The initial interaction of insulin with insulin-sensitive cells probably occurs on the outer face of the plasma membrane. Kono and Barham (1) and Cuatrecasas (2) have shown that trypsin treatment of fat cells or the plasma membranes of fat and liver cells diminishes the binding of \(^{125}\text{I}\)-insulin, and Cuatrecasas (3) has reported that intact \(^{125}\text{I}\)-insulin can be readily recovered following treatment of the bound material with acid. Furthermore, various lines of evidence suggest that this interaction triggers the characteristic biological response. Several workers have reported that trypsin-treated fat cells show diminished insulin responsiveness (2, 4-6), and Freychet et al. (7, 8), Gliemann and Gammeltoft (10), and Simon et al. (11) have shown that the relative abilities of insulin analogues to compete with labeled insulin for insulin binding sites in various systems correspond well to their relative biological potencies. Also, Crofford has demonstrated that the stimulatory effect of insulin on fat cells can be reversed by extensively washing the cells or by the addition of anti-insulin serum (12).

Many insulin-sensitive tissues, notably the liver, also degrade the hormone, and several studies have suggested that insulin degradation occurs intracellularly. The data of Rubenstein et al. (13) and of Mortimore and Tietze (14) suggest that liver homogenates are more active at degrading insulin than is the isolated cyclically perfused liver, and several insulin-degrading enzymes isolated from different tissues have been assigned intracellular localizations (15-18). Intracellular degradation of insulin would necessitate transposition of insulin into the cell, a process requiring prior association of insulin with the plasma membrane. In accordance with this, Crofford et al. have shown that insulin degradation, as well as binding, is diminished in extracts of plasma membranes obtained from trypsin-treated fat cells (19). Although the data of Freychet et al. (20) suggest that the insulin receptor site of rat liver plasma membranes is not itself a site of insulin degradation, they do not preclude the possibility that binding of insulin to a receptor may be the initial step of the degrading process in the intact cell. These considerations prompted us to investigate the relationship between the binding and degradation of insulin in isolated hepatocytes. A preliminary report of portions of this investigation has been presented (21).

**EXPERIMENTAL PROCEDURE**

*These studies were supported by United States Public Health Service Grant AM 15914, University of Chicago Cancer Research Center Project III-B-7, and by the Lolly Coustan Memorial Fund.

†Recipient of a United States Public Health Service Predoctoral Trainee Fellowship (Pediatrics Training Grant HD-00001).
chains of insulin were obtained from Boehringer Mannheim. Guinea pig and chicken insulins were gifts of the Lilly Research Laboratories. A1,B29-suberyl, B1-PTC, A1,B29(Gly)2, and A1,B29(Boc,Gly)2 insulins1 were generous gifts of Dr. D. Brandenburg (Aachen, Germany); their preparation has been described elsewhere (23). Desalanyl-desasparagyl insulin was a generous gift of Dr. F. H. Carpenter (24).

Preparation of 125I-Insulin—Insulin was iodinated using a modification of the method of Freychet et al. (25). Chloramine T (Eastman Kodak, Rochester, N. Y.; 1.3 nmol in 10 μl of 0.3 M sodium phosphate buffer, pH 7.0) was added to 70 μl of buffer containing 2.0 to 2.5 nmol of porcine insulin and 2.0 to 2.3 nmol of carrier-free Na211 (Industrial Nuclear, St. Louis, Mo.). After 2 to 3 min, 300 μl of 4% crystalline bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.) were added, and the mixture was chromatographed over a Bio-Gel P-30 column (1 x 50 cm) equilibrated with 0.01 M Tris-HCl, 0.05 M NaCl, and 0.25% bovine serum albumin (Fraction V; Miles Laboratories) at pH 7.0, at 4°C for purification and determination of the degree of iodination. The central part of the protein peak was collected, diluted 8-fold with phosphate Krebs-Ringer buffer containing 1% crystalline albumin and stored at 20°C for use within 1.5 months. At least 97% of the preparation containing 0.1-0.2 g atom of 125I/mole of insulin (specific activity: 35 to 70 μCi/μg of insulin) co-eluted with the native protein on gel filtration over Sephadex G-50F, eluted with 6 M urea, 1 M acetic acid, and 0.15 M NaCl. The radioactivity measured through polyacrylamide gel electrophoresis (pH 4.5, 8 M urea).

