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IMMUNOELECTROPHORETIC ANALYSIS OF RENAL AND INTESTINAL BRUSH BORDER CONVERTING ENZYME

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Abstract—Antibodies raised against purified hog renal or intestinal brush border protein or against purified hog kidney angiotensin I converting enzyme (ACE) were used to characterize renal and intestinal brush border ACE by techniques of differential solubilization, fused-rocket, line absorption and crossed-immunoelectrophoresis. Renal ACE is immunologically identical to intestinal ACE. ACE is present as a major intrinsic protein of renal brush border and a minor intrinsic protein of intestinal brush border. Renal and intestinal brush border ACE could be solubilized by detergent and/or papain. The electrophoretic mobilities of the papain-treated forms of ACE were greater than the detergent-treated forms. This increased mobility was associated with the removal of a small, non-antigenic component of the enzyme. Thus, like several other intrinsic brush border peptidases, ACE is bound to renal and intestinal brush border by a small hydrophobic anchor.

Angiotensin I converting enzyme (peptidyl dipeptidase; ACE; EC 3.4.15.1) cleaves dipeptides from the C-terminal end of a variety of peptides including bradykinin and angiotensin I [1, 2]. Thus, the same enzyme can inactivate vasodepressors (kinins) and form a vasopressor (angiotensin II). ACE is present on the surface membrane of vascular endothelial cells [3–5] and renal and intestinal epithelial cells [6, 7]. The association of ACE with the vascular plasma membrane may be related to the reported effects of hypoxia on enzyme activity [8]. Nevertheless, little is known about the association of ACE with either endothelial plasma membrane or epithelial brush border.

Recently, Booth *et al.* [9] have utilized procedures of immunoelectrophoresis to separate, identify and characterize numerous brush border enzymes. They have established that several brush border peptidases (e.g. aminopeptidases M and A) are major intrinsic membrane proteins bound to the membrane by a hydrophobic domain or anchor associated with the lipid bilayer.

The present study was carried out using immunoelectrophoretic techniques to study the relationship of ACE with renal and intestinal epithelial brush border. ACE was found to be an intrinsic major (renal) or minor (intestinal) membrane component of epithelial brush border. Like several other brush border peptidases [9], it is bound to the lipid bilayer of the membrane by a hydrophobic anchor. Similar analysis of the ACE/endothelial plasma membrane association should facilitate our understanding of the function of membrane-bound ACE.

MATERIALS AND METHODS

Materials. [^{3}H]Benzoyl-Gly-Gly-Gly 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 the Squibb Institute (Princeton, NJ). Monospecific antibody to purified hog kidney converting enzyme was donated by Dr. E. Erdös of the University of Texas Health Science Center (Dallas, TX). Papain (Type III), L-amino acid oxidase (Type I), leucine 2-naphthylamide, α-glutamyl 2-naphthylamide, γ-glutamyl 2-naphthylamide, Fast Garnet GBC salt, Fast Red TR salt, *o*-dianisidine, naphtol AS-MX phosphate (sodium salt), MTT tetrazolium bromide, phenazine methosulfate, thymidine 5′-monophosphate naphthyl ester, glucose, lactose, maltose and isomaltose were from the Sigma Chemical Co. (St. Louis, MO). Glycyl-prolyl 2-naphththylamide and benzyloxycarbonylprolylmethionine were from Bachem, Inc. (Torrance, CA). Crowle's Double Stain was from Polysciences, Inc. (Warrington, PA) and the nylon sieves were from Tetko, Inc. (Elmsford, NY). All other reagents were of the highest purity available. Hog kidney and intestine were obtained from freshly slaughtered animals. Tissues were immediately rinsed in 0.9% saline (4°). Kidney cortex was dissected free and frozen. Small intestine was opened lengthwise, blotted, and frozen in sheets.

Brush border purification. Hog kidney brush border was purified according to Booth and Kenny [10]. Hog intestinal brush border was purified according to our modification [7] of the procedure of Schmitz *et al.* [11]. Purification was determined by marker enzyme enrichment as previously described [7]. ACE enrichment was determined using [^{3}H]benzoyl-Gly-Gly-Gly as substrate according to Ryan *et al.* [12]. Enzyme activity is expressed as mUnits per mg where 1 unit represents the hydrolysis of 1 μmole of substrate per min.

Brush border solubilization. Purified kidney and intestinal brush border were solubilized by treatment with detergent (Triton X-100) and/or papain according to Booth *et al.* [9].

Brush border immunoglobulin preparation. Purified kidney or intestinal brush border (2 mg/ml) was

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solubilized in Triton X-100 (2%, v/v), and the suspension was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were injected intracutaneously with 400–600 μg of protein every other week and bled 1 week after the fourth injection.

Crossed-immunoelectrophoresis. Electrophoresis was performed in a BioRad model 1415 flat bed electrophoresis apparatus using 1.0 mm thick 1% (w/v) agarose gels. The technique was carried out as described by Booth *et al.* [9] except that 1% (v/v) Triton X-100 was used in place of Emulphogen BC 720. Usually 20 μl of each sample was applied to the gel. After electrophoresis (5 V/cm for 3 hr) in the first dimension, a window was cut for the second dimension gel. This second dimension gel was of the same composition as the first but contained anti-kidney brush border, anti-intestinal brush border or anti-ACE immunoglobulin. The gel was then electrophoresed in the second dimension at 2 V/cm for 18 hr.

