The internalization and degradation of cell-associated $^{125}$I-insulin was studied by kinetic and steady state analyses at 37 °C in primary cultures of adult rat hepatocytes. Discrimination between membrane-bound and internalized insulin was based on the ability of an acid pH to rapidly dissociate membrane-bound insulin.

Receptor-mediated insulin internalization had a halftime of 6–8 min. Cell-associated $^{125}$I-insulin, at apparent steady state, was made up of equal amounts of membrane-bound and internalized insulin. Scatchard analyses of cell-associated $^{125}$I-insulin and membrane-bound insulin demonstrated a curvilinear plot. The curvilinear plot could be explained by binding sites with heterogenous affinity. This interpretation seems likely since negative cooperative interaction between insulin binding sites was absent at 37 °C.

The velocity of insulin internalization was nearly proportional to the concentration of membrane-bound insulin indicating that the reaction approximates first order kinetics with a $K_o$ of $2.5 \times 10^{-11}$ M. However, when the concentration of free insulin in the medium rather than membrane-bound insulin was considered as substrate, the rate of internalization was complex and the $K_o$ of internalization increased ($7 \times 10^{-10}$ M to $1.7 \times 10^{-8}$ M) with increasing concentrations of insulin. These $K_o$ values were similar to the $K_o$ of high and low affinity insulin binding sites, respectively. This indicated that insulin binding is the rate-limiting step for insulin internalization and that once insulin is bound, regardless to which receptors, it will have an equal probability of being internalized.

Over 99% of insulin degradation was cell-mediated. However, half of the degraded insulin was found in the intracellular compartment and the other half at plasma membrane sites. The insulin degradation system at the plasma membrane may be involved in the termination of insulin action and regulate the fraction of bound insulin available for internalization.

Evidence suggesting insulin penetration of rat liver cells came from earlier studies showing that intravenously injected $^{125}$I-labeled insulin was recovered in significant quantities in subcellular extracts of liver (4, 5). These observations have been confirmed and expanded using subcellular fractionation techniques (6, 7). However, possible artifacts produced by homogenization made it difficult to quantitatively compare the fraction of insulin bound to plasma membranes with that internalized. Other evidence for internalization of labeled insulin was provided by optical and electron microscopic studies (8–11). These techniques are sensitive and accurately indicate the location of hormone within the cell; however, the functional relationship between cellular insulin binding, internalization, and degradation is best evaluated by kinetic and steady state experiments as described herein.

Recently, chemical methods have been developed that permit a clear quantitative discrimination between cell surface-bound and internalized $^{125}$I-epidermal growth factor (12, 13), $^{125}$I-$\alpha_2$-macroglobulin (13), $^{125}$I-transferrin (14), $\alpha_2$-macroglobulin, $^{35}$S-trypsin complexes (15), and $[^3H]$asialoorosomucoid (16) in a variety of eukaryotic cell types. These methods are based on the ability of acid pH to decrease the affinity of ligand to its cellular receptor (12, 13, 17) proteases to remove ligand-bound membranes (13, 14) or the calcium dependence of some ligands for binding (15, 16).

This study was performed using an acidification technique (12, 13, 17) to distinguish and quantitate surface-bound and internalized $^{125}$I-labeled insulin in rat liver. Primary cultures of adult rat hepatocytes were used for these experiments. This cell system is particularly well suited to study the relationships between insulin binding, internalization, and degradation and offers distinct advantages over freshly isolated cells (18, 19).

The cultured cells are given time to recover from the trauma associated with isolation. Only viable cells adhering to collagen as a monolayer, responsive to insulin, and leaking only minimal amounts of insulin-degrading activity into the medium were utilized. Unlike cell preparations leaking large quantities of insulin-degrading activity into the medium causing insulin degradation prior to interaction with the insulin receptor, the cell cultures used allow the normal sequence of insulin processing to take place.