Hepatocyte Isolation—Hepatocytes were isolated from male Sprague-Dawley rats weighing 220 to 250 g and fed ad libitum using a modification2 of the method of Berry and Friend (26). After isolation of the cells, the cell pellet was washed three times in ice-cold Hank’s buffer (GIBCO, Grand Island, N.Y.), pH 7.4, containing nonessential amino acids (GIBCO No. 114), 1 mM pyruvate, 1 mM glutamate, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Calbiochem), 10 mM N-tris(hydroxymethyl)methylglycine (Calbiochem), 1 mM bovine serum albumin fraction V, 0.5 g/100 ml of glucose, and 0.17% sodium bicarbonate, and preincubated for 30 min at 30°C in a Dubnoff introduction buffer under an atmosphere of 85% O2 and 15% CO2. Cells were routinely preincubated in all of our experiments, since this procedure increased and stabilized cell viability during the final experimental incubation period. The cells were then collected and resuspended in minimal essential medium for immediate experimental use.

The cell preparation consisted entirely of hepatocytes by phase and light microscopy. Cell viability, determined by vital dye exclusion, was never less than 95% and was well maintained over the 60-min experimental incubation period. The cells incorporated [%C]leucine into trichloroacetic acid-p precipitable material linearly over 1 hour at 30°C. Furthermore, the addition of 10-8 M porcine insulin maintained the leucine incorporation into the trichloroacetic acid precipitable fraction at 100% to 200% of the well mixed control medium (GIBCO, Grand Island, N.Y.), pH 7.4, containing nonessential amino acids (GIBCO No. 114), 1 mM pyruvate, 1 mM glutamate, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Calbiochem), 10 mM N-tris(hydroxymethyl)methylglycine (Calbiochem), 1 mM bovine serum albumin fraction V, 0.5 g/100 ml of glucose, and 0.17% sodium bicarbonate, and preincubated for 30 min at 30°C in a Dubnoff introduction buffer under an atmosphere of 85% O2 and 15% CO2. Cells were routinely preincubated in all of our experiments, since this procedure increased and stabilized cell viability during the final experimental incubation period. The cells were then collected and resuspended in minimal essential medium for immediate experimental use.

Incubation Procedure—One-milliliter aliquots of cell suspensions containing 0.5 to 1.5 x 106 cells were distributed among silanized 25-ml Erlenmeyer flasks containing 100 μl of the experimental agent or appropriate control buffer and 125I-insulin at final concentrations of 5 x 10-11 to 1 x 10-9 M. The flasks were then gassed with 95% O2/5% CO2, stopped, and placed in a shaking metabolic incubator operated at 88 revolutions/min and maintained at 30°C. At intervals up to 60 min after introduction of the cells, 100- to 200-μl aliquots of well mixed cell suspensions were transferred to 0.5 ml of 0.1% Triton X-100 containing 6 M urea, 3 M acetic acid, and 0.15 M NaCl and stored at -20°C until used for analysis of insulin degradation. These aliquots contained the same ratio of medium to cells as those found in the experimental cell suspension. To determine the amount of 125I-insulin bound to hepatocytes, the remainder of the 1-ml cell suspension was diluted with 14 ml of ice cold Hanks’ buffer containing 1% bovine serum albumin, swirled rapidly, and quantitatively transferred to 15-ml centrifuge tubes. The tubes were centrifuged at 100 x g for 10 min at 3°C, by which time the supernatant was cell-free. At 1-3, 30-sec, and 60-sec periods, aliquots of 1 ml from cell suspension versus time using no fewer than four time points. The mean

1 The abbreviations used are: A1,B29-suberyl, insulin in which the Glyα4 a amino and Lysα5 ε amino groups are intramolecularly cross-linked with an 8-carbon aliphatic dicarboxylic acid; B1-PTC, insulin containing a phenylthiocarbamyl substituent on the Pheε α amino group; A1,B29(Gly)2, insulin containing glycyl substituents in the Glyα4 a amino and Lysα5 ε amino groups of A1,B29(Boc,Gly)2; A1,B29(Boc,Gly)2 contains tert-butyloxycarbonylmethyl substituents on the Glyα4 a amino and Lysα5 ε amino groups.

