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# Binding and Degradation of $^{125}$I-Insulin by Isolated Rat Renal Brush Border Membranes: Evidence for Low Affinity, High Capacity Insulin Recognition Sites

Elias Meezan, Dennis J. Pillion, and Ada Elgavish
Department of Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294

**Summary.** The kidney plays a major role in the handling of circulating insulin in the blood, primarily via reuptake of filtered insulin at the luminal brush border membrane. $^{125}$I-insulin associated with rat renal brush border membrane vesicles (BBV) in a time- and temperature-dependent manner accompanied by degradation of the hormone to trichloroacetic acid (TCA)-soluble fragments. Both association and degradation of $^{125}$I-insulin were linearly proportional to membrane protein concentration with virtually all of the degradative activity being membrane associated. Insulin, proinsulin and desoctapeptide insulin all inhibited the association and degradation of $^{125}$I-insulin by BBV, but these processes were not appreciably affected by the insulin-like growth factors IGF-I and IGF-II or by cytochrome *c* and lysozyme, low molecular weight, filterable, proteins, which are known to be reabsorbed in the renal tubules by luminal endocytosis. When the interaction of $^{125}$I-insulin with BBV was studied at various medium osmolarities (300–1100 mOsM) to alter intravesicular space, association of the ligand with the vesicles was unaffected, but degradation of the ligand by the vesicles decreased progressively with increasing medium osmolarity. Therefore, association of $^{125}$I-insulin to BBV represented binding of the ligand to the membrane surface and not uptake of the hormone or its degradation products into the vesicles. Attempts to crosslink $^{125}$I-insulin to a high-affinity insulin receptor using the bifunctional reagent disuccinimidyl suberate revealed only trace amounts of an $^{125}$I-insulin-receptor complex in brush border membrane vesicles in contrast to intact renal tubules where this complex was readily observed. Both binding and degradation of $^{125}$I-insulin by brush border membranes did not reach saturation even at concentrations of insulin approaching $10^{-5}$ M. These results indicate the presence of low-affinity, high-capacity binding sites for $^{125}$I-insulin on renal brush border membranes which can clearly distinguish insulin from the insulin-like growth factors and other low molecular weight proteins and polypeptides, but which do not differentiate insulin from its analogues as do the biological receptors for the hormone. The properties and location of these binding sites make them attractive candidates for the sites at which insulin is reabsorbed in the renal tubule.

**Key Words** insulin · brush border membranes · kidney · receptors · binding · degradation · tubules · protein reabsorption

## Introduction

The kidney has a major role in the handling of circulating insulin in the blood. About 40% of the insulin carried to the kidneys by the blood is extracted and metabolized by this organ [9, 26]. Insulin is constantly being carried to the glomerular capillaries where a portion of it is filtered and gains access to the tubular lumen and its highly developed systems for the reabsorption, transport and degradation of proteins [8, 33–35, 47]. Another fraction of the circulating insulin continues through the peritubular capillaries where some may be taken up at the contraluminal surface of the proximal tubules [26, 44]. Eng and Yalow [15] have found endogenous insulin levels to be higher in the kidney than in any other organ, being several times higher than plasma levels. They suggested that the kidney contains a large concentration of nonsaturable receptor sites for insulin, perhaps greater than that of any other organ. The presence of specific high-affinity insulin receptors in preparations of renal cell membranes from dog and rat has been demonstrated [3, 11]. We [39–41] and Kurokawa and co-workers [28–30] simultaneously and independently demonstrated for the first time that specific high-affinity insulin receptors are present in both isolated renal glomeruli and tubules. Subsequently, others have reported that both purified renal tubular brush border and basolateral membranes have insulin receptors with similar binding properties [17, 18, 45, 48, 57, 58].

Insulin receptors found in the various renal nephron segments and membrane fractions appear to have similar characteristics in specificity and affinity to those previously characterized in adipocytes and hepatocytes and their respective membranes. A notable exception is our finding that the affinity for $^{125}$I-insulin of the tubular receptor(s) is

about tenfold less than that of the glomerular receptor(s) [39–41]. We speculated that tubular insulin receptors of lower affinity and higher capacity might possibly mediate the reabsorption and degradation of insulin [41] in agreement with the known function of the renal tubule in reabsorption and proteolysis of filtered proteins [7, 8, 33, 34, 47]. Recently Hammerman and Gavin [18] found that renal brush border membranes only had one-tenth the insulin binding capacity of renal basolateral membranes and that they lacked the insulin-stimulated phosphorylation of the receptor found with basolateral membranes, suggesting an asymmetrical distribution of specific insulin-binding sites in the renal tubule. In addition, Hjelle et al. [23] failed to find support for the results of previous investigators [48, 57, 58] that renal brush border membranes actively degrade insulin into trichloroacetic acid (TCA)-soluble fragments. Since both uptake and degradation of insulin are believed to be mediated by luminal endocytosis in the renal proximal tubule [4, 5, 7, 8, 26, 34, 47, 49], these results showing a paucity of insulin receptors and a lack of degradative activity in brush border membranes appeared paradoxical in view of the known physiological functions of this membrane. We have reinvestigated the interaction of $^{125}$I-insulin with isolated renal brush border membranes with the objective of distinguishing insulin binding from uptake of the hormone and its degradation products in this system and of establishing what relationship, if any, insulin degradation has to its association with the membrane. We now report that insulin association with isolated renal brush border membrane vesicles almost totally represents a low-affinity, high-capacity binding of the hormone to the membrane surface, accompanied by the membrane-associated degradation of the hormone to TCA-soluble fragments.

## Materials and Methods

### Materials

$^{125}$I-insulin receptor grade (specific activity 2200 Ci/mmol) and $^{14}$C-sucrose (sp act 4 mCi/mmol) were obtained from New England Nuclear. Insulin-like growth factor-I (IGF-I) was obtained from Amgen, Inc. Rat insulin-like growth factor-II (IGF-II), also called multiplication stimulating activity (MSA peak II, $M_r$ 8,700 and MSA peak III-2, $M_r$ 7,400) was a kind gift of Dr. S.P. Nissley, National Cancer Institute, NIH, Bethesda, MD. Insulin, desoctapeptide insulin and proinsulin were kindly provided by Eli Lilly. Bacitracin and dibutylphthalate oil were obtained from the Aldrich Chemical Co., and dinonylphthalate oil was a product of MCB Manufacturing Chemists. Disuccinimidyl suberate was purchased from Pierce Chemical Co. Electrophoresis reagents including molecular weight marker proteins were obtained from Biorad Co. Cytochrome *c* from horse heart, lysozyme from egg white (3 × recrystalized), insulin A chain (oxidized), insulin B chain (oxidized), aprotinin, ethylene diaminetetraacetic acid (tetrasodium salt), phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), and Triton X-100 were all products of the Sigma Chemical Co.

### Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles were isolated from the kidney cortex of Sprague-Dawley rats as described previously [2, 14]. In brief, minced renal cortex was homogenized first with a Potter Elvehjem homogenizer and then with a Polytron followed by precipitation with 10 mM $CaCl_2$. After a low-speed centrifugation and discarding of the pellet the supernatant solution was centrifuged at 35,000 × *g* for 20 min to bring down a crude brush border membrane preparation. This membrane pellet was resuspended by hand homogenization in a Ten-Broeck glass homogenizer in the loading solution, consisting of 100 mM sucrose, 100 mM KCl, 5 mM Tris-HEPES at pH 7.5 and centrifuged again. This procedure was repeated once more with the loosely packed brush border membrane carefully washed off the small densely packed mitochondria-enriched remnant pellet. This latter pellet was found to be threefold enriched in succinic dehydrogenase activity and eightfold enriched in alkaline phosphatase activity, indicating that it represented a fraction of the brush border membranes heavily contaminated with mitochondria, and it was therefore discarded. The purified membranes were dispersed in the loading medium and kept on ice prior to experimentation.

The purity of the brush border membrane preparation was determined by analysis of marker enzymes to assess the extent of contamination by other subcellular fractions (Table 1). Standard methods were used to measure the activities of alkaline phosphatase [59], succinic dehydrogenase [24], $Na^+K^+$-ATPase [60] and lactate dehydrogenase [6] in the renal cortical homogenate and purified brush border membrane preparation. Alkaline phosphatase, a marker enzyme for the brush border, was enriched 10-fold in the BBV preparation over the activity in the renal cortical homogenate. The activities of $Na^+K^+$-ATPase, succinic dehydrogenase and lactate dehydrogenase showed no enrichment in the brush border preparation and the activities obtained indicated a contamination of the BBV of approximately 2% by basolateral membranes, 5% by mitochondria, and 3% by cytosol. The purity of the brush border preparation was comparable to those used by other investigators [2, 14, 52].