**MATERIALS AND METHODS**

**Chemicals—** Carrier-free Na$^{35}$I and $^{14}$Cr were obtained from New England Nuclear. Crystalline porcine insulin was kindly provided by Dr. Ronald Chance of Lilly, crude collagenase (CLS111, 40P102P, 138 units/mg) came from Worthington, and Fraction V bovine serum albumin from Reheis Chemical Co., Kankakee, IL. All other chemicals were reagent grade.

**Preparation of Hepatocytes—** Hepatocytes were isolated from adult male Sprague-Dawley rats (200–250 g) fed ad libitum. The rats were anesthetized with sodium pentobarbital (8 mg/100 g body weight). Liver perfusion was performed using the method of Exton (20) with minor modifications (21) in a liver perfusion cabinet designed and made at Vanderbilt University, Nashville, TN. The per-

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The liver is the major site of insulin degradation, as well as a major site of its action (1). Initial interaction of insulin at the cellular level occurs at the plasma membrane to specific insulin receptors (2). The insulin-receptor complex is then processed including internalization and degradation of the hormone or hormone-receptor complex (3).

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fused solution was Krebs-Ringer bicarbonate buffer free of calcium and albumin and supplemented with 5 mM glutamate, 11 mM glucose, 5 mM sodium pyruvate, and 10% (v/v) washed outdated human erythrocytes at pH 7.4. The erythrocytes were washed three times with 0.9% (w/v) NaCl. After equilibration of the perfusion medium for 30 min at 37 °C with O2/CO2 (95:5, v/v), livers were perfused in a nonrecirculating system for 15 min at 4 °C. The system was then set to recirculate and 20 mg of collagenase was added to the remaining 125-150 ml of perfusion medium. After 35 min, the liver cells were isolated by the method of Feldhoff et al. (22) with minor modifications (23). The liver was removed, minced gently with scissors, suspended in 50 ml of perfusion buffer (without erythrocytes), and filtered through two layers of muslin. The cells were centrifuged at 50 × g for 10 min in polycarbonate centrifuge tubes in a Dynac table-top centrifuge and washed three times with perfusion medium without erythrocytes, but containing 1 g of bovine serum albumin/100 ml and 2.4 mM CaCl2. All buffers were maintained at 37 °C and equilibrated with O2/CO2 (95:5, v/v). After three washes, cells were suspended in the same buffer containing 3 g of albumin/100 ml to yield 3.0 × 10^6-6.0 × 10^6 cells/ml and were placed in 250-ml polycarbonate Erlenmeyer flasks with a maximum of 60 ml of suspended cells in one flask. After 30 min of incubation at 37 °C with a constant flow of O2/CO2 mixture, the cells were centrifuged again and resuspended at 1:15 dilution in serum-free medium (Hanks-Hepes buffer supplemented with gentamycin [50 μg/ml], 13 mM sodium bicarbonate, and bovine albumin [Fraction V, 0.25%]) to prepare primary cultures of isolated hepatocytes by the method of Kretzien et al. (24) with minor modifications (23). One and one-half ml of this cell suspension (5 × 10^4 cells/ml) was plated in a sterile plastic Petri dish (60 mm) coated with rat tail collagen (100 μg/dish). The culture plates containing hepatocytes were placed in an incubator at 37 °C with an atmosphere of 95% N2/5% CO2. The culture dishes were then rinsed four times with 5 ml of phosphate-saline buffer, pH 7.4, at 25, or 37 °C as indicated in each experiment. Following the desired incubation period, the cells were washed four times with 5 ml of phosphate-buffered saline, pH 7.4. As described previously. Following this incubation period, the cells were washed four times with 5 ml of phosphate-saline buffer, pH 7.4, at 4 °C to separate bound from free insulin. The cells were then treated for 6 min at 4 °C with Hanks-Heps buffers of decreasing pH to remove the fraction of cell-associated 125I-insulin that was not bound.