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3 The abbreviations used are: A1,B29-suberyl, insulin in which the Glyα4 a amino and Lysα5 ε amino groups are intramolecularly cross-linked with an 8-carbon aliphatic dicarboxylic acid; B1-PTC, insulin containing a phenylthiocarbamyl substituent on the Pheε α amino group; A1,B29(Gly)2, insulin containing glycyl substituents in the Glyα4 a amino and Lysα5 ε amino groups of A1,B29(Boc,Gly)2; A1,B29(Boc,Gly)2 contains tert-butyloxycarbonylmethyl substituents on the Glyα4 a amino and Lysα5 ε amino groups.
deviation of ordinate determinations from the regression line was never more than 5% of the range of ordinate values.

RESULTS

Concentration Dependencies of Binding and Degradation

Fig. 1 shows the time course of binding (upper) and of degradation (lower) of 125I-insulin at concentrations of native porcine insulin between 3.3 x 10^{-10} and 9.9 x 10^{-7} M. The binding of 125I-insulin was depressed with increasing concentrations of native insulin. In all of the binding curves, binding reached a steady state by 20 to 30 min at 30^°C. Cell densities greater than 1 x 10^6 cells/ml and at total insulin concentrations less than or equal to 9.0 x 10^{-10}, binding began to decline after 45 min. Therefore, although all data were taken from time courses, steady state binding was measured at 30 min. More than 90% of the iodinated material associated with cells at all times during the incubation period was intact insulin, as judged both by gel filtration and by polyacrylamide gel electrophoresis at pH 4.5.

The velocity of degradation of 125I-insulin was depressed by the addition of native insulin at concentrations between 3.3 x 10^{-10} and 9.9 x 10^{-7} M. The binding of 125I-insulin is shown in Fig. 1, lower). Final degradation products are unmeasurable before 10 min and then increase linearly with time. In all experiments, plots of final degradation product versus time intercepted the abscissa between 7 and 10 min at 30°. This lag was not shortened by the addition of native insulin. At total insulin concentrations less than or equal to 5 x 10^{-10} M, the slight depression in 125I-insulin binding after 45 min was accompanied by a commensurate depression of 125I-insulin degradation velocity. Therefore, at all concentrations tested, the degradation velocity was measured over a time period when degradation velocity was linear (15 to 45 or 60 min). The amount of 125I-insulin bound at steady state as well as the degradation velocities were linearly related to cell density between 0.3 and 2.0 x 10^6 cells/ml.

Fig. 2 shows gel filtration patterns of extracts of incubation...
medium at 15, 30, and 45 min after the addition of 125I-insulin alone. Intact 125I-insulin eluted with native insulin; degradation products eluted with NaCl. The small amount (2 to 3%) of the total applied radioactivity, eluting in the void volume, is attributable to aggregate present in the 125I-insulin preparation. This material was not retained by cells. Labeled material eluting between native insulin and the salt peak, including the A chain which eluted immediately after insulin, was never seen.

**Competition with Insulin, Insulin Analogues, and Other Peptides**—In order to measure the affinities of the binding and the degradative processes for native insulin, various insulin analogues and other naturally occurring insulins, competitive binding experiments were performed. The results, shown in Fig. 3 (upper), suggested that both the binding and the degradation velocity of 125I-insulin was inhibited by 50% at native insulin concentrations of $3.5 \times 10^{-8}$ M. Only about 8% of the iodinated insulin was bound in the presence of $10^{-6}$ M native insulin. The experimental points deviate, particularly at high and low concentrations of native insulin, from a curve expected on the basis of a simple reversible interaction between insulin and a homogeneous group of insulin binding sites with a $K_d$ for insulin of $3.5 \times 10^{-8}$ M. In addition, Scatchard plots of these data were nonlinear (not shown).

The concentrations of insulin analogues necessary for 50% inhibition of binding and degradation velocity of 125I-insulin (apparent $K_d$) were read directly from the same type of competitive binding curve (Fig. 3, upper). In order to express the degree of deviation of degradation velocity from the concentration of 125I-insulin bound at steady state and the 125I-insulin degradation velocity to the same extent (Table III). The cell-free medium from suspensions of cells treated with each concentration of protease, however, did not degrade 125I-insulin to any measurable degree.

