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IMMUNOELECTROPHORETIC ANALYSIS OF VASCULAR, MEMBRANE-BOUND ANGIOTENSIN I CONVERTING ENZYME, AMINOPEPTIDASE M, AND DIPEPTIDYL(AMINO)PEPTIDASE IV

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Abstract—Antisera raised against specific renal brush border peptidases have been used to characterize vascular surface membrane angiotensin I converting enzyme (ACE; EC 3.4.15.1), aminopeptidase M (AmM; EC 3.4.11.2), and dipeptidyl(amino)peptidase IV (DAP IV; EC 3.4.14.5) by techniques of differential solubilization, fused-rocket immunoelectrophoresis and crossed immunoelectrophoresis. The vascular membrane-bound enzymes are immunologically indistinguishable from their brush border counterparts and can be solubilized by treatment with detergent and/or papain. The electrophoretic mobilities of the papain-treated forms of each enzyme were greater than those of the detergent-treated forms. This increased mobility is associated with the removal of small, hydrophobic, non-antigenic components of the enzymes. Regardless of the method of solubilization, the electrophoretic mobilities of the vascular enzymes were greater than those of the brush border enzymes. However, after treatment with neuraminidase to remove sialic acid, their respective mobilities were similar. The mobilities of serum AmM and DAP IV were identical to the respective papain-solubilized vascular enzymes both before and after neuraminidase. Thus, like the brush border enzymes, the data presented are consistent with the model that vascular ACE, AmM and DAP IV are intrinsic membrane peptidases bound to their surface membranes by small, non-antigenic, hydrophobic anchors associated with the lipid bilayer. In addition, these vascular surface membrane peptidases are similar to and may be a source of the circulating enzymes.

Angiotensin I converting enzyme (ACE; peptidyl dipeptidase; EC 3.4.15.1) converts angiotensin I to angiotensin II and inactivates kinins [1, 2]. Dipeptidyl(amino)peptidase IV (DAP IV; EC 3.4.14.5) sequentially hydrolyzes the N-terminal Arg^1-Pro^2 and Lys^3-Pro^4 dipeptides of the undecapeptide substance P to produce the biologically active C-terminal hepta(5-11)-substance P [3–5]. Aminopeptidase M (AmM; EC 3.4.11.2) inactivates some angiotensins and hepta(5-11)-substance P [5, 6]. All three enzymes are present on kidney and intestinal brush border. In addition, ACE is localized on vascular endothelial surface membrane [1]. DAP IV and AmM are also localized on the surface membrane of vascular endothelium and/or smooth muscle [5, 7–9].

Although the physiologic significance of vascular membrane-bound DAP IV and AmM remains to be determined, the importance of vascular ACE to *in vivo* metabolism of kinins and angiotensins is well established. Vascular ACE has been reported to be altered by conditions such as hypoxia [10] and captopril therapy [11, 12], but little is known about the manner in which ACE is associated with its vascular surface membrane.

Recently, several studies have utilized procedures of differential solubilization, fused-rocket immunoelectrophoresis, and crossed immunoelectrophoresis to establish that ACE, AmM, and DAP IV are intrinsic enzymes bound to the brush border membrane by small, non-antigenic, hydrophobic anchors associated with the lipid bilayer [13, 14]. Since these studies concerned specialized, extravascular membranes (kidney and intestinal brush border), the conclusions reached cannot necessarily be extended to other surface membranes. Indeed, the topography of numerous ecto-proteins differs from the results obtained with brush border ecto-proteins and even some of the brush border enzymes appear to be bound in different manners [15].

In previous work, we have utilized the procedure of Kwan *et al.* [16] to prepare a purified vascular plasma membrane highly enriched in ACE, AmM and DAP IV [5, 17]. In the present study, immunoelectrophoretic analysis, identical to that previously carried out on renal brush border [13, 14], was performed to study the relationship of these three peptidases to the vascular surface membrane. In addition, the possible relationship of vascular peptidases to the circulating enzymes was investigated because, although several studies have suggested that vascular ACE may be secreted into the circulation [18, 19], purified pulmonary (vascular) and serum ACE are known to differ in sialic acid content [20, 21].

MATERIALS AND METHODS

Materials. Papain (Type III), neuraminidase Type V (0.1 units/mg) and Type X (160 units/mg), dimethylformamide, leucine-2-naphthylamide, Fast Garnet GBC salt, soybean trypsin inhibitor (SBTI),

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ovomucoid trypsin inhibitor (OMTI) and Aprotinin were obtained from the Sigma Chemical Co. (St. Louis, MO). Glycyl-prolyl-2-naphthylamide was obtained from Bachem Inc. (Torrance, CA). [^{3}H] Benzoyl-Phe-Ala-Pro was obtained from Ventrex (Portland, ME). Teprotide (SQ 20881, < Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) was donated by Dr. Z. Horovitz of Squibb Institute (Princeton, NJ). Crowle's Double Stain was from Polysciences, Inc. (Warrington, PA).

Antibody to hog kidney ACE was donated by Dr. E. Erdos of the University of Texas Health Science Center (Dallas, TX). Antibodies to hog kidney brush border and intestinal brush border AmM and DAP IV were obtained as previously described [13, 14]. These antisera specifically precipitate ACE, AmM or DAP IV and do not cross-react with one another or with any other brush border enzymes including aminopeptidase A (EC 3.4.11.7), carboxypeptidase P (EC 3.4.12.__), γ-glutamyltransferase (EC 2.3.2.2), alkaline phosphatase (EC 3.1.3.1) or phosphodiesterase I (EC 3.1.4.1) [14].