### Association and Degradation Studies

All incubations were carried out at 20°C with continuous shaking for 90 min unless otherwise indicated. The standard incubation mixture consisted of 200 μl of brush border membranes dispersed in the loading solution described above, 1.3 ml of uptake medium containing 100 mM sucrose, 100 mM NaCl, 5 mM Tris-HEPES at pH 7.5 and 1.5% bovine serum albumin, and 3.5 × $10^{-10}$ M (~1,000,000 cpm) of $^{125}$I-insulin plus other additions, depending on the particular experiment performed. The final membrane protein concentration in the incubation mixture was about 1–2 mg/ml as determined by analysis of the membrane suspension by the procedure of Lowry et al. [32]. At various times or at the completion of the incubation period 3–5 aliquots (150 μl) of the incubation mixture were removed, layered on top

**Table 1.** Enzymatic characterization of brush border membrane vesicles

| Marker enzyme | Enzyme enrichment[a] | Enzyme recovery[b] (%) | Brush border content[c] (%) |
|---|---|---|---|
| Alkaline phosphatase | 9.71 | 38.4 | 100 |
| $Na^+K^+$-ATPASE | 0.26 | 0.84 | 2.2 |
| Succinic dehydrogenase | 0.64 | 1.8 | 4.7 |
| Lactate dehydrogenase | 0.22 | 4.7 | 2.9 |

[a] Enzyme enrichment = sp act enzyme in BBV/sp act enzyme in cortical homogenate. Sp act = nmol substrate converted/min/mg protein.
[b] Enzyme recovery = (total enzyme activity in BBV/total enzyme activity in cortical homogenate) × 100.
[c] Brush border content = (enzyme recovery of marker enzyme/enzyme recovery of alkaline phosphatase) × 100.

of 100 μl of a 5 : 1 mixture of dibutylphthalate/dinonylphthalate oil in a microfuge tube and centrifuged 5 min at 12,500 rpm in a Beckman microfuge to separate the membrane pellet from the incubation medium. For experiments where the medium osmolarity was varied from 300–1100 mosM with the use of sucrose, this procedure was found to be insufficient to sediment all membrane protein at the high sucrose concentrations, leading to artifactual results [37, 38]. Therefore, in these experiments sedimentation was carried out for 10 min at 105,000 × *g* in a Beckman airfuge at 4°C with silicone oil instead of the phthalate oil mixture. $^{14}$C-sucrose, a molecule impermeant to renal brush border membrane vesicles, was used as a marker for determining extravesicular space trapped in the membrane pellet, and all $^{125}$I-insulin association values were corrected for the extravesicular space at each medium osmolarity. The aqueous and oil layers were aspirated off and the tube tip containing the membrane pellet was cut off and counted in a Beckman gamma counter to determine $^{125}$I-insulin association with the membranes. To determine $^{125}$I-insulin degradation, 2–3 aliquots (150 μl) of the incubation mixture were removed at various time points or after completion of the incubation and pipetted into 150 μl of cold 20% TCA. After 15 min at 4°C the resulting precipitates were sedimented by centrifugation for 2 min at 12,500 rpm in a Beckman microfuge, and 150 μl of the supernatant solution was counted to determine TCA-soluble degradation products. Comparisons of the TCA-precipitation method to determine $^{125}$I-insulin degradation with measurements of degradation by gel chromatography of the reaction mixtures on Sephadex G-50 (*see* Fig. 3) or radioimmunoassay (ICN Micromedic Systems) showed that the relative agreement between all methods was good, with the TCA-precipitation technique tending to underestimate the actual extent of degradation since TCA-precipitable peptides formed as an intermediate in the digestion are unreactive by radioimmunoassay and shift to a more included position in gel chromatography while they are recorded as "undegraded" in the TCA assay [51]. Nevertheless, as in the hands of others [12, 16, 17, 23, 30, 47–51, 53, 54] TCA precipitation proved to be a convenient and useful relative indication of $^{125}$I-insulin degradation by proteolytic activity. For determination of the integrity of membrane-bound $^{125}$I-insulin, the washed membrane pellets were resuspended in 75 μl of uptake medium and 75 μl of 20% TCA added to precipitate intact insulin. Following separation of the pellet from the supernatant by centrifugation both the pellet and aliquots of the supernatant were counted to measure TCA-soluble degradation products and remaining intact insulin. $^{125}$I-insulin was greater than 98% precipitable by TCA at the start of these experiments and was greater than 95% [$^{125}$I-Tyr$^{A14}$]-insulin as established by HPLC run by the manufacturer, and was active in binding and crosslinking studies with liver, renal, colon and retinal tissues. All experiments were performed at least twice and the results of representative experiments are given unless indicated otherwise in the figure legends or tables.

## Crosslinking and Gel Electrophoresis Studies

Proximal tubules were isolated from Sprague-Dawley rats as described previously [41]. The isolated tubules were resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and homogenized by sonic disruption. Membranes were isolated by differential centrifugation [21, 22] and the 35,000 × *g* pellet was used in subsequent cross-linking experiments.

Covalent cross-linking of [$^{125}$I]insulin to renal tubular membranes and to purified renal BBV was performed as described previously [21, 22]. Equivalent amounts of tubular and BBV membrane protein were incubated with 0.5 μCi [$^{125}$I]insulin in the presence or absence of 50 μg/ml unlabeled porcine insulin, and binding of insulin was allowed to equilibrate by incubation at 22°C for 90 min. The membranes were pelleted by centrifugation at 35,000 × *g* for 30 min, washed and resuspended in ice-cold buffer containing no bovine serum albumin. Cross-linking was achieved by the addition of 0.25 mM dissuccinimidyl suberate dissolved in dimethylsulfoxide and the reaction was continued for 15 min on ice. Membranes were then diluted with ice-cold buffer (10 mM Tris-HCl, 1 mM EDTA) to quench the reaction and centrifuged at 35,000 × *g* for 30 min. The resultant pellets were resuspended in 100 μl of sample buffer containing 50 mM dithiothreitol as described by Laemmli [31], boiled for 3 min and applied to a dodecylsulfate polyacrylamide gel (4% acrylamide stacking gel, 6% acrylamide separating gel). Molecular weights were estimated by the inclusion of marker proteins of known size in an adjacent well on the gel. Gels were stained with Coomassie Brilliant blue, dried and autoradiography was performed using Kodak X-AR film and Dupont enhancing screens [21, 22].

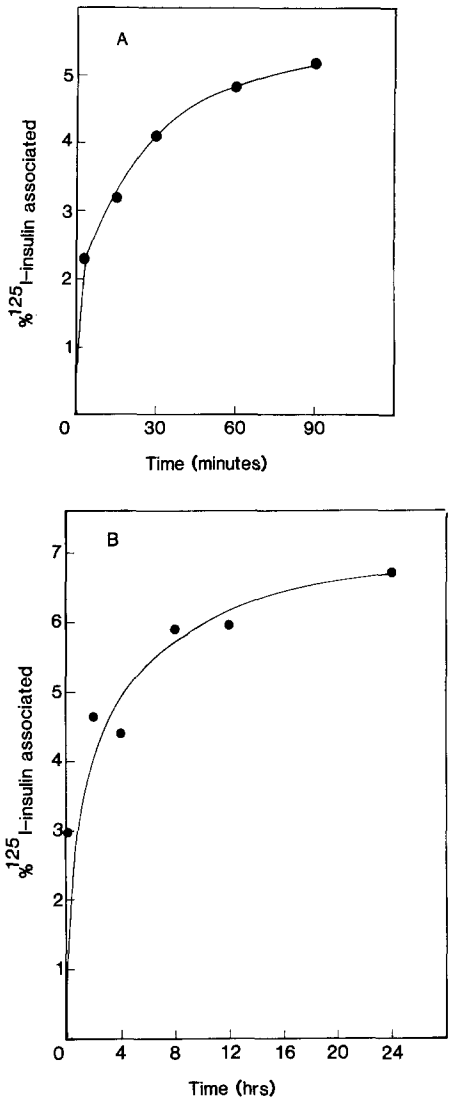


**Fig. 1.** Time course of $^{125}$I-insulin association with isolated rat renal brush border membrane vesicles. Membrane vesicles (*A*: 2.11 mg/ml, *B*: 0.77 mg/ml) were incubated at 20°C (*A*) and at 4°C (*B*) with $^{125}$I-insulin, and aliquots were removed at the indicated time points for determination of $^{125}$I-insulin association. Data show total $^{125}$I-insulin association and are not corrected for degradation

## Results

Initial experiments were focused on characterizing the basic parameters of $^{125}$I-insulin interaction with isolated rat renal brush border membrane vesicles. Association of $^{125}$I-insulin with the vesicles occurred rapidly at 20°C and showed a time-dependent increase to 90 min of incubation (Fig. 1*A*). In these experiments association refers to the total radioactivity found in the membrane pellet. At 4°C the association of $^{125}$I-insulin with the brush border vesicles was appreciably slower and reached a plateau only after 24 hr of incubation (Fig. 1*B*). Initial binding, however, was quite rapid even at 4°C as can be seen by the earliest time point in the association curve which represented almost half of the total $^{125}$I-insulin association achieved at 24 hours. This point represented true association of the $^{125}$I-insulin with the membrane vesicles since centrifugation of the membranes through the oil layer to separate them from the medium minimizes "trapping" of solvent (<10% of total $^{125}$I-association), and blanks run without membranes showed no counts in the tube tips, indicating no leakage or slipping of solvent through the oil layer. A similar rapid association of parathyrin with isolated brush border membranes at 0°C has been previously reported [42].