**Prebound versus Non-Prebound Insulin**—If, as the above results suggest, insulin degradation velocity is linearly dependent upon the concentration of insulin bound to cells, a given concentration of insulin bound to cells should be more rapidly and extensively degraded than an equivalent concentration of insulin newly exposed to fresh cells. Fig. 4 (upper) shows the time course of dissociation of intact 125I-insulin from cells to which 125I-insulin had bound prior to the experimental incubation, as well as the time course of binding of a similar concentration of 125I-insulin to fresh cells at an identical cell density.

>TABLE I

**Inhibition of 125I-insulin binding and degradation by various insulin analogues**

| Insulin analogue                  | $K_d$ binding* (nm) | $K_d$ degradation velocity* (nm) | Regression coefficient | Mean deviation* | Correlation coefficient |
|----------------------------------|---------------------|----------------------------------|------------------------|----------------|------------------------|
| Porcine insulin                  | 3.52 ± 1.71         | 4.93 ± 2.09                      | 1.00                   | 1.18           | 0.999                  |
| Chicken insulin                  | 4.02                | 5.00                             | 1.10                   | 1.89           | 0.997                  |
| B1-FTC                           | 17.7                | 17.3                             | 1.06                   | 1.08           | 0.999                  |
| A1, B29 (Boc, Gly)$_1$           | 45.3                | 36.0                             | 0.95                   | 2.94           | 0.992                  |
| A1, B29 (Gly)$_2$                | 79.4                | 91.1                             | 0.89                   | 3.73           | 0.990                  |
| Guinea pig insulin              | 95.5                | 148.0                            | 1.11                   | 1.58           | 0.994                  |
| A1, B26 auberyol                 | 112.0               | 112.0                            | 1.05                   | 3.06           | 0.961                  |
| Hagfish insulin                  | 112.0               | 135.0                            | 0.93                   | 1.68           | 0.999                  |
| Proinsulin                       | 123.0               | 104.5                            | 0.79                   | 3.25           | 0.988                  |
| Desalanyl-desasparaginyl insulin | 151.0               | 138.0                            | 1.06                   | 3.06           | 0.996                  |

* Data calculated from plot of per cent degradation velocity versus per cent 125I-insulin bound, as in lower panel of Fig. 3.

* Mean deviation of experimental ordinates from regression line along the ordinate from a plot similar to the one shown in Fig. 3 (lower panel).

* Mean of five experiments ± S.D.
### Table II

**Effect of various peptide hormones on the binding and degradation of \(^{125}\text{I}-\text{insulin}**

Hepatocytes (10^6 cells/ml) were incubated with 1 × 10^{-7} \text{M} \(^{125}\text{I}-\text{insulin} in the presence or absence of the stated hormones at concentrations between 10^{-7} and 10^{-5} \text{M} at 30° for 60 min. Binding and degradation velocity of \(^{125}\text{I}-\text{insulin} were measured as described under "Experimental Procedure." Data is expressed as percentage of \(^{125}\text{I}-\text{insulin} binding and degradation velocity in the absence of other hormones. In some cases, the results are expressed as the mean of two separate estimations ± one-half the range.

| Hormone                        | % control | \(^{125}\text{I}-\text{insulin} bound % control | \(^{125}\text{I}-\text{insulin} degradation velocity % control |
|--------------------------------|-----------|-----------------------------------------------|-------------------------------------------------------------|
| None                           | 100.0     | 100.0                                         | 100.0                                                       |
| Oxytocin                       | 100.0     | 100.0                                         | 100.0                                                       |
| Vasopressin                    | 95.4 ± 2.1| 95.8 ± 2.1                                    | 92.0 ± 17.8                                                 |
| Secretin                       | 99.3 ± 1.8| 92.5 ± 12.5                                   | 102.5 ± 5.8                                                 |
| Bovine growth hormone          | 86.5 ± 13.5| 100.4 ± 4.8                                  | 87.4 ± 4.0                                                  |
| Glucagon                       | 84.1 ± 4.2| 84.3 ± 2.3                                    | 94.3 ± 1.0                                                  |
| Nerve growth factor b          | 100.2     | 105.7                                         | 102.3                                                       |
| Thyroid-stimulating hormone    | 100.4     | 95.2                                          | 102.3                                                       |
| Oxidized A chain               | 99.4      | 102.3                                         | 102.3                                                       |
| Oxidized B chain               | 94.3      | 105.8                                         | 102.3                                                       |
| Somatostatin                   | 94.5 ± 2.2| 94.8 ± 1.5                                    | 100.0 ± 0.1                                                 |