Membrane purification. Hog tissues were obtained from freshly slaughtered animals, immediately cleaned with 0.9% saline, and frozen. The plasma membrane-enriched fractions were prepared from both hog aorta (intimal layer) and mesenteric arteries by our modification [5, 17] of the procedure of Kwan *et al.* [16]. Minced tissue was homogenized with a Brinkmann Polytron run at full speed for 20 sec. Homogenates were centrifuged at 900 *g* (10 min) followed by two additional centrifugations at 9000 *g* (10 min). The resulting pellets (P_{1-3}) represent unbroken cells, nuclei, non-vesiculated membrane, lysosomes and mitochondria [5, 16, 17].

After removal of P_3, the supernatant fraction was centrifuged at 105,000 *g* (30 min), and the microsomal pellet was resuspended and separated on a discontinuous sucrose gradient (113,000 *g*; 90 min) into four major bands. After dilution with water and centrifugation (105,000 *g*; 30 min), the resulting pellets (V_{1-4}) were resuspended and assayed. Hog kidney brush border-enriched fractions were prepared as previously [22, 23] according to Booth and Kenny [24] and Schmitz *et al.* [25]. Fresh hog serum or plasma was collected, concentrated with an Amicon B-15 concentrator (Danvers, MA), and frozen.

Membrane solubilization. Purified membranes were solubilized with 2% (v/v) Triton X-100 and/or papain according to Booth *et al.* [13] as previously described [14]. Serum and solubilized membrane enzymes were incubated (2 hr) with 0.02 to 0.2 units neuraminidase (Sigma Type V or X) in 50 mM sodium acetate buffer (pH 5.5) according to Spiro [26]. Samples treated under identical conditions but without neuraminidase were used as controls.

Immunoelectrophoresis. Crossed immunoelectrophoresis was performed using 1.0 mm thick 1% (w/v) agarose gel containing 1% (v/v) Triton X-100. Usually, 7 μl samples were subjected to horizontal electrophoresis (10 V/cm for 2 hr) through agarose gel followed by vertical electrophoresis (10 V/cm for 1 hr) into gel containing appropriate antibody. After repeated dehydration/hydration of the gel to remove soluble protein, individual precipitin lines were identified using specific enzyme stains. Alternatively, gels were stained for protein with Crowle's Double Stain and subsequently destained with 3% (v/v) acetic acid [13, 14].

Enzyme assays. Enrichment and contamination of the plasma membrane fraction was determined by assay of 5'-nucleotidase (EC 3.1.3.5), alkaline phosphatase (EC 3.1.3.1), DNA, lactic dehydrogenase (EC 1.1.1.27), acid phosphatase (EC 3.1.3.2), succinate dehydrogenase (EC 1.3.99.1) and NADPH–cytochrome *c* reductase (EC 1.6.2.4) [17]. ACE, AmM and DAP IV activities were determined using [^{3}H]benzoyl-Phe-Ala-Pro, leucyl-2-naphthylamide and glycyl-prolyl-2-naphthylamide respectively [5, 17]. Specific activity is calculated as milliunits/mg protein where one milliunit equals the hydrolysis of 1 nmole substrate per min (AmM, DAP IV) or 1% substrate per min (ACE). Relative specific activity (RSA) was calculated as (mean specific activity in the fraction)/(mean specific activity in the homogenate) and recovery of activity as percent of total activity in the homogenate.

RESULTS

Membrane purification. As previously found after separation on the sucrose gradient, the top band (V_1) was designated the vascular plasma membrane (VPM)-enriched fraction since the plasma membrane markers 5'-nucleotidase (Fig. 1) and alkaline phosphatase were enriched 18.0- and 12.6-fold, respectively, and contamination by other subcellular organelles was minimal as determined by the lack of enrichment of succinate dehydrogenase (RSA = 0.9) and lactic dehydrogenase (RSA = 0.5) and the absence of detectible levels of NADPH–cytochrome *c* reductase and DNA. A slight enrichment of acid phosphatase activity (RSA = 2.1) was found which may have been due to either marginal lysosomal contamination or activity intrinsic to vascular plasma membrane as suggested by Kwan and co-workers [16]. Gradient layers V_2 and V_3, although also enriched in 5'-nucleotidase, were not used as sources of plasma membrane since, as previously found [16, 17], they were also enriched in succinate dehydrogenase, acid phosphatase, etc. (RSA > 3.0).

As seen in Fig. 1, the purified VPM was similarly enriched in ACE (RSA = 16.7), AmM (RSA = 12.1), and DAP IV (RSA = 19.8). In addition, recovery of total activity in the VPM fraction for 5'-nucleotidase (7%) was the same as that found for the three peptidases (7, 6, and 10% respectively). Although the yield of all four enzymes in the VPM could be substantially increased by longer homogenization, longer times were not used since they resulted in disproportionate increases in contamination.

To rule out the possibility that any enzyme redistribution had occurred during fractionation, suspensions of VPM were incubated (2 hr, 37°) with homogenization buffer, plasma or cytosol, centrifuged (100,000 *g*, 30 min), and assayed. Recovery of ACE, AmM and DAP IV activities ranged from 92 to 103%, indicating that the mechanics of homogenization and fractionation had not resulted in