Fused-rocket immunoelectrophoresis. Fused-rocket immunoelectrophoresis against monospecific swine kidney converting enzyme antibody was performed as previously detailed [7, 9].

Enzyme and protein staining. After repeated dehydration/hydration of the gels to remove soluble protein [13], individual precipitin lines were identified using specific enzyme stains. Aminopeptidase M (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), γ-glutamyltransferase (EC 2.3.2.2), dipeptidylaminopeptidase IV (EC 3.4.14.-), carboxypeptidase P (EC 3.4.12.-), alkaline phosphatase (EC 3.1.3.1) and phosphodiesterase I (EC 3.1.4.1) were stained according to Booth *et al.* [9]. Lactase (EC 3.2.1.23), trehalase (EC 3.2.1.28), α-1,4-glucanglycohydrolase (EC 3.2.1.3) and the sucrase–isomaltase complex (EC 3.2.1.48/10) were stained according to Danielsen *et al.* [14]. Alternatively, gels were stained for protein with Crowle's Double Stain and subsequently destained with 0.3% acetic acid.

RESULTS

Brush border purification. As previously found [7, 15], renal and intestinal brush border were enriched 10- and 15-fold, respectively, in the brush border marker enzyme alkaline phosphatase. Contamination by other subcellular organelles was minimal, as indicated by the low enrichments of DNA, succinate dehydrogenase, acid phosphatase, NADPH–cytochrome *c* reductase and lactic dehydrogenase (<0.2). Electron microscopic examination of both preparations revealed membrane vesicles essentially identical to those previously reported [7, 10]. Visible contamination by other subcellular organelles was minimal.

ACE, determined by [^{3}H]benzoyl-Gly-Gly-Gly hydrolysis, was enriched 7.9-fold on renal brush border and 9.2-fold on intestinal brush border. The purified brush border ACE activity was completely inhibited by SQ 20881 (10^{-7} M).

Fused-rocket immunoelectrophoresis. We have determined previously that hog intestinal ACE cross-reacts with antibody to purified hog kidney ACE [7]. In the present study, fused-rocket immunoelectrophoresis was carried out to compare ACE in both preparations. Detergent-solubilized (1% Triton X-100) renal homogenate, brush border, intestinal homogenate, and brush border (each sample containing five mUnits ACE activity) were placed into gel wells in close proximity to one another (≅2 mm). After 1 hr for diffusion, the samples were vertically electrophoresed into gel containing monospecific antibody to ACE. Similar amounts of ACE in each sample, if immunologically identical, should produce similar sized, fused, rocket-shaped precipitates with no evidence of spurring [9]. Indeed, after staining for protein, each sample had formed an identically sized precipitin line which was continuous with the other three (not shown). The immunologic identity of renal brush border and intestinal brush border converting enzyme is clearly demonstrated in Fig. 1. The experiment was carried out as above except that 20 and 10 mUnits, respectively, of renal and intestinal brush border converting enzyme were used (Fig. 1). Thus, the fused-rocket experiments confirmed the enzymatic distribution of ACE and established the immunologic identity of the renal and intestinal enzymes.

Crossed-immunoelectrophoresis. Crossed-immunoelectrophoresis utilizing antibody to purified brush border has been used by Booth *et al.* [9] and Danielsen *et al.* [14] to separate and characterize specific

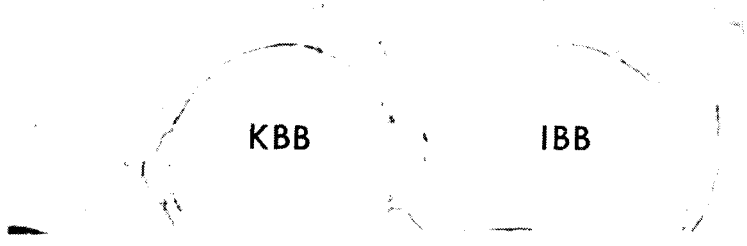


Fig. 1. Fused-rocket immunoelectrophoresis of detergent-solubilized purified kidney brush border (KBB) and intestinal brush border (IBB). After 1 hr for diffusion, samples were vertically electrophoresed in gel containing anti-ACE immunoglobulin at 2 V/cm for 18 hr.

enzymes intrinsic to hog kidney and intestinal brush border respectively. Approximately half of the major precipitin lines have been identified. The present experiments were carried out in an attempt to identify one of the as yet unidentified major precipitin lines as ACE.

Kidney brush border. Crossed-immunoelectrophoresis of detergent-solubilized kidney brush border against the corresponding immunoglobulin revealed at least ten visible precipitates when stained with Crowle's Double Stain. Identification of individual precipitin lines by staining for enzyme activity established that the overall precipitin pattern of the detergent-solubilized (not shown) and papain/detergent-solubilized (Fig. 2, panels A and B) kidney membrane was essentially identical to that reported by Booth *et al.* [9]. After papain treatment, the electrophoretic mobilities of all the identified enzymes except neutral endopeptidase increased. In addition, papain treatment generated bimodal precipitates in the case of dipeptidylaminopeptidase IV and carboxypeptidase P (Fig. 2, A and B). The identified precipitates (Fig. 2A), listed in decreasing order of intensity (protein stain), were aminopeptidase M, dipeptidylaminopeptidase IV, neutral endopeptidase, carboxypeptidase P and aminopeptidase A. This sequence corresponds to the known levels [9, 16] of these enzymes in kidney brush border (aminopeptidase M ≅ 8% of the membrane protein; dipeptidylaminopeptidase IV and neutral endopeptidase ≅4%). Enzymes associated with a low percentage of brush border protein (e.g. γ-glutamyltransferase) could only be detected by enzyme staining (Fig. 2B). Alkaline phosphatase, which represents only 0.04% of brush border protein, could not be detected at all.