### Table III

**Depression of binding and degradation velocity of \(^{125}\text{I}-\text{insulin following protease treatment of hepatocytes**

Hepatocytes (2 to 3 × 10^6 cells/ml) were incubated in minimal essential medium/bovine serum albumin containing the stated concentrations of trypsin or chymotrypsin at 30° for 20 min (Experiment 2) or 30 min (Experiments 1 and 3). Soybean trypsin inhibitor (30 μg/ml) was then added to the trypsin-treated cells, and the cells (0.5 ml packed volume) were washed three times in 50 ml of ice-cold Hank’s buffer. After resuspension in minimal essential medium/1% bovine serum albumin, the cells were incubated with 1 × 10^{-7} \text{M} \(^{125}\text{I}-\text{insulin as described under "Experimental Procedure."} and \(^{125}\text{I}-\text{insulin binding and degradation velocity were measured.**

| Protease   | \(^{125}\text{I}-\text{insulin} bound μM} | \(^{125}\text{I}-\text{insulin} degradation velocity μM} | % control | % control |
|------------|----------------------------------------|------------------------------------------------------|------------|------------|
| Experiment 1: trypsin | 100.0 | 100.0 | 100.0 | 100.0 |
| 10 | 43.8 | 45.2 | 104.0 | 105.4 |
| 20 | 10.4 | 15.4 | 95.6 | 60.9 |
| Experiment 2: trypsin | 100.0 | 100.0 | 100.0 | 100.0 |
| 5 | 72.2 | 74.0 | 105.0 | 109.0 |
| 10 | 60.8 | 59.5 | 98.5 | 91.0 |
| 15 | 35.7 | 35.3 | 85.0 | 75.0 |
| Experiment 3: chymotrypsin | 100.0 | 100.0 | 100.0 | 100.0 |
| 5 | 74.9 | 74.0 | 100.0 | 100.0 |
| 10 | 52.5 | 56.1 | 95.5 | 92.0 |
| 15 | 28.7 | 33.4 | 85.0 | 76.0 |

between the two experimental conditions at the beginning of the final incubation is that in the one case, all of the \(^{125}\text{I}-\text{insulin is bound to cells, whereas in the other case, none of the \(^{125}\text{I}-\text{insulin is bound} to cells. Fig. 4 (lower) shows the time course of degradation in each case. A comparison of the areas under the two degradation curves shows that the total concentration of degradation products formed from prebound insulin is greater than that formed from a comparable concentration of non-prebound insulin. Furthermore, there is no appreciable time lag in the degradation of prebound insulin at the intervals measured, in contrast to the lag in the degradation of non-prebound insulin (Fig. 4, lower; see also Fig. 1, lower). Furthermore, over the time interval when the amount of prebound insulin is greater than the amount of non-prebound insulin (0 to 30 min; Fig. 4, upper panel), the degradation velocity of the prebound insulin (slope of degradation products versus time, lower panel) is higher than the degradation velocity of non-prebound insulin. In contrast, during the 40- to 60-min time interval when the amount of prebound insulin is less than the amount of non-prebound insulin (upper panel), the degradation velocity of prebound insulin is lower than that of the non-prebound insulin.

Two aspects of the above experiment should be noted. First, when the cell-free medium from the cell suspension which had been incubated without insulin for the duration of the entire experiment was incubated with \(^{125}\text{I}-\text{insulin for an additional hour at 30°}, less than 1.3% of the insulin was degraded; that is, at least 98% of the insulin degradation was attributable to the cells and not to degradative enzymes which had leaked from the cells. Second, more than 95% of the total prebound radioactivity at the beginning of the experimental incubation period eluted with intact insulin on gel filtration. Therefore, the final degradation products were newly formed during the experimental incubation period and do not represent release of preformed degradation products from the cells.