Degradation of $^{125}$I-insulin also proceeded rapidly at 20°C and was linear for up to 90 min of incubation after a slight initial lag period (Fig. 2*A*). Typically 20–25% of the $^{125}$I-insulin was converted to TCA-soluble products during a 90-min incubation. Since the TCA-precipitation method may underestimate the extent of degradation compared to radioimmunoassay, chromatographic, or rebinding procedures, insulin degradation was also examined by gel chromatography of the reaction mixtures on Sephadex G-50 (Fig. 3) and by radioimmunoassay and was compared to the results obtained with the TCA-precipitation procedure. After 30, 60 and 90 min of incubation at 20°C with brush border membranes, $^{125}$I-insulin, which primarily chromatographed as a large peak I on Sephadex G-50 (0 time incubation), progressively shifts to a more included position, peak II, representative of $^{125}$I-insulin degradation products (Fig. 3). The peak eluting at the void volume does not represent free monomeric $^{125}$I-insulin, but has been observed previously in iodinated samples of insulin [13, 36]. Quantitation of the area under peak II indicated that 32.8, 46.3 and 53.6% of the $^{125}$I-insulin had been degraded after 30, 60 and 90 min, respectively, compared to 23.9, 35.7 and 44.5% by the TCA-precipitation procedure. Therefore the TCA-precipitation technique underestimated the $^{125}$I-insulin degradation somewhat compared to estimation of the degradation products by gel chromatography. In another experiment degradation of unlabeled insulin estimated by radioimmunoassay was 17.8, 23.4 and 35.8% after 30, 60 and 90 min of incubation, respectively, compared to 8.0, 13.6 and 21.6% degradation of $^{125}$I-insulin at the same time points under identical conditions by the TCA-precipitation procedure. Therefore, actual proteolytic cleavage of $^{125}$I-insulin was somewhat greater than measured in our experiments. However, the TCA-precipitation procedure is simple to perform for routine monitoring and in comparative studies under different conditions gives the same

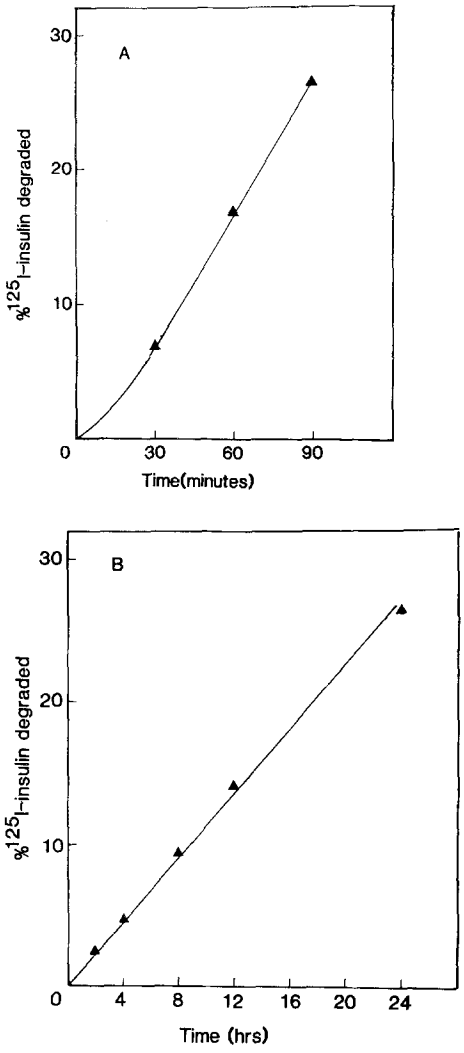


**Fig. 2.** Time course of $^{125}$I-insulin degradation by isolated rat renal brush border membrane vesicles. Membrane vesicles (*A*: 2.11 mg/ml, *B*: 0.77 mg/ml) were incubated at 20°C (*A*) and at 4°C (*B*) with $^{125}$I-insulin, and aliquots of the total incubation mixture were removed at the indicated time points for determination of $^{125}$I-TCA soluble products

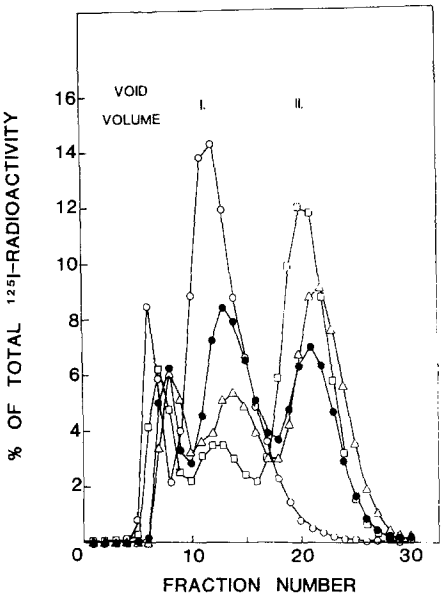


**Fig. 3.** Chromatography on Sephadex G-50 of incubation mixtures containing $^{125}$I-insulin and degradation products. Brush border membranes were incubated with $^{125}$I-insulin (*see* Methods) and 150 $\mu$l aliquots removed at 0, 30, 60 and 90 min and pipetted into 20% TCA for estimation of degradation (*see* Methods and text) or 750 $\mu$l 1 M acetic acid for chromatography. After centrifugation the acetic acid supernatants were chromatographed on Sephadex G-50 and 1-ml fractions eluted with 1 M acetic acid were assayed for radioactivity. 0 min (○); 30 min (●); 60 min (△); 90 min (□)

relative values as other procedures [30, 48]. Degradation of $^{125}$I-insulin proceeded even at 4°C, although at a much slower rate (Fig. 2*B*) than at 20°C. Nevertheless, production of TCA-soluble products was linear for up to 24 hr at 4°C, at which time over 25% of the total $^{125}$I-insulin had been degraded. These findings confirmed the known degradative capacity of the renal brush border membrane for peptides and proteins and are in agreement with the substantial content of several proteases in these membranes [27].

Both $^{125}$I-insulin association (Fig. 4*A*) with and degradation (Fig. 4*B*) by brush border membrane vesicles were linearly related to the amount of membrane protein in the incubations up to concentrations of at least 1.8 mg/ml protein. Of interest was the observation that at vesicle membrane concentrations below 100 $\mu$g/ml, both association and degradation of $^{125}$I-insulin by brush border preparations was very small. This may account for the results of other investigators who concluded that renal brush border membranes possessed negligible insulin-degrading activity, but who used membrane protein concentrations of well under 100 $\mu$g/ml in their assays [23, 46]. For our subsequent experiments, membrane protein concentrations of 1–1.5 mg/ml were generally employed.

Because mitochondria and cytoplasm have been reported to contain proteolytic activity [12, 20], some of which is directed towards insulin [23], it was important to exclude these possible contaminants as the source of $^{125}$I-insulin-degrading activity observed in the renal brush border preparations. Therefore, the ability of purified brush border membranes to degrade $^{125}$I-insulin was compared with the degradative capacity of the underlying reddish brown mitochondria-enriched remnant and the membrane pellet and supernatant fractions derived from the purified membrane preparation by a final

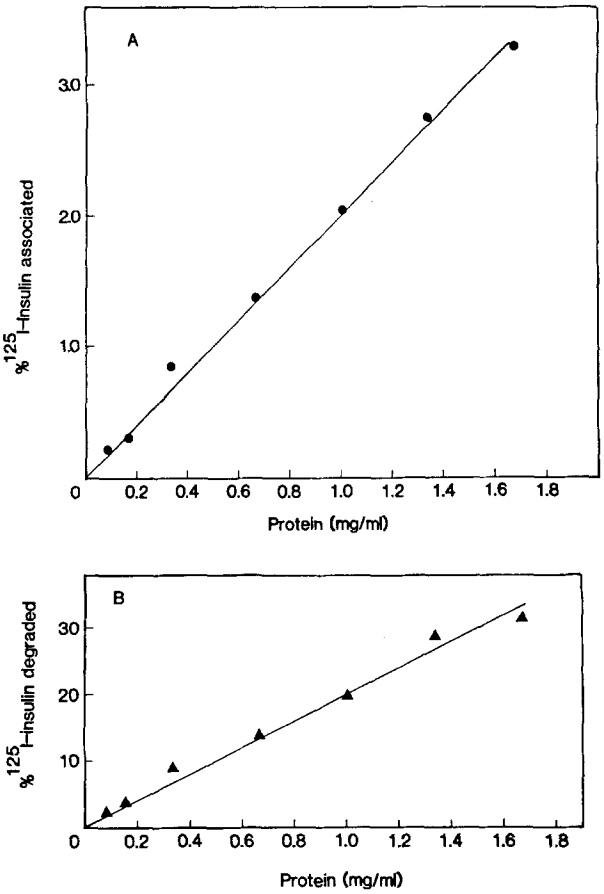


**Fig. 4.** Association and degradation of $^{125}$I-insulin as a function of renal brush border membrane protein concentration. Membrane vesicles were incubated with $^{125}$I-insulin at 20°C for 90 min with different concentrations of membrane protein and association (*A*) and degradation (*B*) of $^{125}$I-insulin determined at the completion of the incubation. Data show total $^{125}$I-insulin association and degradation, and the association data are not corrected for degradation