After excluding the known precipitin lines, the remaining lines were considered candidates for ACE since they did not stain for any of the enzymes listed in Materials and Methods. After crossed-immunoelectrophoresis, using monospecific antibody to ACE, only one precipitin line was visible. The detergent form (Fig. 2D) migrated further after papain treatment (Fig. 2C), did not stain for any of the known enzymes, and was positioned and shaped similarly to one of the unknown brush border precipitin lines (Fig. 2, A and C).

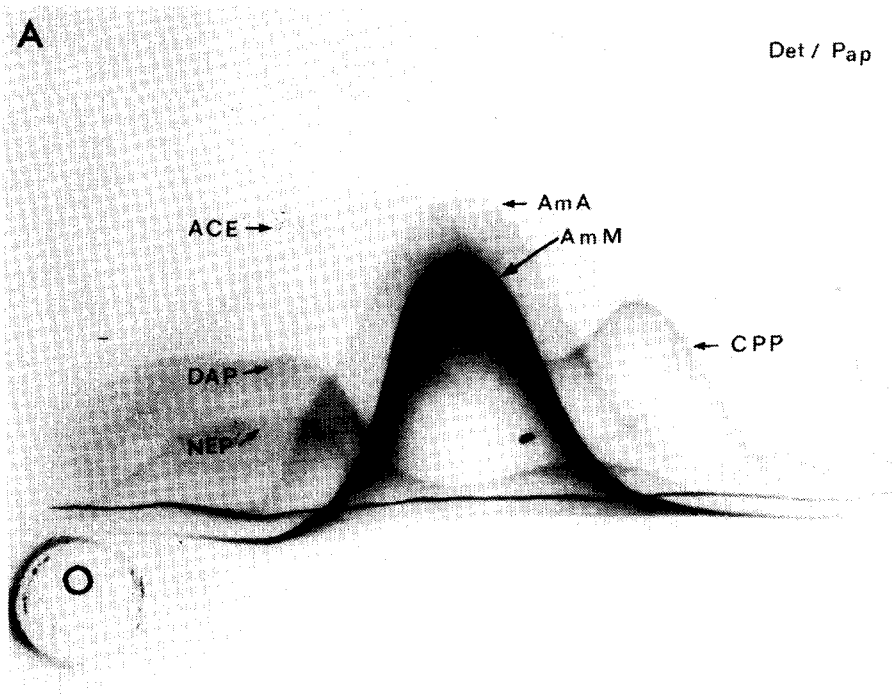
Line absorption immunoelectrophoresis. To establish the identity of this line with ACE, line absorption immunoelectrophoresis was carried out. Detergent/papain-solubilized renal brush border was electrophoresed as before except that the second-dimension gel contained two windows: the first with anti-ACE immunoglobulin and the second with anti-brush border immunoglobulin. The first window precipitates out (absorbs) only ACE while allowing the remaining brush border enzymes to pass through and precipitate in the second window. The resultant precipitin pattern in the second window (Fig. 3A) was identical to the control gel (Fig. 3B) except for the prior precipitation of the one previously mentioned unidentified line. Confirmation of this identity with ACE was also obtained by an increase of the height of this precipitin line by prior addition of purified ACE to the brush border sample (not shown). These results demonstrate ACE to be a major component of renal brush border that exists as a single species which migrates faster after papain treatment. In addition, the papain-treated form migrates further into antibody containing gel (Fig. 4). This is not due to removal of antigenic determinants because fused-rocket immunoelectrophoresis of the detergent-, detergent/papain- and papain-solubilized forms of ACE demonstrated immunologic identity (Fig. 4). Thus, like aminopeptidase M and aminopeptidase A [9], the material removed by papain from detergent-solubilized ACE does not bear detectable antigenic determinants. However, it is the presence of this non-antigenic component that is responsible for the relative retardation of the detergent form. Finally, the intensity of the ACE precipitin line (Fig. 2A) was significantly less than the precipitin lines for aminopeptidase M (≅8%) and dipeptidylaminopeptidase IV (≅4%) and similar to that of aminopeptidase A (≅1–2%). This suggests that ACE makes up approximately 1–2% of the protein of renal brush border membrane. Table 1 summarizes the characteristics of renal brush border ACE determined in the present study with those of several other brush border enzymes (established by Booth *et al.* [9] and confirmed in the present study).

Intestinal brush border. Crossed-immunoelectrophoresis of detergent- or detergent/papain- (Fig. 5A) solubilized intestinal brush border against the corresponding immunoglobulin revealed at least twelve visible precipitates. Identification of six precipitin lines by staining for enzyme activity (Fig. 5B, three shown) established that the overall precipitin pattern was similar to that reported by Danielsen *et al.* [14]. Bimodal precipitates were produced by papain treatment in the case of aminopeptidase M and aminopeptidase A (Fig. 5, A and B).