A closer examination of the handling of prebound insulin showed that at least 90% of the iodinated material retained by the cells was intact insulin throughout the final 60-min incubation period (Fig. 5, upper panel). The finding that most of the bound insulin is intact is comparable to the findings of others (20, 27) and suggests that final degradation products are not retained by the cells. The lower panel shows that the early loss of iodinated material from the cells (0 to 15 min) represents release of degradation products as well as dissociation of intact \(^{125}\text{I}-\text{insulin. By 15 min, the amount of intact} \(^{125}\text{I}-\text{insulin has dissociated and rebound. The slight lag in the initial appearance of degradation products in the supernatant contrasts with the immediate release of degradation products from prebound \(^{125}\text{I}-\text{insulin shown in Fig. 4, and is**
A specific dissociation rate constant of insulin, calculated on the basis of the early (up to 10 min) loss of intact $^{125}$I-insulin from the cells (as in Fig. 5, upper panel) was estimated to be $0.0385 \pm 0.0075 \text{ min}^{-1}$ (mean $\pm$ S.D. from eight experiments). However, analysis of the cell-free supernatant (Fig. 5, lower panel) suggests that a significant fraction of the radioactive material dissociating at early times from the cells is iodinated degradation products. In view of this observation, the physical significance of the specific rate constant of dissociation is unclear. It should be emphasized that the cell-free medium in these experiments did not degrade more than 1.5% of the added iodinated insulin, so that more than 98% of the iodinated degradation products formed resulted from degradation by the cells and not from degradation of released intact insulin by the medium.

**Quantitative Relationship between Insulin Binding and Degradation Velocity**—In order to express our results over a wide range of insulin concentrations, we plotted the binding and degradation data in log-log form. Plots of the log total insulin bound or of the log degradation velocity versus log total insulin concentration in the medium were linear over total medium insulin concentrations of $5 \times 10^{-11}$ to $10^{-4} \text{ M}$ (Fig. 6, left and right panels, respectively). Dilutions of $^{125}$I-insulin were used to measure binding and degradation velocity at insulin concentrations lower than $10^{-8} \text{ M}$, whereas final insulin concentrations greater than $10^{-8} \text{ M}$ were achieved by the addition of appropriate concentrations of native porcine insulin. Therefore, the binding at concentrations lower than $10^{-8} \text{ M}$ in Fig. 6 (left) represents primarily binding which is displaceable by $10^{-8} \text{ M}$ native insulin, whereas the binding of $^{125}$I-insulin at concentrations greater than $10^{-8} \text{ M}$ represents primarily binding which is not displaceable by $10^{-8} \text{ M}$ native insulin.

The fact that plots of insulin binding and degradation velocity versus total insulin concentration are parallel (Fig. 6) suggests that degradation velocity and the concentration of bound insulin are related to one another in a simple way. Accordingly, the log of the degradation velocity of total insulin was plotted against the log of the total insulin bound at steady state in the presence of insulin concentrations of $7 \times 10^{-10}$ to $10^{-4} \text{ M}$. As seen in Fig. 7, this plot is linear and therefore can be expressed in the following form:

$$\log \frac{dP}{dt} = \sigma \log (IR) + \log k_{sp}$$

or, taking antilogs,

$$\frac{dP}{dt} = k_{sp} (IR)^{\sigma},$$

where $P$ is the instantaneous total concentration of insulin degradation product; $dP/dt$, the instantaneous degradation velocity; $(IR)$, the concentration of total insulin bound at steady state corrected only for the $^{125}$I-insulin trapped in the cell pellet; $k_{sp}$, the apparent rate constant relating degradation velocity to the amount of insulin bound; and $\sigma$, the reaction order of degradation with respect to total insulin bound at steady state. In a plot of log $dP/dt$ versus log $(IR)$ (equation 1), $\sigma$ is evaluated directly from the slope of the line and was found to be $0.994 \pm 0.082$ (mean $\pm$ S.D. from five experiments), suggesting that degradation velocity is first order with respect to total amount of insulin bound. The log $k_{sp}$ is equal to the log $dP/dt$ when log $(IR)$ is 0, and is $0.030 \pm 0.011 \text{ min}^{-1}$ (mean $\pm$ S.D. from five experiments).