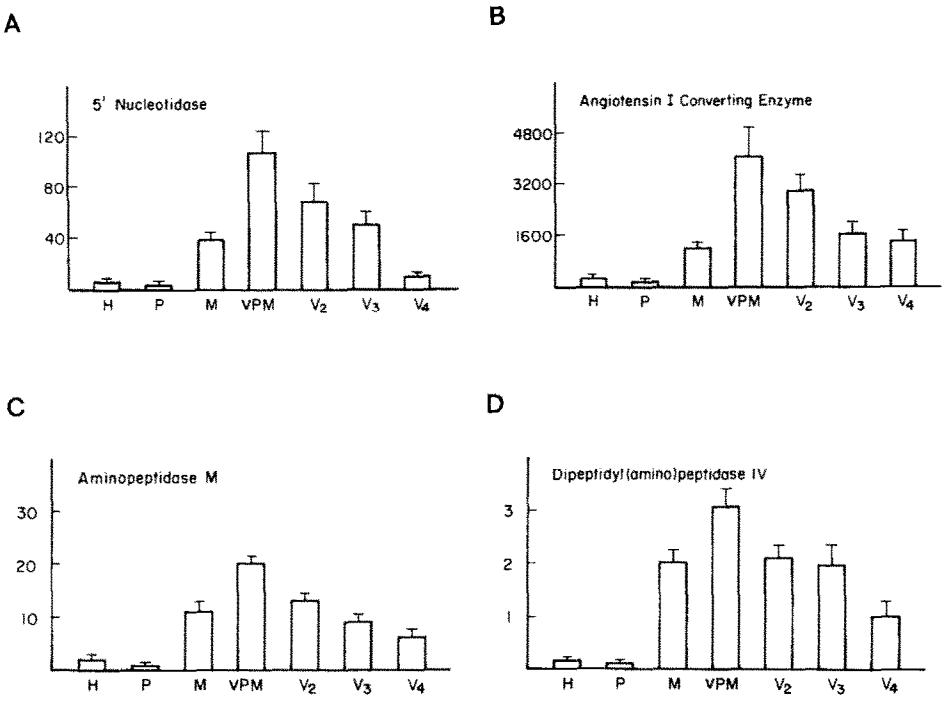


Fig. 1. Distribution of 5'-nucleotidase (A), angiotensin I converting enzyme (B), aminopeptidase M (C) and dipeptidyl(amino)peptidase IV (D) in hog mesenteric artery homogenate (H), low speed pellets (P), microsomes (M), vascular plasma membrane (VPM), and subsequent membrane fractions (V_2, V_3, V_4). Specific activity is given on the ordinate as milliunits per mg protein.

either loss of membrane bound or absorption of soluble (plasma) activity.

Fused-rocket immunoelectrophoresis. Solubilized VPM and kidney brush border (KBB) were placed in gel wells in close proximity to one another (= 2 mm). After 1 hr for diffusion, the samples were vertically electrophoresed into 1% agarose gel containing the appropriate antibody. After staining for protein (ACE, Fig. 2A) or enzyme activity (AmM, Fig. 2B), fused, rocket-shaped precipitates could be visualized with no evidence of spurring. In the case of AmM, the shadowed areas of the precipitin lines result from overstaining in an effort to detect any minimal spurring. Thus, as previously shown for DAP IV [5], vascular plasma membrane ACE and AmM are immunologically indistinguishable from their kidney brush border counterparts. In similar experiments, enzymes immunologically indistinguishable from vascular plasma membrane AmM and DAP IV were also detected in concentrated serum (Fig. 3).

Enzyme-membrane association. Differential solubilization of the vascular plasma membrane by detergent, detergent/papain, or papain alone and subsequent crossed immunoelectrophoresis with appropriate antibody established that the papain and detergent/papain forms of ACE, AmM and DAP IV exhibited greater electrophoretic mobilities than the detergent forms (Fig. 4, right side). Nevertheless, the papain forms retained immunologic identity with their respective detergent forms as shown by fused-rocket immunoelectrophoresis (Fig. 4, left side). Thus, as previously seen with brush border ACE, AmM and DAP IV [13, 14], these results are consistent with the binding of all three enzymes to vascular plasma membrane by hydrophobic domains or anchors associated with the lipid bilayer. When solubilized with a non-ionic detergent, the hydrophobic, non-antigenic anchors become associated with large detergent micelles which decrease electrophoretic mobility. Removing the hydrophobic, non-antigenic anchor/detergent complex produces a species of the enzyme exhibiting a greater electrophoretic mobility without affecting immunologic identity. Although the experiments shown in Figs. 2–4 are the results obtained with single antisera, other experiments using different antisera to renal AmM and DAP IV and antisera to intestinal AmM [14] also failed to detect any antigenic differences.

Crossed immunoelectrophoresis of concentrated serum AmM and DAP IV established that their respective electrophoretic mobilities were the same as the papain-treated forms of the VPM enzymes. Treatment of the serum enzymes with detergent did not alter their mobilities, presumably because they do not have hydrophobic anchors which bind detergent.

Sialic acid content. In spite of the immunologic identity and similar enzyme/membrane association of the brush border and vascular plasma membrane enzymes, the electrophoretic mobilities of the vascular enzymes were consistently greater than those of the brush border-bound enzymes regardless of the method of solubilization (Fig. 5, AmM shown). These differences were particularly apparent with the papain forms of the enzymes. As shown in