The identified precipitates, listed in decreasing order of intensity, were α-1,4-glucanglycohydrolase, aminopeptidase M, aminopeptidase A, the sucrase–isomaltase complex, and dipeptidylaminopeptidase IV. Alkaline phosphatase, representing a small percentage of intestinal brush border, was only faintly detectable (dotted line).

After crossed-immunoelectrophoresis using monospecific antibody to ACE, only one extremely faint precipitin line was formed (not shown). Five times as much membrane protein was required to give a strong precipitin line (Fig. 5D). After papain treatment, the precipitin line migrated further into the gel and into the antibody containing gel (Fig. 5C) and was positioned similarly to several marginally detectable, unidentified brush border precipitin lines (Fig. 5A broad arrow). Line absorption immunoelectrophoresis (not shown) did not alter the pattern of the major precipitin lines seen in the control gel (Fig. 5A).

These results demonstrate ACE to be a minor component of intestinal brush border that exists as a single species which migrates faster after papain treatment. Again, the faster mobility is not due to the removal of antigenic determinants because fused-rocket immunoelectrophoresis of the detergent-, detergent/papain- or papain-solubilized forms of ACE demonstrated immunologic identity (Fig. 6).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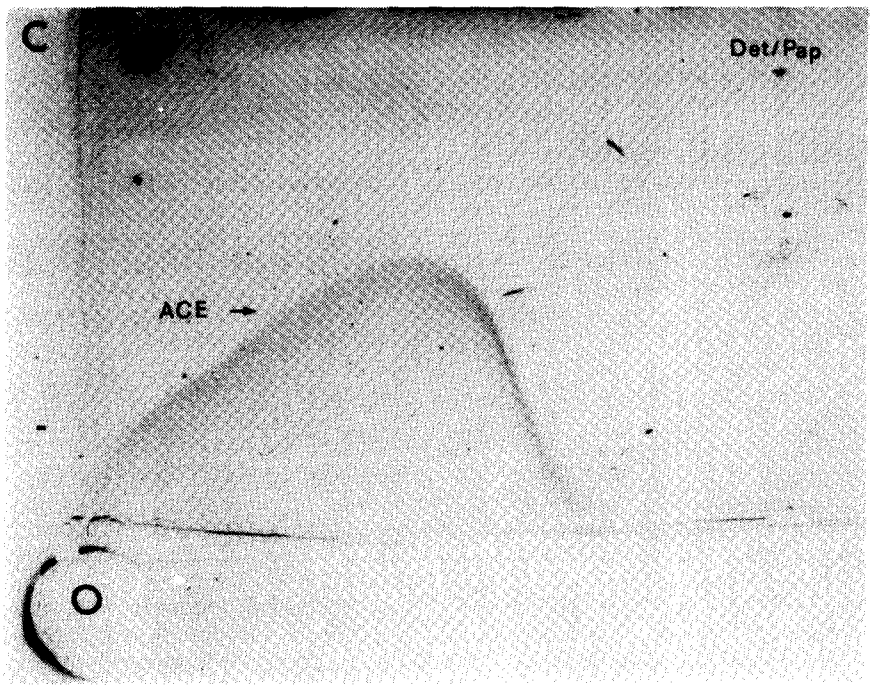
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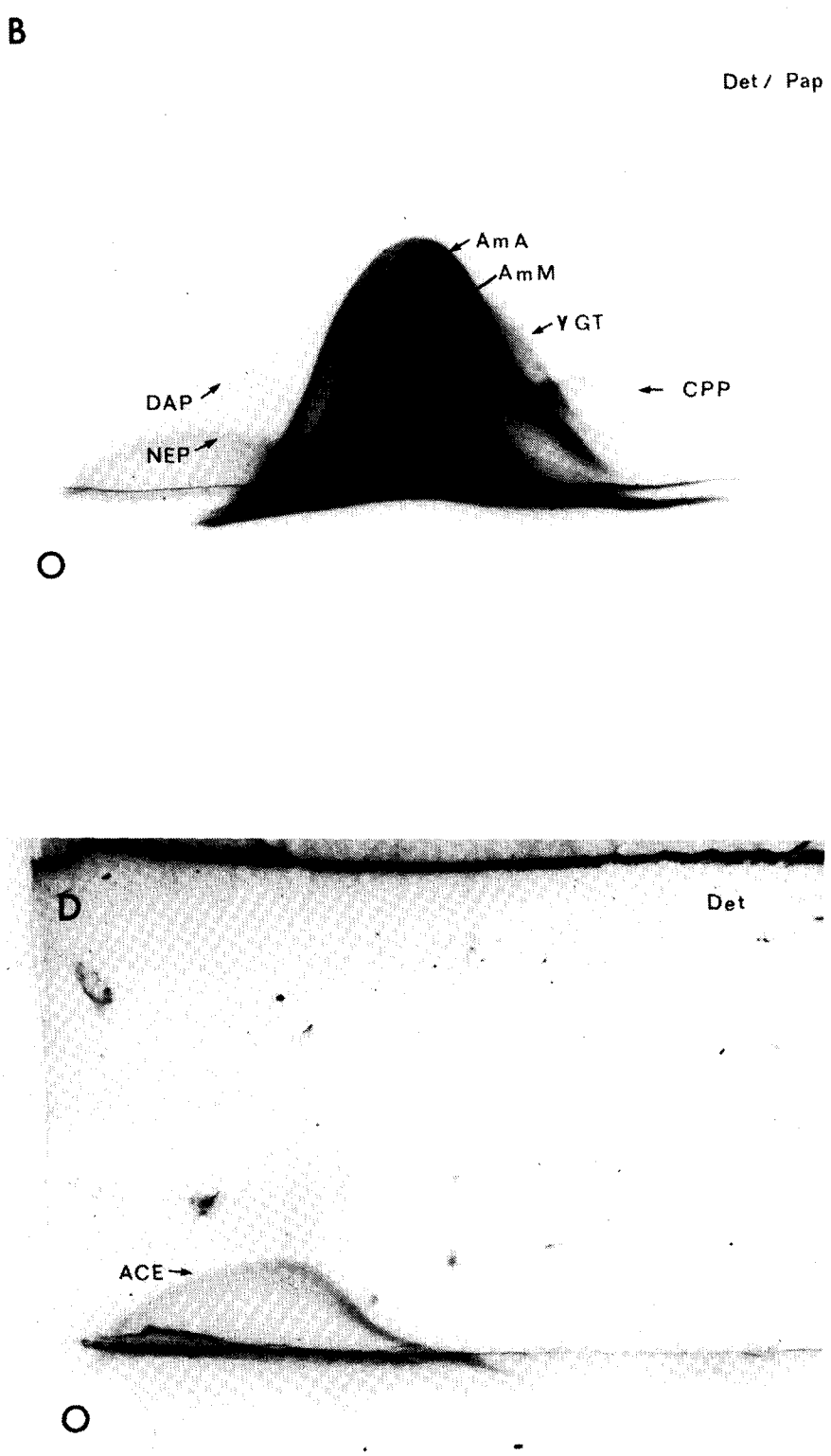