The relationship between insulin degradation velocity and the amount of insulin bound at steady state persisted even at low $^{125}$I-insulin concentrations ($5 \times 10^{-11}$ to $1.6 \times 10^{-8} \text{ M}$), at
FIG. 6. Concentration dependence of insulin binding (left) and degradation velocity (right). Hepatocytes (10^6 cells/ml) were incubated with various dilutions of ^125I-insulin (5 x 10^{-11} to 10^{-9} M) or with ^125I-insulin plus varying concentrations of native porcine insulin (5 x 10^{-10} to 10^{-6} M). Insulin binding and degradation were measured at regular intervals as described under “Experimental Procedure.” Each symbol represents a separate experiment. ^125I-Insulin at various dilutions, □—□, ○—○; ^125I-insulin and various concentrations of native porcine insulin, ■—■, ●—●. The lines were determined by the method of least squares (unweighted). The regression equation of y on x, where y represents the ordinate and x, the abscissa, in Fig. 6 (left) is: y = 0.649x + 5.392. The correlation coefficient is 0.999, and the mean deviation of experimental ordinates from the regression line along the ordinate is 0.014. The regression equation in Fig. 6 (right) is: y = 0.050x + 1.805; the correlation coefficient is 0.994, and the mean deviation of experimental ordinates from the regression line is 0.009.

FIG. 7. Plot of log degradation velocity versus log of the concentration of total insulin bound at total insulin concentrations of 7 x 10^{-10} to 1 x 10^{-6} M from data pooled from two experiments. The order of the dependence of the degradation velocity on the total insulin bound at steady state, determined from the slope of this line, is 0.97. The k_{ap} is 0.027 min^{-1} (see Equation 1). ○—○, Experiment 1; ■—■, Experiment 2.

which only about 7.5% of the bound ^125I-insulin was not displaced by the addition of high concentrations of native insulin. At these low concentrations, a plot of insulin degradation velocity versus the amount of insulin bound is linear and has a slope, equal to the k_{ap}, of 0.0385 min^{-1}, which is within the error of the k_{ap} mentioned above (Fig. 8). The ordinate intercept is -0.0026, not significantly different from 0. A plot of these data as log dP/dt versus log (IR) (not shown here) was linear with slope, equal to 1.08 (correlation coefficient of 0.99), implying a reaction order of 1.

FIG. 8. Dependence of degradation velocity on concentration of insulin bound at ^125I-insulin concentrations of 5 x 10^{-11} to 1 x 10^{-9} M. Cells (1 x 10^6) were incubated with various dilutions of ^125I-insulin. Binding and degradation velocity were estimated as described under “Experimental Procedure.” This figure represents the data pooled from two experiments. The line was determined by the method of least squares. The ordinate intercept is -0.0026; the mean deviation of the experimental ordinates from the regression line along the ordinate is 0.0037. ○—○, Experiment 1; O—O, Experiment 2.

DISCUSSION

We have measured total insulin binding in order to avoid a priori assumptions about the class or classes of insulin binding proteins on the cell surface which might be involved in the degradative process. The linearity of the log-log plot of insulin bound versus total insulin concentration (Fig. 6, left) is in part
a reflection of the inclusion of two different types of insulin binding sites which can be distinguished operationally by their interaction with 
\[ ^{125}I \] insulin in the presence and absence of \( 10^{-8} \) M native insulin. At total insulin concentrations between \( 5 \times 10^{-11} \) and \( 9 \times 10^{-10} \) M, over 90% of the binding of \( ^{125}I \) insulin is inhibited by \( 10^{-8} \) M native insulin; at concentrations greater than \( 10^{-8} \) M, most of the binding of \( ^{125}I \) insulin is not inhibited. However, the observed linearity (Fig. 6) must in addition reflect deviation of the saturable component of insulin binding from the behavior expected on the basis of a simple reversible association between insulin and a binding protein (Fig. 3). The peculiar properties of this association have been implicitly recognized in the literature in the form of attempts to rationalize insulin binding in terms of multiple orders of binding sites (9, 28, 29), insulin-insulin interactions (30), and negative homotropic interactions among binding sites (31). Linear plots of log total insulin bound versus log total insulin concentrations (as in Fig. 6, left) are obtained upon similar analysis of much of the actual data appearing in the literature from a variety of systems (7, 12, 27, 29, 32–35).