**RESULTS**

**Effect of pH in the Dissociation of Cell-associated 125I-Insulin and Cellular Integrity**—The dose-response relationship between pH of the incubation medium and the amount of dissociation of cell-associated 125I-insulin (Fig. 1) was investigated by incubating primary cultures of rat hepatocytes with 125I-insulin, 1 × 10^-10 M, in the absence or presence of unlabeled insulin, 1 × 10^-6 M, for 30 min at 25 °C, as described previously. Following this incubation period, the cells were washed four times with 5 ml of phosphate-saline buffer, pH 7.4, at 4 °C to separate bound from free insulin. The cells were then treated for 6 min at 4 °C with Hanks-Heps buffers of decreasing pH to remove the fraction of cell-associated 125I-insulin that was not bound.

![Figure 1](image-url)

**Fig. 1. Effect of pH on removal of cell-associated 125I-insulin (upper panel) and cell protein (lower panel).** Primary cultures of rat hepatocytes were first incubated with 125I-insulin, 1 × 10^-10 M, in the absence and presence of unlabeled insulin, 1 × 10^-6 M, pH 7.4, for 30 min at 25 °C. Cells were washed four times with 5 ml of phosphate-saline buffer, pH 7.4, at 4 °C. Cells were then incubated for 6 min at 4 °C with Hanks-Heps buffer of decreasing pH and the amount of cell-associated 125I-insulin removed by the acid wash and the total protein per plate were determined. The data represent the mean ± S.E. from three different experiments.
After acid wash treatments, cultures were washed with 10 ml of phosphate-saline, pH 7.4, and solubilized with 1 N NaOH. Total cellular protein was then measured by the method of Miller (27). Results showed that total cellular protein remained relatively unchanged in spite of the varying pH of the acid wash indicating that low pH did not damage the cells with resultant intracellular protein release.

Further evidence that the acid wash treatment did not disrupt cellular structure was obtained by incubating hepatocytes with $^{51}$Cr (2.5 µCi/plate) for 16 h at 37 °C. Following the incubation period, the cells were washed four times with 5 ml of phosphate-saline buffer, pH 7.4, at 4 °C and the cells were then incubated in Hanks-Hepes buffer, pH 7.4 or 3.5, at 4 °C. The amount of $^{51}$Cr released into the medium was 2.0 ± 0.7% and 2.6 ± 1.2% (mean ± S.E., n = 3) of the total at pH 3.5 and 7.4, respectively. In addition, electron microscopic study of the hepatocytes exposed to buffers at pH 7.4 or 3.5 for 6 min revealed that this treatment did not change cellular architecture (Fig. 2).

The time course of insulin dissociation by acid pH was analyzed by incubating $^{125}$I-insulin and cultured hepatocytes as previously described and then determining the percentage of $^{125}$I-labeled material removed with time by pH 7.4 and 3.5 at 4 °C. Fig. 3 shows that less than 5% of membrane-bound $^{125}$I-insulin was dissociated in 15 min at pH 7.4 while the majority of insulin dissociated at pH 3.5 (70%) was removed by 5 min. Prolonging the acid wash for up to 15 min did not increase insulin removal. Therefore, for subsequent experiments, 6 min was chosen as an optimal incubation time for membrane-associated $^{125}$I-insulin removal by acidic medium at 4 °C.

**Effect of Temperature on Cell-associated $^{125}$I-Insulin**—The effect of temperature on cell-associated $^{125}$I-insulin and the fraction that is membrane-bound (removed by the acid wash) and internalized (resistant to the acid wash) was studied by incubating hepatocytes with $^{125}$I-insulin, $1 \times 10^{-10}$ M, in the presence and absence of unlabeled insulin, $1 \times 10^{-6}$ M, at 4 and 25 °C (Fig. 4) and 37 °C (Fig. 5). At different intervals indicated in each experiment, cell-associated $^{125}$I-insulin, membrane-bound, and internalized insulin were measured as previously described.