Fig. 2. Crossed-immunoelectrophoresis of purified renal brush border. First dimension is from the origin (○), left to right at 5 V/cm for 3 hr, and in the second dimension, bottom to top at 2 V/cm for 18 hr. The sample applied was detergent/papain-solubilized (A, B and C) or detergent-solubilized (D) membrane. The second dimension gel contained antibody to renal brush border (A and B) or antibody to ACE (C and D). Precipitin lines were visualized by staining for protein (A, C and D) or enzyme activity (B). Key: dipeptidylaminopeptidase IV (DAP), neutral endopeptidase (NEP), aminopeptidases M and A (AmM and AmA), carboxypeptidase P (CPP), and γ-glutamyltransferase (γGT).

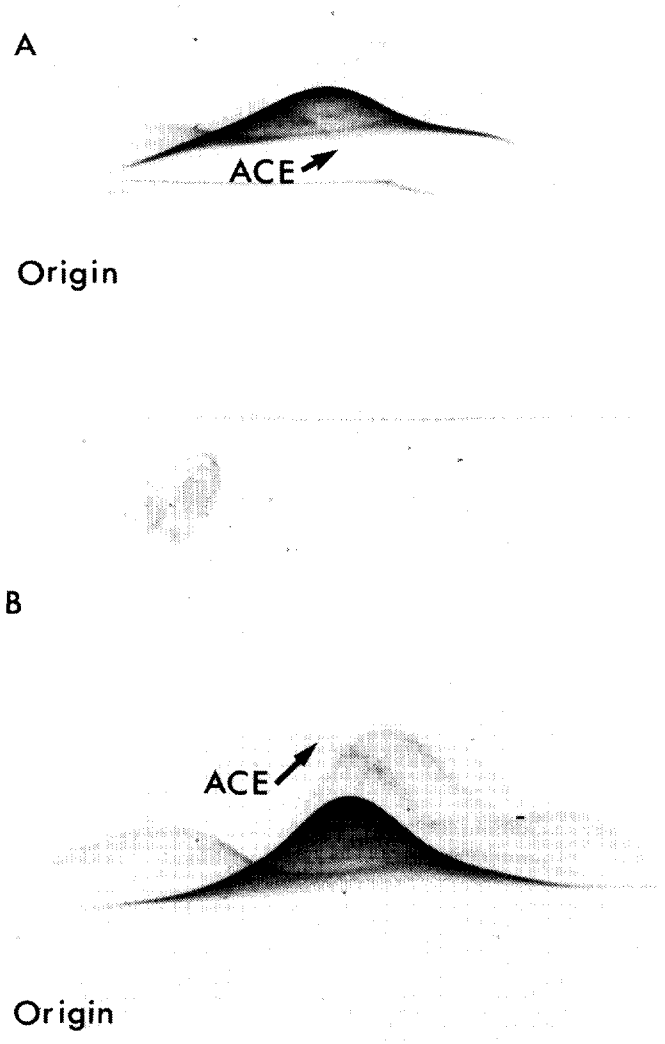


Fig. 3. Line absorption crossed-immunoelectrophoresis. The second dimension gel contained first antibody to ACE and then antibody to renal brush border (A) or just antibody to renal brush border (B). Electrophoresis conditions were the same as noted in the legend for Fig. 2A.