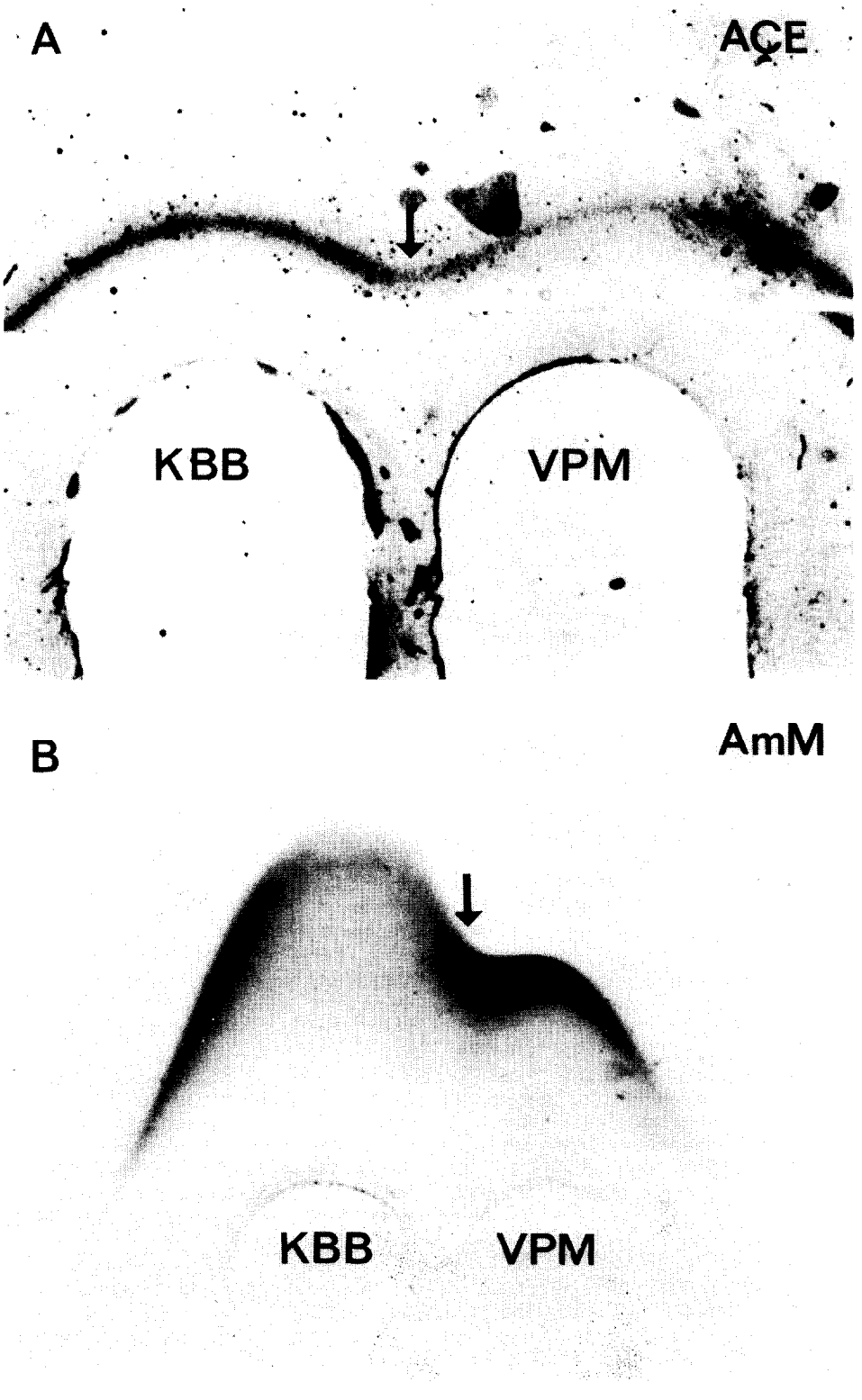


Fig. 2. Fused-rocket immunoelectrophoresis of detergent/papain-solubilized kidney brush border (KBB) and vascular plasma membrane (VPM). After 1 hr for diffusion, samples were vertically electrophoresed into gel containing anti-ACE (A) or anti-AmM (B) immunoglobulin at 2 V/cm for 18 hr. Precipitin lines were visualized by staining for protein (A) or enzyme activity (B). Vertical arrows indicate areas of fusion.