The upper panel of Fig. 4 indicates that at 4 °C the amount membrane-bound. The upper panel of Fig. 1 demonstrates that the percentage of cell-associated $^{125}$I-insulin removed was a function of the pH of the acid wash. Less than 5% of membrane-bound insulin was removed at pH 7.4 and over 80% at pH 4. The fraction of labeled insulin resistant to acid wash may be internalized into a cellular compartment where it cannot be dissociated by acid pH.

That the dose-response relationship of dissociation of membrane-bound insulin is a reflection of the labeled material removed by acid wash and not a reflection of release of cellular protein due to cell destruction is demonstrated in the lower part of the figure.
of cell-associated $^{125}\text{I}$-insulin increased with time reaching a maximum at 120 min and then plateaued for up to 180 min. The great majority of $^{125}\text{I}$-insulin was removed by the acid wash showing that internalized insulin was minimal at 4 °C. Cell-associated $^{125}\text{I}$-insulin at 25 °C was maximum at 60 min and plateaued for up to 120 min (lower panel, Fig. 4) and its concentration was over 100% of that at 4 °C. This was partially due to the fact that at 25 °C approximately 4% of the cell-associated $^{125}\text{I}$-insulin was internalized.

The upper panel of Fig. 5 shows that at 37 °C cell-associated $^{125}\text{I}$-insulin increased rapidly reaching a maximum at 15 min and plateaued for up to 60 min (data shown up to 30 min). Internalized insulin increased linearly for up to 15 min and then plateaued. The lower panel of Fig. 5 demonstrates that the percentage of membrane-bound insulin and that internalized changed reciprocally with time and equilibrated after 15 min. Thus, the cell-associated $^{125}\text{I}$-insulin, at apparent steady state, at 37 °C, was equally made up of membrane-bound and internalized insulin.

**Kinetic State Experiments of Internalized Insulin**—Fig. 5 demonstrates that internalized insulin resistant to the acid wash increased linearly up to 15 min. Therefore, experiments were performed at 10 min to evaluate the effect of a wide range of insulin concentrations on hormone internalization. Cultured hepatocytes were incubated at 37 °C with $^{125}\text{I}$-labeled insulin, $1 \times 10^{-10}$ M, and increasing concentrations of unlabeled insulin, for 10 min. Membrane-bound (removed by the acid wash) was separated from internalized insulin as previously described.

The upper panel of Fig. 6 shows a plot of the velocity of insulin internalization versus the concentration of insulin bound to the membrane which was the substrate for internalization. This linear plot ($r = 0.998, p < 0.001$) demonstrates that the velocity of insulin internalization is nearly proportional to the concentration of membrane-bound insulin indicating that the reaction approximates first order kinetics. Furthermore, when these data are replotted in a double reciprocal plot, a straight line is obtained, as shown in the inset of the upper panel of Fig. 6. A single $K_{\text{m}}$ value of $2.5 \times 10^{-11}$ M for this reaction can be calculated from this plot.

The lower panel of Fig. 6 shows a plot of the velocity of
Insulin Processing by the Liver

Fig. 6. Kinetic analysis of 125I-insulin internalization. Cultured hepatocytes were incubated with 125I-insulin, 1 x 10^{-10} M, with increasing concentrations of unlabeled insulin at 37°C for 10 min. The amount of internalized insulin (resistant to acid wash) and membrane-bound insulin (removed by acid wash) was determined as described in Fig. 4. The upper panel is a plot of the velocity (V) of insulin internalization versus the concentration of insulin bound to the membrane ([S]). The inset is a double reciprocal plot of the same data. The lower panel is a plot of the velocity of insulin internalization versus the concentration of free insulin in the medium. The inset is a double reciprocal plot of the same data. The data represent the mean ± S.E. from three or six different experiments.

insulin internalization versus the concentration of free insulin in the medium. The kinetics of insulin internalization when free insulin rather than bound insulin was considered as substrate for internalization was complex. When these data are replotted in a double reciprocal plot, as shown in the inset of the lower panel of Fig. 6, it appears that KIncrease value of insulin internalization increased with increasing concentrations of insulin. The KIncrease was 7 x 10^{-10} M at lower insulin concentrations and 1.7 x 10^{-9} M at higher insulin concentrations. These values are similar to the KIncrease of the high and low affinity insulin binding sites, respectively (18).