Table 1. Comparison of hog renal brush border converting enzyme with other renal brush border enzymes*

		After papain treatment		
	Major component	Increased mobility	Uni-modal	Immunologic identity
Angiotensin converting enzyme	\	\	\	\
Aminopeptidase M	\	\	\	\
Aminopeptidase A	\	\	\	\
Neutral endopeptidase	\	No	\	\
Dipeptidylaminopeptidase IV	\	\	No	\
Carboxypeptidase P	\	\	No	\
γ-Glutamyltransferase	No	\	\	\
Alkaline phosphatase	No			
Phosphodiesterase I	No	\	\	\

* Data were taken from the results of the present study and those of Booth *et al.* [9].

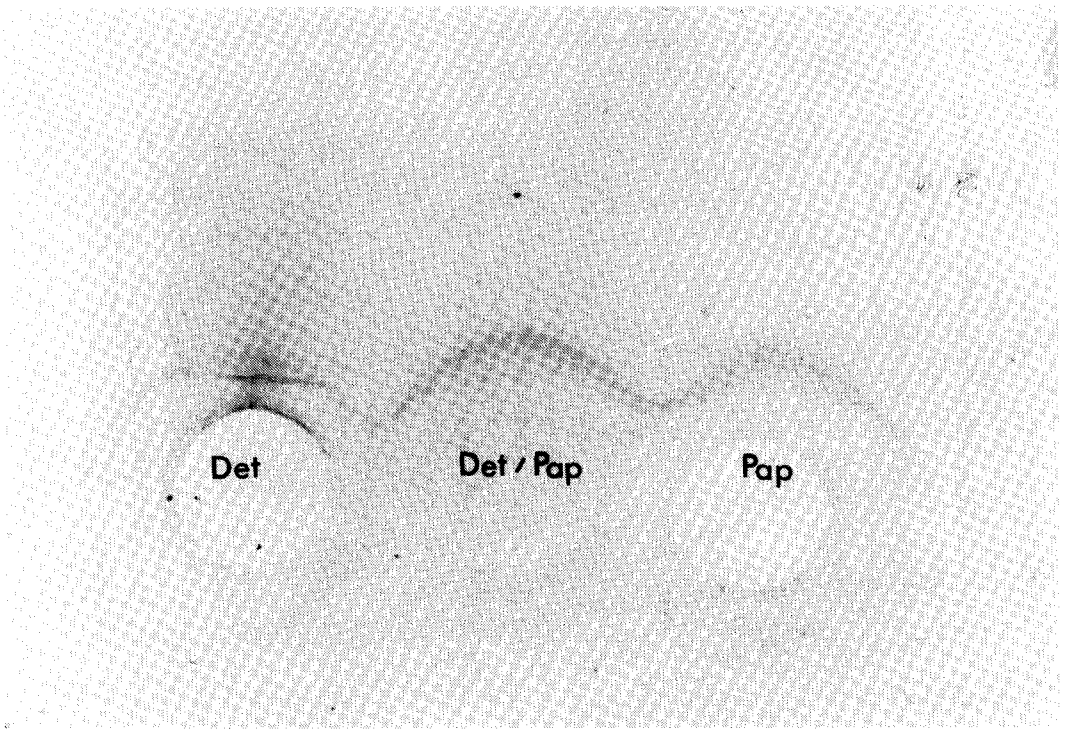


Fig. 4. Fused-rocket immunoelectrophoresis of purified renal brush border solubilized by detergent (Det), detergent and papain (Det/Pap) or papain alone (Pap). After 1 hr for diffusion, samples were vertically electrophoresed in gel containing anti-ACE immunoglobulin at 2 V/cm for 18 hr.