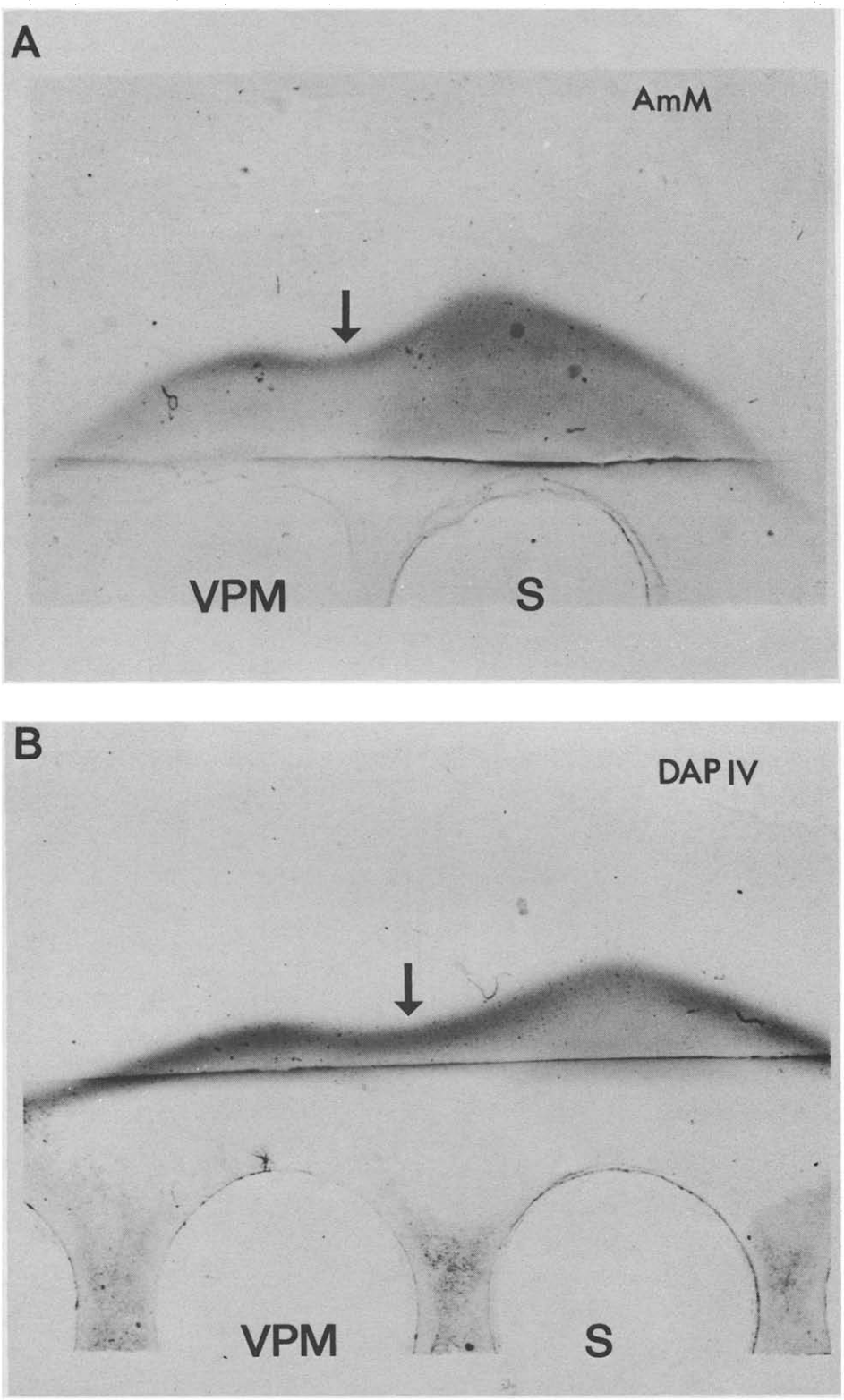


Fig. 3. Fused-rocket immunoelectrophoresis of vascular plasma membrane (VPM) and concentrated serum (S) as described in Fig. 2. Precipitin lines were visualized by staining for AmM (A) or DAP IV activity (B). Vertical arrows indicate areas of fusion.

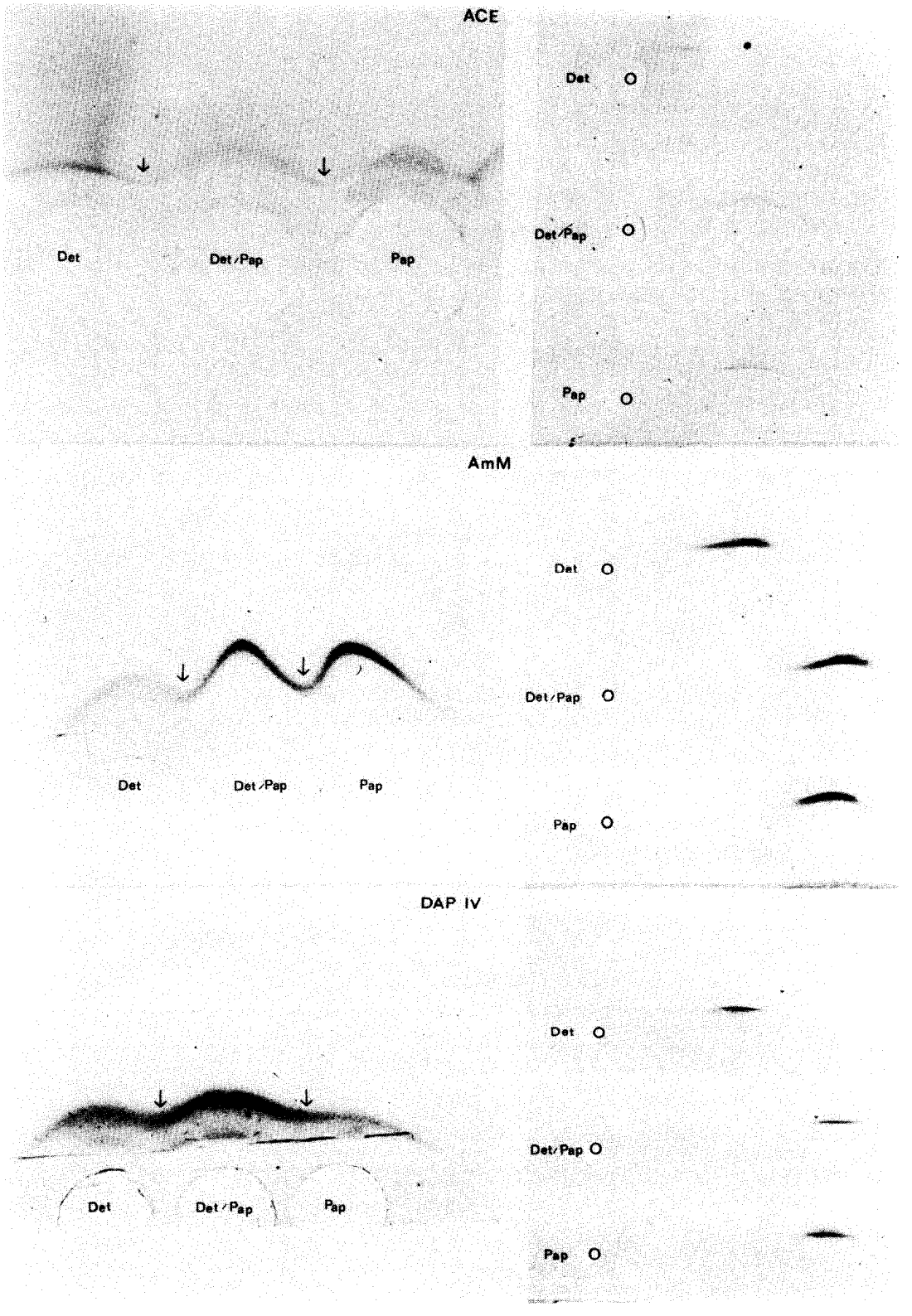


Fig. 4. Fused-rocket (left-hand side) and crossed (right-hand side) immunoelectrophoresis of detergent (Det), detergent/papain (Det/Pap) and papain (Pap)-solubilized vascular plasma membrane. For crossed immunoelectrophoresis, the first dimension is from the origin (○), left to right at 10 V/cm for 2 hr and in the second dimension, bottom to top at 10 V/cm for 1 hr. Precipitin lines were visualized by staining for protein (ACE) or enzyme activity (AmM, DAP IV). Vertical arrows indicate areas of fusion.