Steady State Experiments of Membrane-bound Insulin—Fig. 5 reveals that cell-associated 125I-insulin at 37°C reached apparent steady state after 15 min of incubation for at least 60 min. Thus, Scatchard analyses of cell-associated 125I-insulin and membrane-bound insulin (removed by acid wash) were performed at 20 min. Hepatocytes were incubated with 125I-insulin, 1 x 10^{-10} M, and increasing concentrations of unlabeled insulin at 37°C for 20 min. Cell-associated 125I-insulin and the amount of membrane-bound insulin were measured as previously described.

Fig. 7 shows (closed triangles) the Scatchard plot of cell-associated 125I-insulin. The plot is curvilinear, consistent with either the negative cooperativity theory (29) or the possibility of multiple binding sites with different affinity for insulin. Also shown is the Scatchard plot of membrane-bound insulin (open circles). This plot parallels and has similar curvilinearity but is displaced to the left. The curvilinearity of the Scatchard analyses of insulin binding data was studied to see if it might be due to negative cooperativity between binding sites. Hepatocytes were incubated with 125I-insulin, 1 x 10^{-10} M, for 2 h at 4°C. Then the free insulin was removed as previously described and the plates were then incubated at 37°C in the presence and absence of unlabeled insulin, 1 x 10^{-6} M, to study the amount of cell-associated 125I-insulin dissociated under either condition. Fig. 8 reveals that a large excess of unlabeled insulin did not increase the dissociation of cell-associated 125I-insulin under the experimental conditions used.

Insulin Degradation Studies—Insulin degradation in the medium and the characteristics of the radioactive material associated with the cells that were removed (membrane-bound) or resistant (internalized) to acid wash at 37°C were analyzed by gel chromatography as described under "Materials and Methods." In each experiment, appropriate amounts of insulin were added containing either unlabeled insulin, 1 x 10^{-10} M, and then Hanks-Hepes buffer, pH 7.4, for 6 min at 4°C. Then, they were exposed to Hanks-Hepes buffer, pH 7.4, for 6 min at 4°C. The amount of cell-associated 125I-insulin (closed triangles) and that removed by acid wash (open circles) was determined as described in Fig. 4. The data represent the mean ± S.E. of three different experiments.

Fig. 8. Dissociation of cell-associated 125I-insulin. Primary cultures of rat hepatocytes were incubated with 125I-insulin, 1 x 10^{-10} M, at 4°C for 2 h. Cells were washed four times with 5 ml of phosphate-saline buffer, pH 7.4, and then Hanks-Hepes buffer, pH 7.4, was added containing either unlabeled insulin, 1 x 10^{-6} M (●—●), or no insulin (○—○). The cells were then incubated at 37°C and at the times indicated the cell-associated 125I activity was determined and expressed as the percentage of initial binding. The data represent the mean ± S.E. from three or the mean of two different experiments.
control plates were prepared that were identical with experimental plates in all respects except that liver cells were omitted. That percentage of degraded insulin present in the control plates was subtracted from that present in the experimental ones to quantitate cell-mediated insulin degradation. Fig. 9 indicates that chromatography of medium, membrane-bound, and internalized fractions revealed three elution peaks. Peak 1 represented material eluting in the void volume, peak 2 co-eluted with intact insulin, and peak 3 was the final degradation products that eluted with or shortly before Na\(^{131}\)I. Each sample of chromatographed material was dissolved in 4 M urea, 1 M acetic acid, and 0.1\% Triton X-100 and subjected to gel filtration on Sephadex G-50 in the same buffer. The inset in the middle panel is a chromatograph of \(^{131}\)I-insulin utilized in these studies.