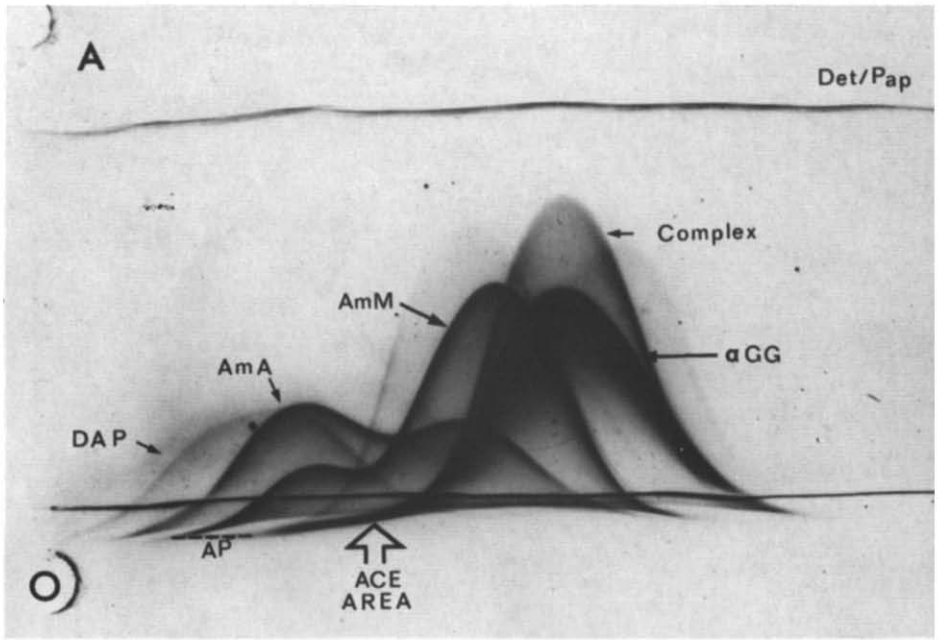
DISCUSSION

The results of the present experiments confirm and extend those of Booth *et al.* [9] and Danielsen *et al.* [14] with regard to numerous renal and intestinal brush border enzymes. Hog renal and intestinal ACE are immunologically identical. ACE is an intrinsic major component of renal brush border and an intrinsic minor component of intestinal brush border. The effects of detergent and/or papain solubilization of ACE with regards to electrophoretic mobility and immunologic identity are the same as that reported for several other brush border peptidases including aminopeptidases M and A. As Kenny and Booth have pointed out [17], several well documented brush border enzymes have been shown to be inserted into the membrane lipid bilayer by non-antigenic, hydrophobic anchors. When solubilzed with non-ionic detergents, the hydrophobic anchors become associated with large detergent micelles which decrease electrophoretic mobility. The increase in electrophoretic mobility after papain (without affecting immunologic identity) is due to the removal of this hydrophobic anchor/detergent complex [9].

The reason for production of bimodal precipitates after treatment with papain in the cases of renal brush border dipeptidylaminopeptidase IV and carboxypeptidase P (Fig. 2) and intestinal brush border aminopeptidases M and A (Fig. 5) is not clear. However, as pointed out by Booth *et al.* [9], it clearly indicates a difference in susceptibility to cleavage by papain between the membrane-bound and the detergent-solubilized forms of the enzymes.

As mentioned previously, ACE is also present on the surface membrane of vascular endothelial cells [3–5]. Utilizing the procedure of Kwan *et al.* [18], we have purified a vascular surface membrane from rat that is highly enriched in ACE activity [19]. Application of this purification technique to hog vasculature and subsequent immunoelectrophoretic analysis with antibody raised to the purified vascular plasma membrane will allow a similar analysis of the vascular plasma membrane/ACE relationship. Comparative studies of the relationship of ACE with both epithelial brush border and endothelial plasma membrane should increase our understanding of the manner in which membrane-bound ACE activity may be secreted [20] and/or altered by conditions such as hypoxia [8] or captopril therapy [21, 22].