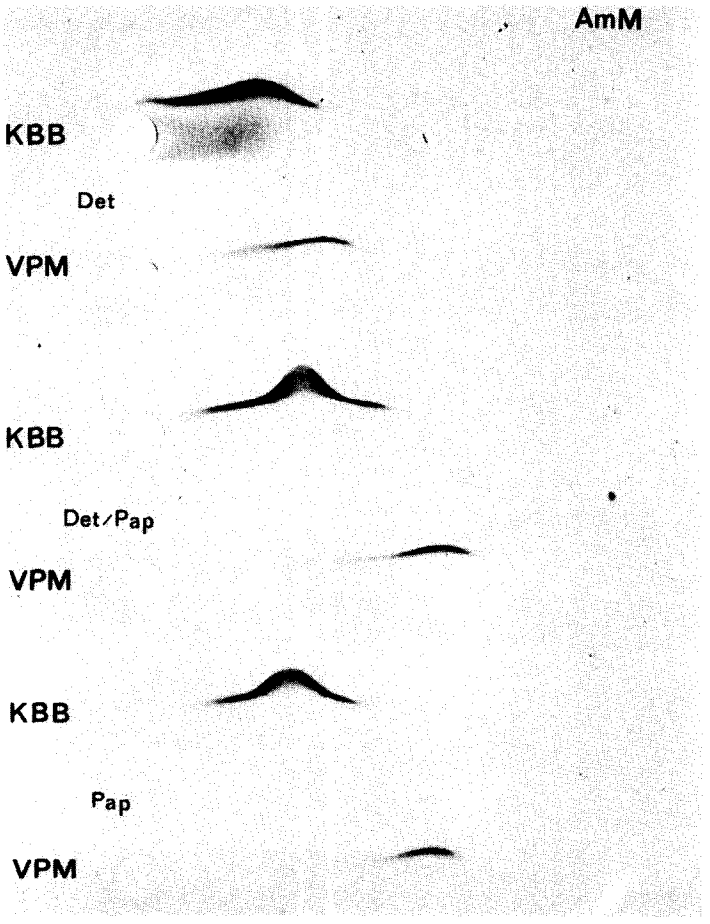


Fig. 5. Crossed immunoelectrophoresis of kidney brush border (KBB) and vascular plasma membrane (VPM) as described in Fig. 4 and subsequent visualization of AmM precipitin lines after solubilization with detergent (Det), detergent and papain (Det/Pap), or papain alone (Pap).

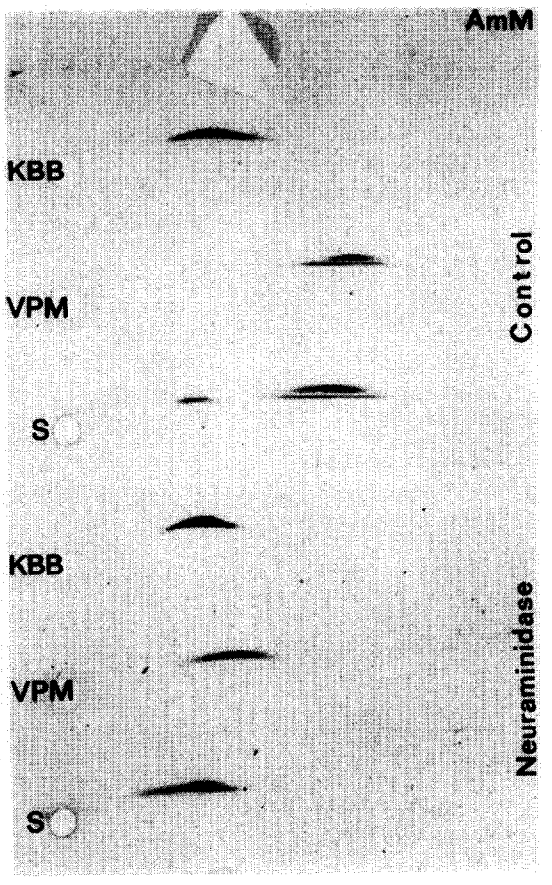
Fig. 6A, the electrophoretic mobilities of papain-solubilized vascular plasma membrane (VPM) AmM and serum (S) AmM (major component) were the same and about twice that of papain-solubilized kidney brush border (KBB) AmM. However, after incubation with neuraminidase to remove sialic acid, the electrophoretic mobilities of both the vascular plasma membrane and serum AmM decreased to positions similar to the kidney brush border AmM (which was relatively unaffected by neuraminidase treatment). The effect of neuraminidase treatment on vascular plasma membrane and serum DAP IV was essentially the same (Fig. 6B). Due to the lower sensitivity of the protein stain used to detect ACE, we were unable to measure the mobility of serum ACE. However, as shown in Fig. 6C, neuraminidase also decreased the mobility of the vascular plasma membrane ACE to a position similar to kidney brush border ACE. Since Sigma's Type V neuraminidase (0.1 units/mg) may contain proteases, experiments were also carried out using highly purified Type X neuraminidase (160 units/mg) with and without a mixture of inhibitors including SBTI (50 μg/ml), OMTI (50 μg/ml) and Aprotinin (600 KIU) which confirmed that the changes in mobility were due only to removal of sialic acid.