In Fig. 10, time course of \(^{131}\)I-insulin degradation. At different times, as indicated in the figure, samples from the medium, cell-associated material removed by acid wash, and cell-associated material resistant to acid wash were chromatographed as in Fig. 9. Peaks 1, 2, and 3, as defined in Fig. 9, are shown in the upper, middle, and lower panel, respectively. The data represent the mean ± S.E. from three different experiments.

15% of the total counts by 60 min of incubation.

DISCUSSION

Insulin processing by primary cultures of rat hepatocytes was analyzed with a method used previously to study the processing of other ligands in different tissues (12, 13, 17) based on the ability of acid pH to dissociate surface-bound ligands. This method allows separation of insulin associated with the cell into two components. That removed by acid pH is surface-bound insulin and that resistant to acid pH is internalized insulin. This method was evaluated by a series of experiments. Firstly, it was shown that cellular integrity was not damaged by acidic pH. Three studies showed: 1) unchanged cellular protein/plate following acid treatment (Fig. 1), 2) electron microscopically intact cells at acid pH, and 3) failure of acid pH to release \(^{51}\)Cr incorporated into hepatocytes in a quantity significantly greater than that released at physiologic pH. Secondly, it was shown that membrane-associated insulin removal was a function of pH (Fig. 1). The dose-response curve is a classic illustration of the functional relation of the independent variable to the dependent variable. In addition, it was demonstrated that this dissociation is rapid (Fig. 3). Finally, in any viable biological system there is a
These differences in results could be explained by variations particularly important when "specific insulin binding" at internalization by its inhibition of transglutaminase activity. Fasting causes insulin resistance and a decreased rate of insulin degradation. Insulin binding is a necessary and rate-limiting step of insulin degradation. It has not been known whether insulin has to be internalized prior to degradation or if degradation can take place at or close to its binding site in the plasma membrane. The chemical method used in this study which discriminates between internalized and membrane-bound insulin allowed us for the first time to examine by gel chromatography the nature of the radioactive material in membrane-bound or internalized components (Fig. 9). The nature of the large molecular weight peak (void volume) of the chromatograph has not been definitely identified. In the internalized component this peak increased linearly with time, and represented a significant portion of total counts during the 60 min of incubation (Figs. 9 and 10) contrasted to the small percentage of this peak in the medium or membrane-bound component. It is attractive to postulate that this large molecular weight material could be insulin-receptor complexes as suggested by Saviolakis et al. (41).

The site of insulin degradation is a complex and incompletely understood process. After considerable controversy, the concept of a close relationship between insulin binding and degradation has been well established (18, 34, 35, 42, 43). Insulin binding is a necessary and rate-limiting step of insulin degradation. It has not been known whether insulin has to be internalized prior to degradation or if degradation can take place at or close to its binding site in the plasma membrane. Lysosomal enzymes and glutathione insulin transhydrogenase located primarily in microsomes and insulin protease mainly in cytosol are the three known pathways for insulin degradation. Insulin protease is the only one of these three systems found in fully purified plasma membranes (1). We have shown that in the intact liver cell insulin is degraded both intracellularly and at the plasma membrane (Figs. 9 and 10). The chemical method used in this study which discriminates between internalized and membrane-bound insulin allowed us for the first time to examine by gel chromatography the nature of the radioactive material in membrane-bound or internalized components (Fig. 9). The nature of the large molecular weight peak (void volume) of the chromatograph has not been definitely identified. In the internalized component this peak increased linearly with time, and represented a significant portion of total counts during the 60 min of incubation (Figs. 9 and 10) contrasted to the small percentage of this peak in the medium or membrane-bound component. It is attractive to postulate that this large molecular weight material could be insulin-receptor complexes as suggested by Saviolakis et al. (41).

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ibly, approximately half of the insulin degradation products associated with the cell are found at the plasma membrane. Insulin degradation taking place at the membrane could be explained by the presence of insulin protease in purified plasma membrane (1). The plasma membrane insulin degradation system may have an important function in the termination of insulin biological activity and in the regulation of the fraction of bound insulin available for internalization.

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