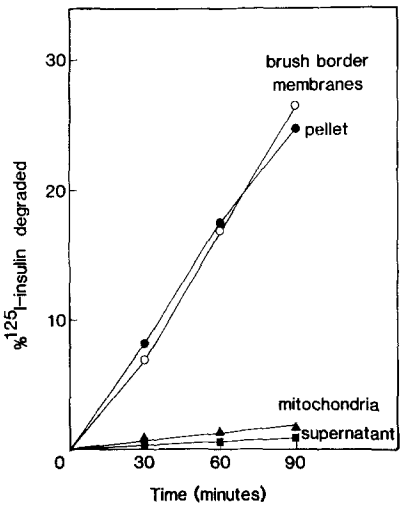


**Fig. 5.** Degradation of $^{125}$I-insulin by purified brush border membranes and potential contaminating fractions. A brush border vesicle suspension (1.76 mg/ml) was prepared and pellet (1.65 mg/ml) and supernatant (0.013 mg/ml) fractions were derived from the suspension by centrifugation at 30,000 × *g* for 15 min. The mitochondria-enriched pellet (0.033 mg/ml) was separated from the brush border membranes during the final step of the isolation procedure. All membrane and supernatant fractions were adjusted to volumes equivalent to that of the dispersed brush border membrane preparation, incubated with $^{125}$I-insulin at 20°C and aliquots removed at the indicated time periods for determination of $^{125}$I-TCA-soluble products. Brush border vesicle suspension (○—○), membrane pellet from vesicle suspension (●—●), supernatant fraction from vesicle suspension (■—■), mitochondria-enriched pellet from brush border isolation (▲—▲)

centrifugation at 30,000 × *g* for 15 min. When the entire mitochondria-rich pellet was suspended in an equivalent volume of incubation medium to that used for brush border membranes, simulating the maximum mitochondrial contamination of the brush border membranes which could occur during isolation, the degradation of $^{125}$I-insulin by the mitochondria-enriched suspension was negligible compared to that seen with the brush border preparation (Fig. 5). In addition, the $^{125}$I-insulin-degradative activity was found to reside almost completely in the brush border vesicles, with little soluble activity in the supernatant solution, following sedimentation of the membranes by centrifugation.

The presence of definite degradative activity toward $^{125}$I-insulin in the purified brush border membrane preparation and its effective separation, during membrane isolation, from the large amount of soluble degradative activity present in the cytoplasm was confirmed by assaying intermediate fractions from the membrane isolation for degradative activity. The results (Table 2) indicate that the highest specific activities for $^{125}$I-insulin degradation are found in the soluble fractions (*see* low speed supernatant) and that this activity is steadily decreased during the washing procedures so that the final supernatant solution (high speed supernatant 3) has only one-fiftieth the specific activity of degradation of the original homogenate. Given the specific activities of degradation of the potential mitochondrial and cytosolic contaminants removed from the brush border membrane during the final centrifugation step in the preparation procedure, these fractions combined could account for no more than 5% of the total degradative activity observed in the brush border preparation (*see* Table 1).

Since lysosomes are a potential contaminant of brush border membrane preparations and are known to contain proteolytic activity capable of de-

**Table 2.** Distribution of $^{125}$I-insulin degradative activity during brush border membrane isolation

| Renal fraction[a] | Specific activity degradation[b] | Relative specific activity degradation[c] |
|---|---|---|
| Homogenate | 28.86 | 1.00 |
| Low speed pellet | 4.50 | 0.16 |
| Low speed supernatant | 51.21 | 1.77 |
| High speed supernatant 1 | 39.47 | 1.37 |
| High speed supernatant 2 | 12.80 | 0.44 |
| High speed supernatant 3 | 0.67 | 0.02 |
| Mitochondria-enriched remnant | 3.31 | 0.11 |
| Purified brush border membranes | 3.06 | 0.11 |

[a] Renal fractions at different stages of brush border membrane isolation as described in Methods.
[b] Specific activity degradation = % total $^{125}$I-insulin degraded/100 $\mu$g protein in a 90-min incubation at 20°C.
[c] Relative sp act degradation = sp act degradation of renal fraction/sp act degradation of homogenate.

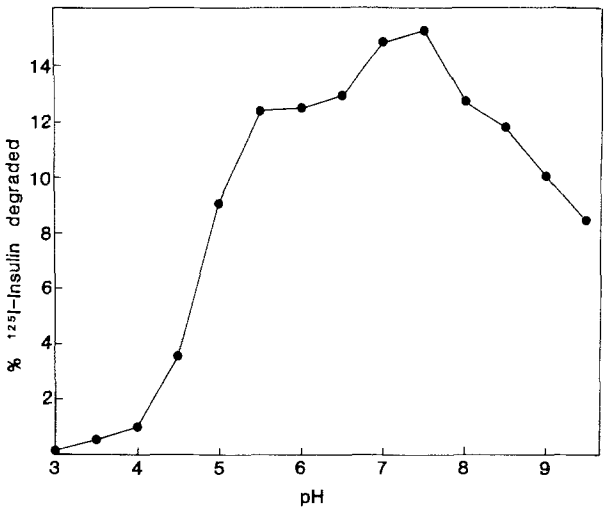


**Fig. 6.** pH profile of $^{125}$I-insulin degradation by brush border membranes. Brush border membranes were incubated with $^{125}$I-insulin at 4°C for 16 hr in 100 mM Tris, 100 mM sodium acetate, 100 mM $NaH_2PO_4$ adjusted to pH ranging from 3.0 to 9.5. Degradation was determined by the TCA-precipitation technique (*see* Methods). The experiment shown is representative of three such experiments. Similar results were obtained in incubations for 90 min at 20°C

grading $^{125}$I-insulin [23], the pH profile of $^{125}$I-insulin degradation in the brush border membranes was determined to establish if appreciable activity was seen in the acidic pH range where lysosomal protease activity is optimal. As can be seen (Fig. 6), most degradative activity occurs over a broad range of pH from 5.5 to 8.5 with a marked decline in activity below pH 5.5 and little or no degradation occurring at pH 4.0 or below. This pH profile of $^{125}$I-insulin degrading activity is not consistent with an appreciable contribution of lysosomal proteases to the total activity of brush border membranes.

Further support for the membrane-bound nature of both the association and degradation of $^{125}$I-insulin by brush border membrane vesicles was provided by examining these processes in native membranes or in the presence of the nonionic detergent Triton X-100. In the presence of the detergent, membrane association of $^{125}$I-insulin was virtually abolished while degradation was markedly increased (Table 3). Evidence that Triton X-100 was solubilizing the degradative activity rather than releasing it from intravesicular sites (e.g., trapped cytosol) was the failure of several cycles of freeze-thawing or lysis in distilled water to release degradative activity from the membrane vesicles. Further investigation indicated that 1% Triton X-100 could extract all of the insulin-degrading activity from the membranes, leaving no activity remaining in the residual pellet (*data not shown*). Therefore, the detergent solubilizes all of the membrane-associated degradative activity, some of which may be in cryptic membrane sites. The

**Table 3.** Association and degradation of $^{125}$I-insulin by untreated and detergent-treated brush border membrane vesicles

| | Association | | Degradation | |
|---|---|---|---|---|
| | cpm | % total cpm | cpm | % total cpm |
| Membranes | 3828 | 3.51 | 30230 | 27.7 |
| Membranes + NEM | 3409 | 3.13 | 21500 | 19.7 |
| Membranes + Triton | 621 | 0.57 | 95521 | 87.6 |
| Membranes + Triton + NEM | 661 | 0.61 | 25198 | 23.1 |
| No membranes | 79 | 0.07 | 2979 | 2.7 |

Membrane vesicles (1.21 mg/ml) were incubated at 20°C for 90 min with $^{125}$I-insulin (109,000 cpm) in the presence or absence of 4.2 mM N-ethylmaleimide (NEM) and/or 0.2% Triton X-100, and aliquots of the total incubation mixture were removed for determination of $^{125}$I-insulin association and degradation.

thiolprotease inhibitor, N-ethylmaleimide (NEM), which has been reported to be a very effective inhibitor of renal degradative activity towards $^{125}$I-insulin [11, 28, 30], only diminished brush border associated degradation by about one-third, but inhibited virtually all of the increased degradative activity uncovered in the presence of detergent (Table 3). These results suggest that the major portion of the detergent-solubilized degradative activity is located at sites which are inaccessible to NEM or which may not participate in the membrane-associ-

**Table 4.** Effect of protease inhibitors on $^{125}$I-insulin association and degradation by brush border membrane vesicles

| Protease inhibitor | % association | Relative % association | % degradation | Relative % degradation |
|---|---|---|---|---|
| None | 4.39 | 100 | 19.09 | 100 |
| EDTA (2.5 mM) | 3.02 | 68.8 | 7.79 | 40.8 |
| NEM (5 mM) | 3.91 | 89.2 | 12.45 | 65.2 |
| PMSF (0.5 mM) | 4.29 | 97.9 | 19.07 | 99.9 |
| EDTA + NEM | 4.20 | 95.7 | 0 | 0 |
| EDTA + PMSF | 3.22 | 73.4 | 7.34 | 38.4 |
| NEM + PMSF | 4.44 | 101.2 | 13.57 | 71.1 |
| EDTA + NEM + PMSF | 2.20 | 50.2 | 0.82 | 4.3 |

Membrane vesicles (1.58 mg/ml) were incubated at 20°C for 90 min with $^{125}$I-insulin in the presence or absence of 2.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM N-ethylmaleimide (NEM) and/or 0.5 mM) phenylmethylsulfonyl fluoride (PMSF), and aliquots were removed for determination of $^{125}$I-insulin association and degradation.