DISCUSSION

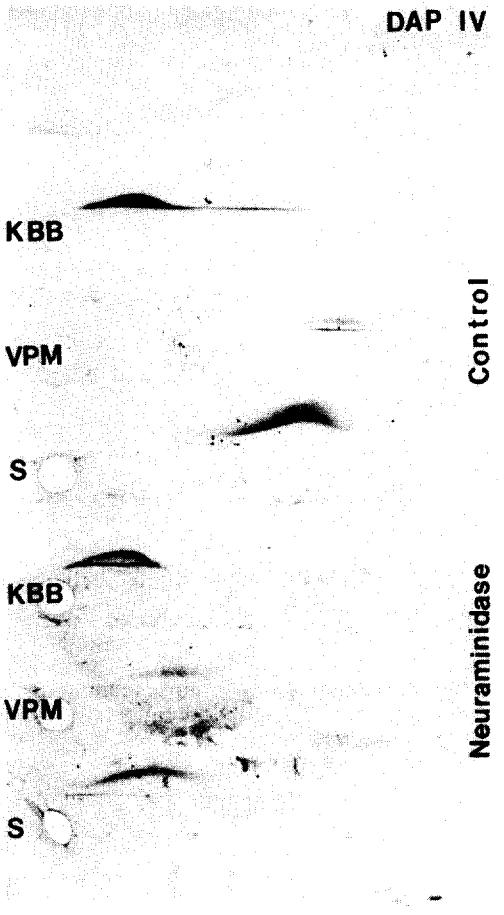
Enzymes may be classified as either extrinsic or intrinsic membrane proteins. Previous studies by Booth *et al.* [13], Danielsen *et al.* [27], and Ward and Sheridan [14] have established that brush border ACE, AmM and DAP IV are not ionically bound extrinsic brush border proteins which can be solubilized with simple salt solutions or EDTA but intrinsic membrane components bound by small, non-antigenic, hydrophobic anchors associated with the lipid bilayer. In several instances, these hydrophobic anchors have been shown to be located within the N-terminal amino acid sequence of the enzymes and to project through the membrane [15, 28, 29]. Kenny and Booth [30] have suggested that such enzymes represent a unique group of transmembrane proteins with physiologic functions not limited to simple substrate hydrolysis.

The data presented are consistent with the model that vascular ACE, AmM and DAP IV are also intrinsic surface membrane components bound by small, non-antigenic, hydrophobic anchors associated with the lipid bilayer and suggest that these vascular peptidases may share with the above renal enzymes a common mode of membrane insertion and

A


AmM
KBB
VPM
S
KBB
VPM
S
Control
Neuraminidase

B


DAP IV
KBB
VPM
S
KBB
VPM
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Neuraminidase

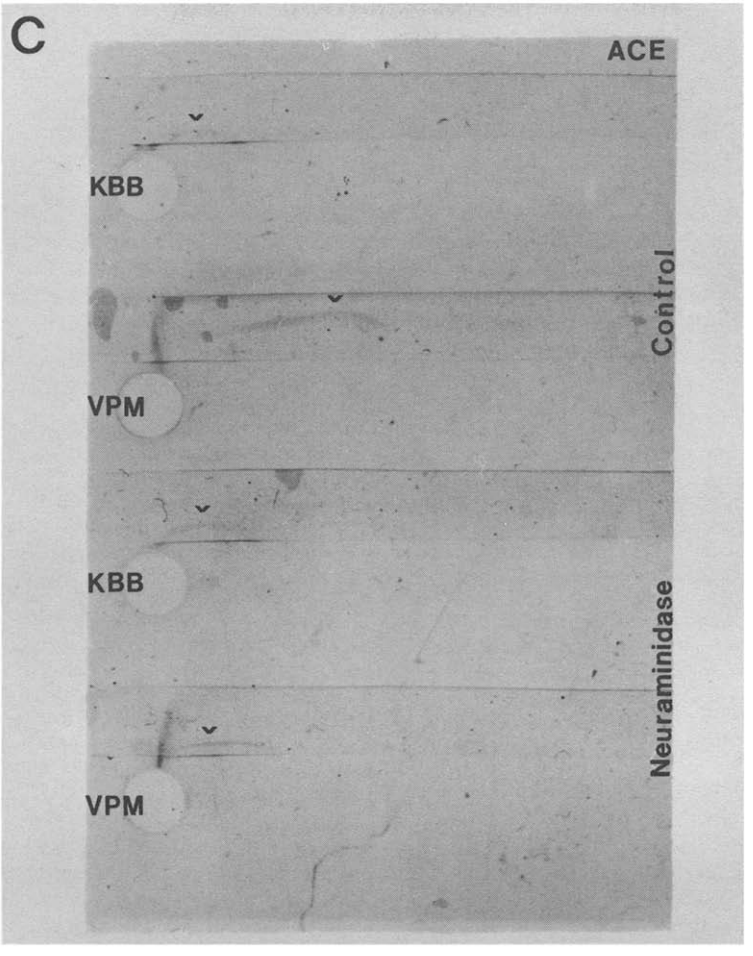

Fig. 6. Crossed immunoelectrophoresis of detergent/papain-solubilized kidney brush border (KBB) and vascular plasma membrane (VPM) or untreated, concentrated serum (S) both before (control, top half) and after (neuraminidase, bottom half) treatment with neuraminidase to remove sialic acid. Precipitin lines were visualized by staining for AmM (A), DAP IV (B) or ACE (C). Faint ACE precipitin lines are marked (V).

topography. However, this suggestion must remain tentative until more extensive analyses of the vascular peptidases are carried out, including asymmetric membrane labeling and N-terminal analysis of the detergent and papain forms of the enzymes [15, 29]. Nevertheless, Bretscher and Raff [31] have proposed that all intrinsic membrane glycoproteins have a transmembrane topography.

