of mouse pancreas: labeling of endothelial cell surface by the cHUP$_p$ of pI 10.6 (a similar labeling pattern was obtained with cytochrome *c*, pI 10.6). (*a*) Peroxidatic reaction product decorates plasma membrane (*p*) in a relatively unhomogeneous fashion, as well as plasma vesicles (*v*), junctional infundibulum (*i*), and, variably, the fenestral diaphragms (*f*). (*b*) Plasmalemmal vesicles (*v*) open on or close to the luminal front are marked by cHUP$_t$. (*c*) Labeling of a two-vesicle transendothelial channel (*c*). (*d*) Heavy decoration of a coated pit (*cp*). *ec*, Endothelial cell; *l*, lumen; *ps*, pericapillary space. (*a*) × 81,000; (*b*) × 80,000; (*c*) × 99,000; (*d*) × 81,000.

ular species to another and from one vascular bed to another in different physiologic and pathologic conditions. With respect to the range of molecular size at which charge discrimination becomes effective (22), our results indicate that this size limit, if it exists, should be < 2 nm, corresponding to an M_r of ~2,000.

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