**Table 5.** Determination of integrity of bound and free $^{125}$I-insulin in incubation mixtures

| | Total % binding[a] | Specific % binding[b] | Total % degradation[c] | Supernatant % degradation[d] | Pellet % degradation[e] | Pellet % intact[f] |
|---|---|---|---|---|---|---|
| Membranes −NEM-EDTA −insulin | 10.35 | 6.08 | 14.98 | 14.34 | 38.9 | 61.1 |
| Membranes −NEM-EDTA +insulin | 4.27 | 0 | 4.46 | 4.21 | 35.4 | 64.6 |
| Membranes +NEM-EDTA −insulin | 10.16 | 6.67 | 3.09 | 2.91 | 7.0 | 93.0 |
| Membranes +NEM-EDTA +insulin | 3.49 | 0 | 0.49 | 0.20 | 8.0 | 92.0 |

Membrane vesicles (0.60 mg/ml) were incubated at 20°C for 90 min with $^{125}$I-insulin in the presence or absence of 2.5 mM EDTA, 5 mM NEM and with or without 50 μg/ml unlabeled insulin, and aliquots were removed for determination of binding and degradation as described in Methods.

[a] Represents (cpm bound/total cpm) × 100.

[b] Represents (% bound − insulin) − (% bound + insulin).

[c] Represents (cpm degraded in total incubation mixture/total cpm) × 100.

[d] Represents (cpm degraded in supernatant/total cpm) × 100.

[e] Represents (cpm degraded in pellet/total cpm in pellet) × 100.

[f] Represents (cpm intact in pellet/total cpm in pellet) × 100.

ated degradation of $^{125}$I-insulin, since the former is completely sensitive to inhibition by NEM while the latter is largely resistant. N-ethylmaleimide had little effect on the association of $^{125}$I-insulin with the brush border membrane vesicles.

To further examine the nature of the membrane-associated degradative activity towards $^{125}$I-insulin, association and degradation of the hormone were investigated in the presence of three commonly employed protease inhibitors: ethylenediaminetetraacetic acid (EDTA), a metalloprotease inhibitor, N-ethylmaleimide (NEM), an inhibitor of thiol proteases, and phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor (Table 4). Both EDTA (2.5 mM) and NEM (5 mM) were partially effective in blocking brush border degradation of $^{125}$I-insulin while PMSF (0.5 mM) had no effect on association or degradation (Table 4). However, when EDTA (2.5 mM) and NEM (5 mM) were used in combination, all degradation of $^{125}$I-insulin was abolished with little or no effect on the association of $^{125}$I-insulin with the brush border vesicles. These results demonstrated that the process of degradation of $^{125}$I-insulin in the brush border membrane

can be inhibited independent of effects on membrane binding.

The presence and extent of specific binding of $^{125}$I-insulin to brush border membranes and the integrity of the free and membrane-associated $^{125}$I-insulin in the presence and absence of protease inhibitors and unlabeled insulin were also determined (Table 5). About 60% of the total binding was specific as indicated by its inhibition in the presence of 50 $\mu$g/ml unlabeled insulin. Both total and specific binding were similar whether protease inhibitors were included in the incubations or not, but the integrity of the membrane-bound $^{125}$I-insulin was clearly different under these conditions. In the absence of protease inhibition about two-thirds of the associated $^{125}$I-insulin was TCA precipitable and one-third was TCA soluble. In the presence of protease inhibitors, greater than 90% of the membrane-bound ligand was intact $^{125}$I-insulin. It is interesting that under all the conditions examined the ratio of degraded to intact $^{125}$I label bound to the membranes was greater than the corresponding ratio of $^{125}$I label free in the medium and this ratio was similar at both low and high concentrations of insulin. This result would be expected if a significant fraction of the bound hormone is continually being degraded on the membrane by a virtually nonsaturable process. To ensure that the presence of degradation products did not affect studies on the affinity of binding, all subsequent experiments were performed in the presence of 2.5 mM EDTA and 5 mM NEM unless concomitant measurement of both binding and degradation was desired.

Since the brush border membranes used in these experiments are vesicular in nature, it was possible that part or all of the association of $^{125}$I-insulin with the membranes consisted of uptake of the hormone and/or its degradation products into the vesicles rather than binding of intact hormone to the membrane surface. In analogy with experiments to differentiate the binding vs. uptake of small molecules such as glucose in renal brush border vesicles, the uptake of $^{125}$I-insulin and/or its degradation products by BBV should be sensitive to increasing osmolarity of the incubation medium which reduces intravesicular volume, while $^{125}$I-insulin binding to the membrane surface would be expected to be independent of osmolarity unless the latter influenced the accessibility of the hormone to its binding site. Therefore, we examined this question by determining the association and degradation of $^{125}$I-insulin by brush border membrane vesicles incubated in media of varying osmolarity (300–1100 mOsM). The resultant plot of $^{125}$I-insulin associated vs. the medium osmolarity yielded an essentially unchanged level of ligand association at all medium osmolarities, indicating $^{125}$I-insulin was binding to the external face of the membrane vesicles rather than being taken up into the intravesicular space (Fig. 7).

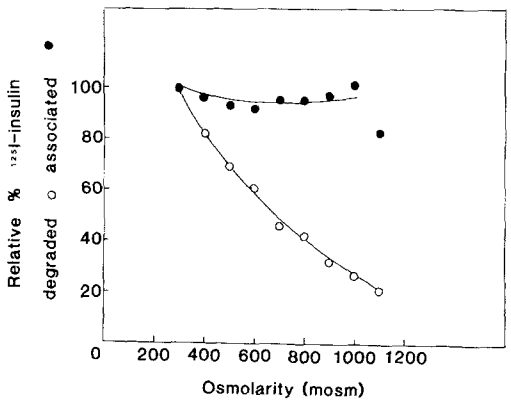


**Fig. 7.** Association and degradation of $^{125}$I-insulin by isolated rat renal brush border membrane vesicles as a function of the osmolarity of the incubation medium. Membrane vesicles (1 mg/ml) were incubated at 4°C for 15 min in incubation media containing different concentrations of sucrose to achieve osmolarities of 300–1100 mOsM, $^{125}$I-insulin was added and the incubation continued at 20°C for 90 min. Aliquots were removed at the completion of the incubation for determination of $^{125}$I-insulin association (●) and degradation (○) as described in Methods, except that since the density of the sucrose media of high osmolarities exceeded that of the oil layer, all of the membrane pellets in this experiment were obtained by sedimentation in a Beckman airfuge at 105,000 × *g* for 10 min and were washed twice with 100 $\mu$l of oil to remove any adhering medium before cutting off the tube tips and counting to determine association. Data show total $^{125}$I-insulin association and are not corrected for degradation, but are corrected for extravesicular space determined in parallel incubations with $^{14}$C-sucrose as a marker. Degradation data are corrected for degradation of a control incubation mixture without membrane vesicles which ranged between 0.95–2.57% of the total $^{125}$I-insulin counts. The data were derived from six separate experiments. Binding at 300 mOsM averaged 6.2% (set to 100%) of total counts and 5.8% at 1000 mOsM. Degradation at 300 mOsM averaged 27.5% (set to 100%) and 6.8% at 1000 mOsM

Concomitant measurement of $^{125}$I-insulin degradation as a function of the osmolarity of the medium gave an inversely proportional relationship which contrasted with that seen with the association data (Fig. 7). The fact that degradation of $^{125}$I-insulin showed a continuous decrease as the osmolarity of the incubation medium increased could imply that the degradation of the hormone by isolated brush border vesicles is dependent upon the intravesicular space. However, the demonstration that $^{125}$I-insulin association with the membrane vesicles represented binding to the vesicles, rather than uptake, did not support this interpretation of the data and suggested a direct inhibitory effect of sucrose or increasing osmolarity on $^{125}$I-insulin degradation. This latter possibility was indeed found to be the case since a