Angiotensin I converting enzyme and several aminopeptidases normally circulate in the blood stream and several studies have suggested that some of the circulating enzymes may originate from the vasculature [18, 19]. In the present study, the vascular plasma membrane enzymes all exhibited greater electrophoretic mobilities than their brush border counterparts regardless of the method of solubilization. These greater mobilities are due, at least in part, to their higher content of sialic acid residues since treatment with neuraminidase resulted in electrophoretic mobilities similar to the brush border enzymes. The electrophoretic mobilities of circulating AmM and DAP IV were identical to the papain-treated forms of the vascular enzymes both before and after neuraminidase.

A model consistent with the results of these experiments is illustrated in Fig. 7. A number of kidney and/or intestinal brush border peptidases have been shown to be bound to the membrane by a non-antigenic, hydrophobic, transmembrane anchor. Vascular ACE, AmM and DAP IV, although containing substantially higher levels of sialic acid, are bound to surface membrane in a similar manner (whether or not the anchors are transmembrane). After solubilization with papain (or papain/detergent), species of the enzymes from both brush border and vascular plasma membrane are produced which are antigenically indistinguishable from the plasma enzymes. However, due to their higher content of sialic acid, the vascular and circulating enzymes migrate substantially faster than the brush border enzymes. Removal of sialic acid with neuraminidase has relatively little effect on the brush border enzymes but decreases the mobilities of both the vascular and circulating enzymes to similar positions.

The high sialic acid content found for plasma AmM and DAP IV and reported for purified serum ACE [21] is not surprising since Ashwell and co-workers

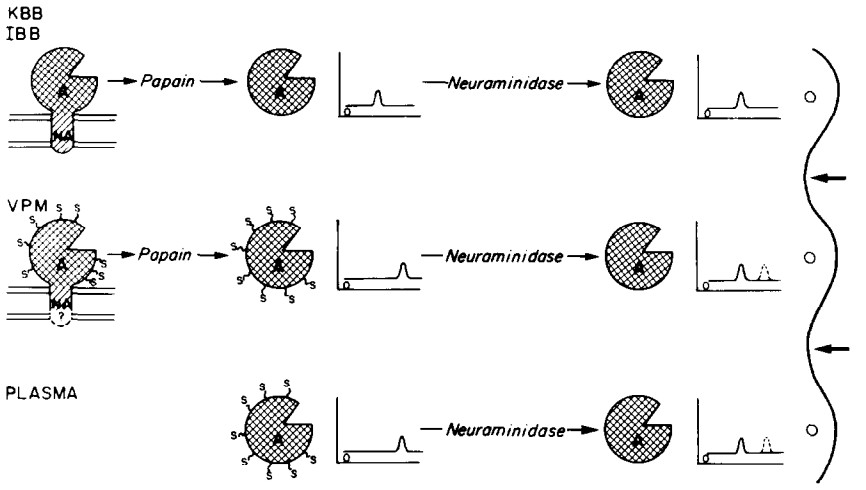

Fig. 7. Hypothetical illustration of the possible relationship of ACE, AmM and DAP IV with kidney and intestinal brush border (KBB, IBB), vascular plasma membrane (VPM) and plasma. The bulk of the enzyme (antigenic portion), the anchor (non-antigenic), and sialic acid are noted as A, NA and S respectively.

[32, 33] have observed that sialic-deficient plasma glycoproteins are rapidly removed from the circulation by a hepatic plasma membrane binding protein specific for asialogycoproteins. In view of the mode of insertion and high sialic acid content of ACE, AmM and DAP IV on the vascular plasma membrane, it is unlikely that the vascular enzymes are "picked up" from the circulation. A more likely hypothesis is that the vascular proteins are synthesized on the endoplasmic reticulum, processed into glycoproteins, and incorporated into the plasma membrane. Subsequently, they may then be released into the circulation by detaching from their hydrophobic anchors. The mechanism of such a detachment is unclear but it should be noted that brush border peptidases allowed to undergo autolysis also lose their hydrophobic anchors [15]. Nevertheless, the possibility that the anchors are removed after release into the circulation should not be ruled out.

Although little data have been published regarding plasma AmM and DAP IV, the above model is supported by the data of Soffer and co-workers [21] who have shown that purified serum and pulmonary (vascular) ACE are immunologically indistinguishable, exhibit identical behavior during gel filtration, gradient centrifugation and disc gel electrophoresis, and display similar catalytic properties. A release of vascular ACE into the circulation is also consistent with the observations of Hayes *et al.* [18] that endothelial cells cultured in serum-free media release a form of ACE into culture medium which is similar to serum ACE.

Since the present study investigated only one subcellular compartment of the vasculature (plasma membrane), the possible presence of multiple pools or populations of vascular ACE (AmM, DAP IV) cannot be ruled out. The difference in sialic acid content between purified serum ACE and vascular ACE purified from a low speed pellet of pulmonary tissue [20, 21] has been interpreted by Soffer and colleagues to suggest that the vasculature may produce microheterogeneous populations of ACE (with respect to sialic acid) which may be sloughed or secreted into the blood stream where sialic acid-deficient molecules are selectively removed [1, 34]. However, since low speed pellets of crude homogenate are known to contain not only plasma membrane, but also endoplasmic reticulum, Golgi, etc., an alternate interpretation of the above is that such multiple pools or populations could represent stages in a single pathway of ACE production beginning with *de novo* synthesis on the endoplasmic reticulum (asialo-form) and ending with incorporation of mature (fully-sialated) enzyme into the plasma membrane. Such an interpretation is in accordance with the "membrane flow" hypothesis of Palade [35] and is consistent with the recent work of Danielsen *et al.* [36] delineating the biosynthetic pathway of intestinal brush border AmM and DAP IV.

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