Finally, crossed-immunoelectrophoresis utilizing antibody to purified vascular plasma membrane may also reveal additional enzymes which metabolize vasoactive peptides. For instance, dipeptidylaminopeptidase IV and aminopeptidase A, clearly detectable in the present studies, are known to metabolize substance P [23] and angiotensin II [24] respectively.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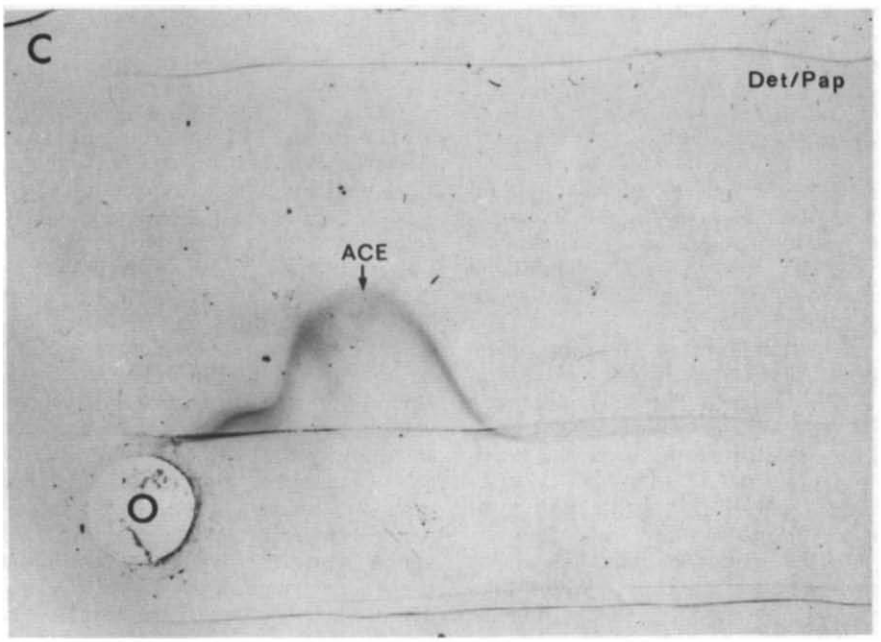
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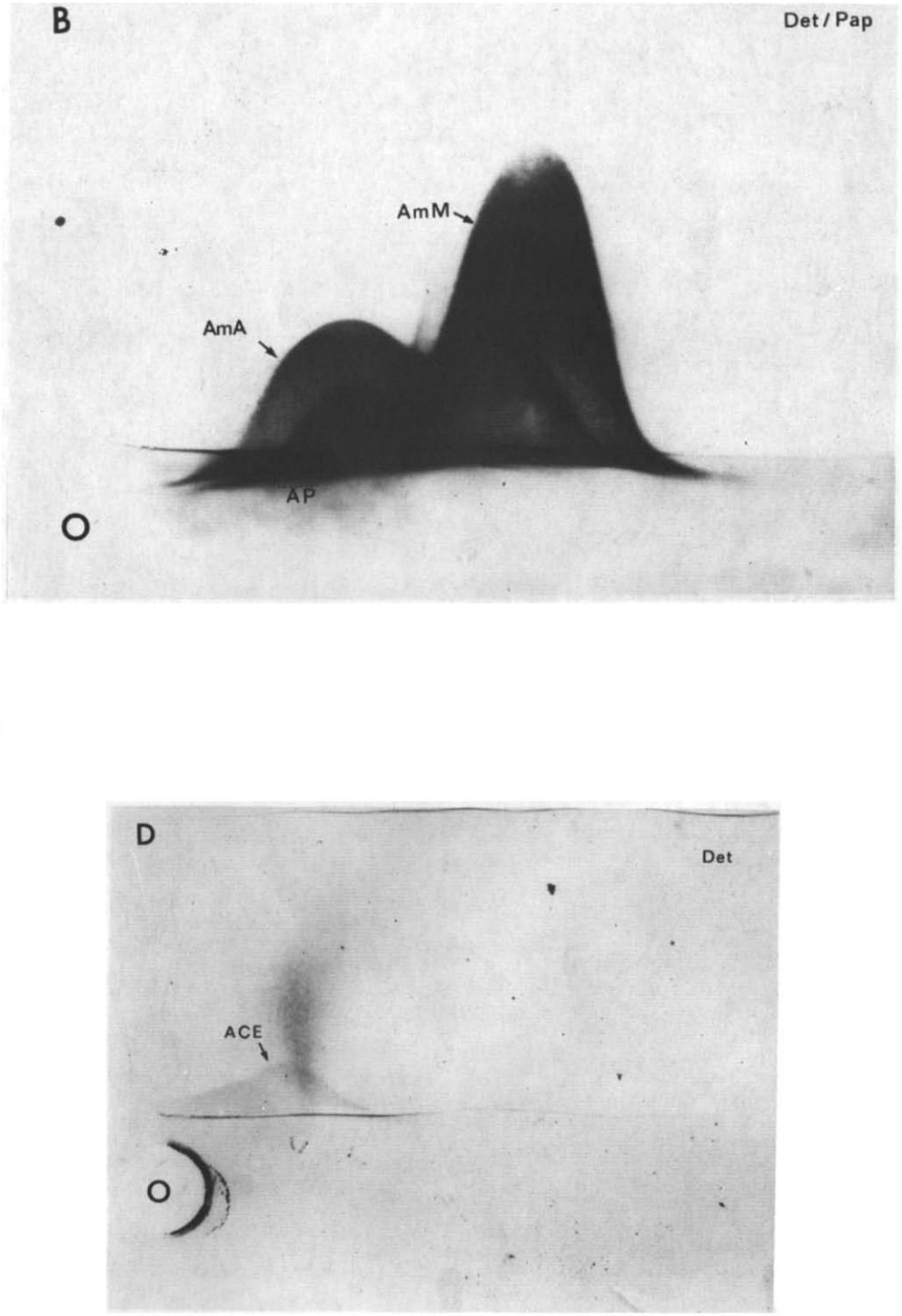

Fig. 5. Crossed-immunoelectrophoresis of purified intestinal brush border. All conditions are as described in Fig. 2 except that the second dimension gel of A and B contained antibody to intestinal brush border, and five times as much solubilized membrane was used in gels C and D. Key: alkaline phosphatase (AP); sucrase–isomaltase complex (Complex); and α-1,4-glucanglycohydrolase (αGG).

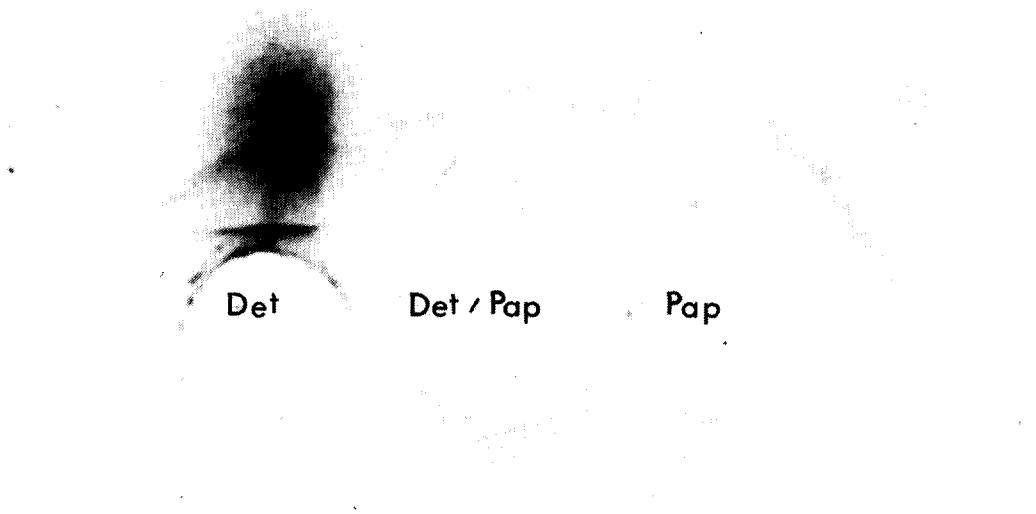

Fig. 6. Fused-rocket immunoelectrophoresis of purified intestinal brush border as described in Fig. 4.

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