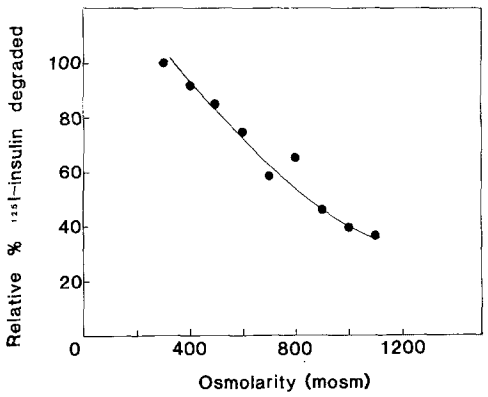


**Fig. 8.** Degradation of $^{125}$I-insulin by a 1% Triton X-100 extract of isolated rat renal brush border membrane vesicles as a function of the osmolarity of the incubation medium. Membrane vesicles were extracted with 1% Triton X-100 for 1 hr at 20°C and the Triton supernatant after centrifugation was incubated (121 $\mu$g protein/ml) with $^{125}$I-insulin at 20°C for 90 min in incubation media containing different concentrations of sucrose to achieve osmolarities of 300–1100 mosM. Aliquots of the total incubation mixture were directly pipetted into 20% TCA at the completion of the incubation for determination of $^{125}$I-insulin degradation as described in Methods. The data is the average of two experiments. Degradation averaged 56.0% of total counts (set to 100%) at 300 mosM and 20.7% at 1100 mosM

similar inhibition of $^{125}$I-insulin degradation with increasing medium osmolarity was observed when 1% Triton X-100 extracts of BBV were tested instead of the membranes themselves (Fig. 8) and also with the soluble degradative activity present in the 35,000 $\times$ *g* supernatant from the membrane isolation procedure (*data not shown*). This result was unexpected since we are unaware of any reports of a direct inhibitory effect of sucrose or medium osmolarity on protease activity, although cells exposed to sucrose have been reported to show decreased protein degradation, presumably due to disruption of the lysosomal system [10]. We have recently extended these findings to show that high osmolarity induced by mannitol and sorbitol has a similar inhibitory effect on $^{125}$I-insulin degradation and that this effect is seen with $^{125}$I-IGF-I degradation, as well (*unpublished observations*). In view of these results it is possible that the high sucrose concentrations in gradients used by other investigators [23] to fractionate renal protease activity may have influenced their results. Because of the concomittant inhibition of $^{125}$I-insulin degradation with the reduction of intravesicular space as medium osmolarity is increased, we can also not exclude the possibility that some $^{125}$I-insulin degradation products may be taken up into the vesicles at normal osmolarity (e.g., *see* Table 5). The appreciable degradation of $^{125}$I-insulin by BBV and derived soluble extracts is consistent with the presence of several proteases comprising a significant proportion of the brush border membrane proteins [27].

In order to determine if the binding of $^{125}$I-insulin by brush border membrane vesicles was a process specific for this hormone or was common to other small proteins and peptides, several substances known to be taken up by luminal endocytosis at the brush border membrane or to directly interact with the membrane [7, 8, 25, 33, 34] were examined for their ability to inhibit $^{125}$I-insulin binding by the vesicles. Of the five proteins and peptides examined, only unlabeled insulin and bacitracin showed a dose-dependent inhibition of $^{125}$I-insulin binding to the vesicles (Fig. 9). The concentration of insulin needed to achieve 50% inhibition of $^{125}$I-insulin association was approximately $1.4 \times 10^{-6}$ M, which is several orders of magnitude greater than the unlabeled insulin concentration needed for equivalent inhibition of binding of $^{125}$I-insulin to high-affinity renal receptors or to those of other tissues. It should be borne in mind that this is the value for half maximal inhibition of total $^{125}$I-insulin binding. If one defines "nonspecific" binding of $^{125}$I-insulin as that occurring in the presence of about $10^{-5}$ M unlabeled insulin, as is commonly done in binding studies, then half-maximal inhibition of specific binding occurs at $2.8 \times 10^{-7}$ M unlabeled insulin which is still much greater than that required for half-maximal inhibition of high-affinity receptor binding. Bacitracin, an antibiotic peptide commonly used to inhibit insulin degradation in binding and metabolic studies, inhibits $^{125}$I-insulin binding by brush border membrane vesicles only at very high concentrations, although these concentrations (0.8–8 mg/ml) are the same as those used to inhibit degradation [50, 58]. The low molecular weight proteins lysozyme and cytochrome *c*, both of which are known to be filtered at the glomerulus and reabsorbed at the renal brush border membrane surface [34], show little or no dose-related inhibition of $^{125}$I-insulin binding. The polypeptide aprotinin, which is therapeutically and experimentally used as an inhibitor of insulin degradation and which has been reported to be strongly bound to renal brush border membranes [25], had no effect on $^{125}$I-insulin binding even at very high concentrations.

Although the association of $^{125}$I-insulin with brush border vesicles was specific for insulin when compared to other proteins and peptides that interact with these membranes, it did not distinguish insulin from the related polypeptides proinsulin and desoctapeptide insulin (Fig. 10). The latter two polypeptides are clearly distinguishable from insulin in both binding affinity and biological activity in a variety of other systems including renal tubules

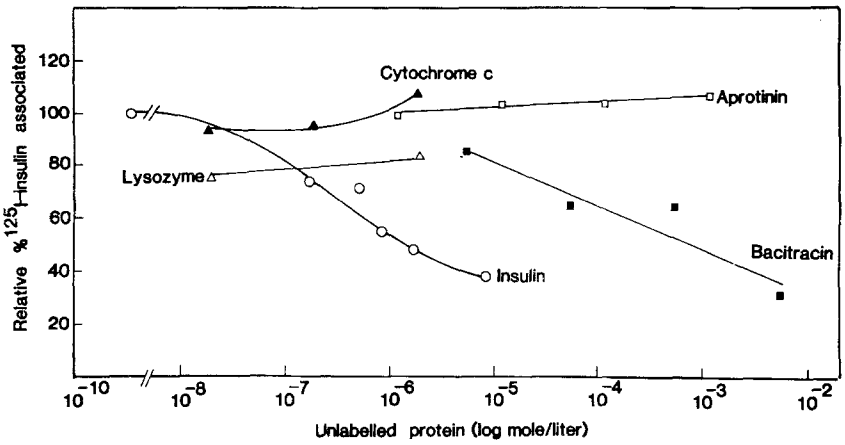


**Fig. 9.** Inhibition of $^{125}$I-insulin association to isolated rat renal brush border membrane vesicles by unlabeled insulin (○—○), lysozyme (△—△), cytochrome *c* (▲—▲), bacitracin (■—■) and aprotinin (□—□). Membrane vesicles (1.28 mg/ml) were incubated at 20°C for 90 min with $^{125}$I-insulin in the absence (control binding) or presence of the unlabeled proteins and peptides at the indicated concentrations. Association is expressed as the percentage of $^{125}$I-insulin associated with the membranes in the presence of the indicated protein relative to that in the absence of added protein (control binding = 100%). All data represent total binding and are not corrected for $^{125}$I-insulin degradation. The data were derived from eight separate experiments

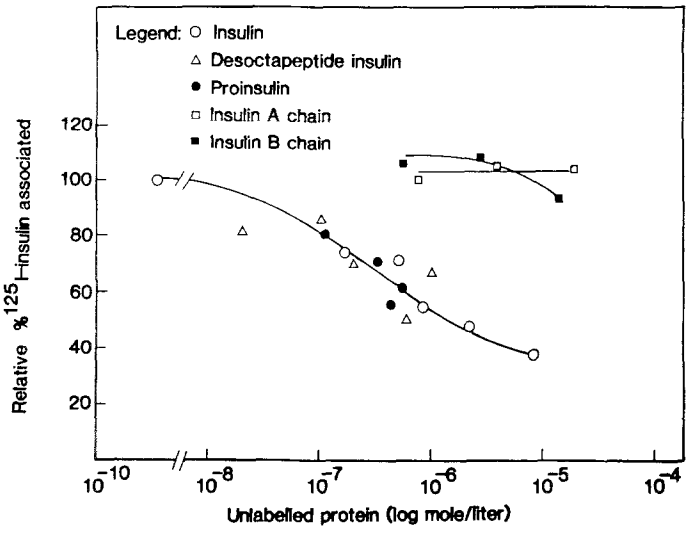


**Fig. 10.** Inhibition of $^{125}$I-insulin association to isolated rat renal brush border membrane vesicles by unlabeled insulin (○—○), desoctapeptide insulin (△—△), proinsulin (●—●), insulin A chain (□—□) and insulin B chain (■—■). Membrane vesicles (1.17 mg/ml) were incubated at 20°C for 90 min in the absence (control binding) or presence of the unlabeled insulin and related polypeptides at the indicated concentrations. Association is expressed as the percentage of $^{125}$I-insulin associated with the membranes in the presence of the indicated protein relative to that in the absence of added protein (control binding = 100%). All data represent total binding and are not corrected for $^{125}$I-insulin degradation. The data were derived from six separate experiments

[19, 39, 41, 43]. It is interesting to note, however, that several inhibition studies of $^{125}$I-insulin binding in renal tissues show that these systems distinguish insulin from related analogues much better at lower concentrations than at higher concentrations [19, 41, 43], suggesting that a portion of the interaction being studied was low-affinity binding of the hormone rather than high-affinity receptor-mediated binding. While brush border membrane hormone binding sites do not differentiate among insulin, proinsulin and desoctapeptide insulin, they clearly distinguish these proteins from the separated insulin A and B chains which have little or no effect on $^{125}$I-insulin binding even at very high concentrations. The effects of the various peptides studied on $^{125}$I-insulin degradation were analogous to their effects on insulin binding, with proinsulin and desoctapeptide insulin having similar inhibitory effects to that of insulin (35% inhibition at a concentration of 5 $\mu$g/ml) while insulin A and B chains cytochrome *c*, and lysozyme had no inhibitory effect on $^{125}$I-insulin degradation (*data not shown*).

Since it was possible that the low-affinity $^{125}$I-insulin binding to brush border membranes could represent a cross-reaction of $^{125}$I-insulin with specific high-affinity receptor(s) for insulin-like growth

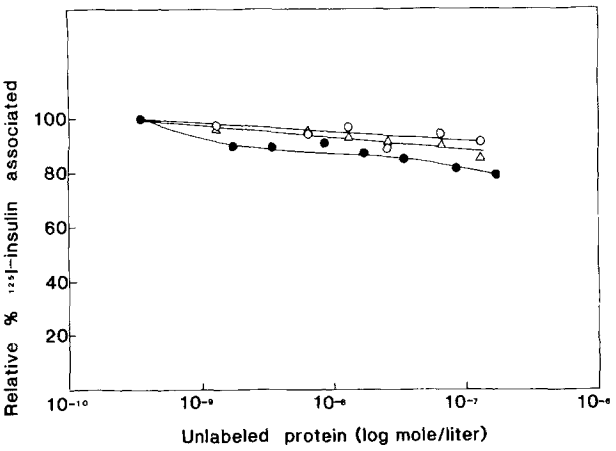


**Fig. 11.** Inhibition of $^{125}$I-insulin association to isolated rat renal brush border membrane vesicles by unlabeled insulin (●—●), IGF-I (○—○) and IGF-II (△—△). Membrane vesicles (3.25 mg/ml) were incubated at 20°C for 90 min in the absence (control binding) or presence of the unlabeled proteins at the indicated concentrations. Association is expressed as the percentage of $^{125}$I-insulin associated with the membranes in the presence of the indicated protein relative to that in the absence of added protein (control binding = 100%). All data represent total binding and are not corrected for $^{125}$I-insulin degradation. The data were derived from four separate experiments

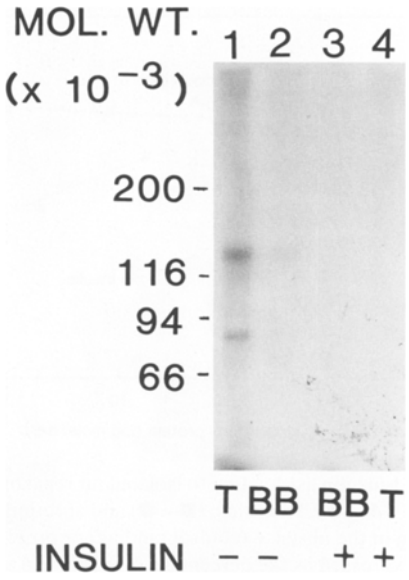


**Fig. 12.** Autoradiogram of $^{125}$I-insulin-labeled subunits of the insulin receptor in tubular membranes (*T*) and brush border membranes (*BB*). Membranes were incubated with $^{125}$I-insulin in the presence (+) or absence (−) of unlabeled insulin, washed, and treated with disuccinimidyl suberate for crosslinking. Crosslinked membranes were washed, dissolved in SDS and subjected to gel electrophoresis followed by autoradiography. Details are described in Methods

factor(s), the ability of IGF-I and IGF-II to compete with $^{125}$I-insulin for binding was determined (Fig. 11). The results indicated that at concentrations of IGF-I or IGF-II as high as $10^{-7}$ M there was only minimal inhibition of $^{125}$I-insulin association with brush border membranes, which did not exceed the less than 25% inhibition given by insulin itself over this concentration range. In contrast, when IGF-I, IGF-II and insulin were compared in their ability to inhibit $^{125}$I-IGF-I binding to brush border membranes, IGF-I gave a pronounced and maximal inhibition of $^{125}$I-IGF-I binding at concentrations below $10^{-7}$ M, while insulin and IGF-II showed little or no inhibition at these concentrations (E. Meezan et al., *in preparation*). Therefore, although specific receptor sites for IGF-I and/or IGF-II may be present on brush border membranes, the failure of IGF-I and IGF-II to inhibit $^{125}$I-insulin association with the membrane indicates that insulin is binding to a site distinct from the receptors for the insulin-like growth factors.

In order to further investigate the molecular nature of the binding site for $^{125}$I-insulin on renal brush border membranes, covalent crosslinking studies were performed. Rat renal tubules, which are known to have high-affinity receptors for insulin, and renal brush border membranes, which appear to lack such sites, were equilibrated with $^{125}$I-insulin, exposed to the crosslinking agent disuccinimidyl suberate, solubilized in dodecylsulfate and subjected to gel electrophoresis and autoradiography (Fig. 12). A prominent band corresponding to the $\alpha$-subunit of the insulin receptor ($M_r$ = 130,000) was present in the tubular sample incubated in the absence of unlabeled insulin (lane *1*) and this band was specifically blocked by the inclusion of unlabeled insulin (lane *4*), confirming that it represented a specific association of an insulin receptor subunit with $^{125}$I-insulin. In contrast, only a trace amount of this band appeared in the brush border membrane sample incubated in the absence of unlabeled insulin (lane *2*). Several other bands in the tubular sample, but not in the brush border sample, which were labeled in the absence, but not in the presence, of cold insulin, may represent aggregates or fragments of receptor subunits. These results are consistent with those of the binding studies which indicate a lack of appreciable amounts of a high affinity, specific insulin receptor in brush border membranes compared to that which can be demonstrated in membranes from intact tubules.

The relatively low affinity and broad specificity of the renal brush border membrane for $^{125}$I-insulin is accompanied by a high capacity of the brush border vesicles to bind and degrade the hormone. When the amount of insulin bound and degraded by

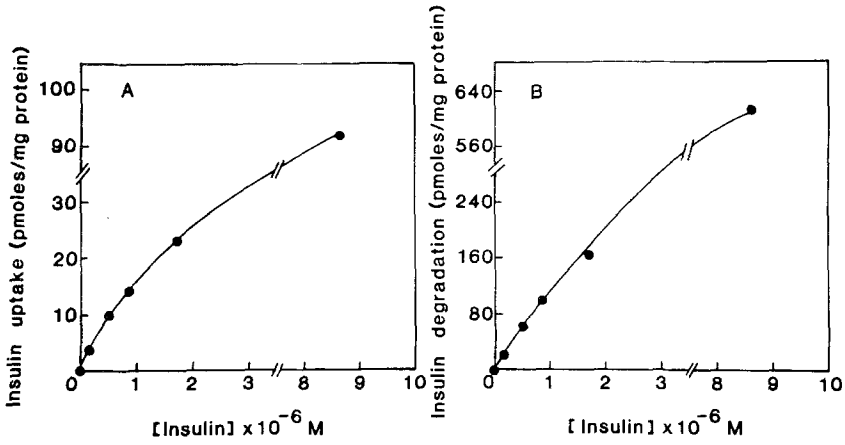


**Fig. 13.** Uptake (*A*) and degradation (*B*) of $^{125}$I-insulin by isolated rat renal brush border membrane vesicles (1.38 mg/ml) as a function of the medium insulin concentration. Membrane vesicles were incubated at 20°C for 90 min with $^{125}$I-insulin and unlabeled insulin at the indicated concentrations and uptake (*A*) and degradation (*B*) of insulin determined at the completion of the incubation. Data show total insulin uptake and degradation, and the uptake data are not corrected for degradation

brush border membrane vesicles is plotted against the concentration of insulin in the incubation medium, neither process approaches saturation even at concentrations of insulin approaching $10^{-5}$ M (Fig. 13*A* and *B*), although both plots are curvilinear. These results are in agreement with the work of other investigators, which indicate that the renal uptake and degradation mechanisms for insulin are virtually nonsaturable [3, 26, 35, 44, 55, 57] and may be contributed to by the continual binding and degradation of $^{125}$I-insulin at the luminal membrane sites followed by dissociation of the degradation products.

## Discussion

The present study strongly supports the presence of low-affinity, high-capacity recognition sites on the renal brush border membrane which specifically bind insulin and insulin analogs, but which have properties that distinguish them from the high-affinity insulin receptors which have been well-characterized in many tissues, including the kidney [3, 17–19, 28–30, 39–41, 43, 45, 48, 57, 58]. The isolated renal brush border membrane vesicle system is an attractive one for the detailed study of the interaction of $^{125}$I-insulin with a well-defined membrane known to interact with and mediate the tubular uptake of insulin in vivo. The use of isolated membrane vesicles provides a simple experimental system in which binding of $^{125}$I-insulin can be clearly distinguished from uptake of the hormone without the complexities of whole cell systems in which energy metabolism and polarized cell surfaces complicate similar studies, as is the case in renal tubules. The use of isolated membrane vesicles also permitted an investigation of the relationship of $^{125}$I-insulin association with the membranes to the degradation of the hormone.

$^{125}$I-insulin association with brush border membrane vesicles was time and temperature dependent and was linearly related to membrane protein concentration. These properties are typical characteristics of $^{125}$I-insulin binding to renal insulin receptors as previously reported by ourselves [39–41] and other investigators [3, 17, 28–30, 45–48, 57, 58]. The present study demonstrates a clear delineation between $^{125}$I-insulin binding and $^{125}$I-insulin uptake in renal brush border membrane vesicles by demonstrating that the association of the hormone with the vesicles is not affected by varying the osmolarity of the incubation medium and therefore represents binding to an external membrane site, rather than uptake of $^{125}$I-insulin or its degradation products into an osmotically sensitive space. The properties of the recognition sites for $^{125}$I-insulin are distinct from those characteristics of high-affinity insulin receptors but agree well with one of the in vivo functions of the renal brush border membrane, that of mediating reabsorption of filtered small proteins by the tubules [4, 7, 8, 33, 34]. This reabsorptive function implies a mechanism for internalization of proteins at the brush border membrane surface. The mechanism of protein reabsorption by the renal tubules has been characterized as a process of luminal endocytosis that is energy dependent and has the properties of a low-affinity, high-capacity uptake system, which is not highly specific in its selectivity [4, 7, 8, 33, 34]. This description is based mainly on electron microscopic examination of the luminal reabsorption of tracer macromolecules and on physiological studies in vivo and in the isolated perfused kidney [4, 5, 7, 8, 33–35, 44, 47]. In these studies it was not clear whether the reabsorption process is receptor mediated.

Our results demonstrate the presence of low-affinity binding sites for $^{125}$I-insulin in renal brush border membrane vesicles which can distinguish insulin from chemically unrelated low molecular

weight proteins and polypeptides, but which do not differentiate insulin from its analogs as do the high-affinity receptors for this hormone. These low-affinity recognition sites examined in this in vitro membrane system may correspond to the luminal membrane sites at which endocytosis takes place in vivo. In this respect our results differ from those of some other investigators, who report a high affinity receptor for insulin in renal brush border membranes [48, 57, 58]. However, a recent study [18] found isolated brush border membranes to have less than one-tenth the specific insulin binding of renal basolateral membranes, which indicates that the amount of classical insulin receptors in the brush border membrane preparation is very small and may even be due to unavoidable contamination by basolateral membranes. The low affinity of $^{125}$I-insulin for its binding sites and the inability to crosslink more than a trace of the hormone to high-affinity receptor sites, in contrast to the positive results with intact tubules, support the view that few if any high-affinity receptors for insulin are present on the luminal side of the tubule and instead that these are localized to the basolateral membrane [17].

These results are also consistent with physiological studies on the in vivo and in vitro properties of the brush border membrane. For example, recently Martineau-Doize et al. [35] reported the results of in vivo radioautography experiments in rat kidneys that showed an intense association of $^{125}$I-insulin with the brush border of the proximal convoluted tubules, which represented binding of labeled insulin to high-capacity sites which they suggested were related to the reabsorption and degradation of the hormone from the urinary filtrate. In addition, Nielsen et al. [44], using isolated perfused proximal tubules from rabbits, have shown that perfused $^{125}$I-insulin associates initially with the brush border membrane of the tubules, from where it is taken up by a high capacity, efficient and largely nonsaturable uptake process. The results of both of these studies are in perfect agreement with the properties we have determined for $^{125}$I-insulin association with isolated brush border membranes and argue against the mediation of this process by high-affinity insulin receptors, which are largely absent from the luminal membrane and which would be expected to be saturated at the concentration of insulin employed.

Our current results support our previous findings [39–41] that the affinity of $^{125}$I-insulin for its renal tubular receptors is significantly lower than that found in other tissues or other renal sites for insulin receptors including glomeruli. This low affinity for insulin observed in renal tubular membranes may reflect the contribution of the low affinity binding of $^{125}$I-insulin at the luminal surface of the tubule, which dilutes the high-affinity insulin binding sites at the basolateral surface and results in binding to tubules having an average lower affinity than that seen with other tissues.

Proteins and peptides that interact with the renal brush border membrane surface may either be reabsorbed intact by luminal endocytosis and then degraded intracellularly or may be initially hydrolyzed to smaller peptides and amino acids at the luminal surface by membrane associated proteases, followed by reabsorption of the degradation products by peptide and amino acid transport systems [4, 7, 8, 33, 34]. The results of our experiments with protease inhibitors and the effects of varying medium osmolarity on the binding and degradation of $^{125}$I-insulin by brush border membrane vesicles suggest that both mechanisms may be operative. Insulin degradation could be inhibited almost completely with a combination of NEM and EDTA, with little or no effect on the extent of $^{125}$I-insulin association with the membranes at the steady state. However, the integrity of bound $^{125}$I-insulin in the presence of protease inhibitors was greater than 90%, while in their absence only two-thirds of the hormone was intact. This implies that binding of the hormone to the membrane is associated with degradation, since all degradation in this experimental system is membrane bound. However, it should be noted that the binding of $^{125}$I-insulin to renal brush border membranes is not to a high-affinity insulin receptor, but to low affinity recognition sites (acceptors) whose function may be to take up and/or degrade the hormone at the luminal surface of the tubule. Although binding can be easily dissociated from degradation by inhibition of the latter with protease inhibitors, all maneuvers that inhibit binding (e.g. presence of unlabeled insulin or insulin analogs, bacitracin) also inhibit degradation but this does not exclude the possibility that these processes take place at separate membrane sites.

Many of the properties of the $^{125}$I-insulin degrading activity, however, suggest the intriguing possibility that both binding and degradation of the hormone on the brush border surface may occur at the same sites. For example: (i) the neutral pH optimum of the degrading activity, (ii) its inhibition by bacitracin and NEM, (iii) its broad specificity as suggested by similar inhibition by insulin and its analogs, but not by the insulin A or B chains, cytochrome *c* or lysozyme and (iv) its low affinity for $^{125}$I-insulin, which is comparable to the binding affinity for $^{125}$I-insulin in brush borders but not to the binding affinity of true insulin receptors, are all properties consistent with an insulin-degrading enzyme recently identified in cell extracts from sev-

eral tissues including kidney [53, 54]. Roth and coworkers [53, 54] have identified this enzyme as insulin-degrading enzyme or insulin protease (E.C. 3.4.22.11, insulinase), and have succeeded in highly purifying it and cross-linking it to $^{125}$I-insulin with the bifunctional crosslinker dissuccinimidyl suberate. Although the reported properties of this enzyme are somewhat variable from tissue to tissue and in the studies of different investigators, they are generally consistent with the brush border $^{125}$I-insulin binding and degrading activity reported here, and this enzyme deserves further consideration for the low-affinity $^{125}$I-insulin association and degradation sites on brush border membranes.

Degradation due to mitochondrial or cytoplasmic contamination of BBV was excluded by the minimal contamination of the preparation with these fractions and by a direct comparison of the activity of BBV, the mitochondria-enriched pellet and the residual cytoplasm. In addition, the mitochondrial protease is stimulated by EDTA and is inactive in the absence of Triton X-100 [20], properties which are both at variance with the $^{125}$I-insulin degrading activity of BBV. Furthermore, the pH profile of the brush border $^{125}$I-insulin degrading activity is consistent with that of a neutral protease and does not support a lysosomal origin for this activity in our preparation. For example, cathepsin D, the major renal lysosomal endopeptidase, exhibits maximal activity at pH 3.0–3.5 [1], a range in which almost no degradative activity is seen in our preparation. In addition bacitracin and NEM do not inhibit insulin degradation by lysosomal proteases [16], whereas they inhibit $^{125}$I-insulin degradation by brush border membranes. It is possible that some of the $^{125}$I-insulin degrading activity seen in isolated brush border membranes reside in endosomes present in this preparation. However, since endosomes are derived from the luminal surface by the budding off of coated pits which are subsequently acidified and directed towards lysosomes [56], it is probable that any proteolytic activity contributed by this subcellular fraction is derived from the brush border membrane and would be small since coated pits comprise only 1–2% of the plasma membrane surface of most cells. In any event, insulin binding was not dependent on its prior or subsequent degradation by the brush border membranes.

Our data are in agreement with the present knowledge of the tubular reabsorptive process for low molecular weight proteins including insulin, via a low affinity, high-capacity system which is virtually nonsaturable [3, 4, 7, 8, 26, 33–35, 44, 55]. Our data are also compatible with the recent results of other investigators, which indicate that brush border membranes have very low levels of high-affinity insulin receptors [17, 18], suggesting that the renal tubular epithelial cell is able to selectively direct the intracellular trafficking of the high-affinity insulin receptor exclusively to the basolateral membrane of the cell. Most importantly, our data, in conjunction with that of others [17, 18, 35, 44], strongly suggest that true high-affinity insulin receptors do not mediate reabsorption of insulin at the tubular luminal brush border surface, but that this process is mediated by low-affinity, high-capacity, broadly specific insulin recognition sites whose exact molecular nature remain to be characterized.

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