In agreement with the data of many others (1, 7, 9, 27, 29, 32–36), treatment of our binding data in the conventional fashion reveals both saturable and nonsaturable components (Fig. 3). The saturable component has an apparent \( K_d \) for insulin (Fig. 3 and Table I) comparable to reported values (1, 33); its relative affinities for insulin analogues, including desaslylsdesasparaginyl insulin (Table I and Appendix 3), are similar to reported values for apparent \( K_d \) and for the relative biological potencies of these analogues (7, 8, 10, 11, 20, 24, 33, 37–42); peptides unrelated to insulin, including A and B chains of insulin, do not inhibit the binding of \( ^{125}I \) insulin to this site (Table II); and the site occurs on the cell surface, as suggested by the protease experiments (Table III and Refs. 1, 2, 4–6). The properties of the nonsaturable site, measured only at high concentrations of native insulin or of insulin analogues, are more difficult to define.

Several aspects of our data suggest that \( ^{125}I \) insulin bound to saturable sites on the plasma membrane is the substrate for insulin degradation. First, at all concentrations tested, native insulin and various insulin analogues inhibit degradation velocity to the same extent that they inhibit \( ^{125}I \) insulin binding and degradation should be extended to include what have been called nonsaturable binding sites as well. For if degradation velocity were dependent only upon binding either to saturable sites or to nonsaturable sites, this plot would not be linear over the entire range of insulin concentrations measured, and its slope would be less than 1 at concentrations greater than \( 10^{-8} \) or less than \( 10^{-4} \) M, respectively. Thus, while there may be a structural or functional differentiation of sites with respect to insulin binding, all of these sites appear to be homogeneous with respect to insulin degradation. Interestingly, Goldstein and Brown have shown that degradation of low density lipoprotein is related to its binding to both saturable and nonsaturable sites on the plasma membranes of cultured fibroblasts (43).

The more rapid degradation of prebound insulin with respect to non-prebound insulin (Fig. 4) is incompatible with a model in which insulin reversibly associates with a receptor protein in an interaction which does not modify the insulin molecule and then is degraded by the cell independently of the receptor. If this were the case, the degradation of prebound insulin would be retarded relative to that of non-prebound insulin because two steps—dissociation and reassociation—would be necessary for the former. Considered alone, the more rapid degradation of prebound insulin is compatible with a model in which pre-bound insulin is released as a slightly modified, presumably less active, species which is then rapidly degraded by the cells in a manner independent of the insulin receptor. Such a scheme would require two recognition sites with similar susceptibilities to protease treatment but with different specificities. While this scheme is consistent with our protease data (Table III), it is inconsistent with our data on the specificities of binding and degradation (Table I and Appendix 3). The simplest model which is compatible with all of our data is that insulin binds to an apparently heterogeneous set of binding sites on the cell surface, some of which are readily saturable and have an apparent \( K_d \) of \( 3.5 \times 10^{-8} \) M. The bound insulin is then either released intact or is subsequently degraded without dissociating from the cell.

Our observations are consonant with the compartmentalization of the binding and degradation of insulin in intact hepatocytes. First, the lag in the initial appearance of iodinated degradation products (Fig. 1) has also been noted by Mortimore and Tietze (14) in cyclic rat liver perfusions with \( ^{125}I \) insulin as well as by the authors following 1-min infusions of \( ^{125}I \) insulin into noncyclically perfused rat livers (44). Our observation that increasing concentrations of insulin do not shorten this lag either in hepatocytes or in perfused liver is consistent with an obligatory translocation of insulin from a binding site to an intramembranous or intracellular degrading site. In contrast, studies with various broken cell preparations (13, 45–49) and isolated enzymes (17, 50–54) do not show a lag. Second, with intact cells, degradation velocity is negligible at temperatures of 0–20°, but rises rapidly between 20 and 30°. 7 In agreement with this, Mortimore has shown that although degradation of insulin by cyclically perfused liver is negligible at 0°, low temperature does not completely inhibit degradation of insulin by liver homogenates (14). It is possible that the rapid rise of insulin degradation velocity between 20 and 30° is similar to the temperature dependence of membrane transport functions in alveolar macrophage and reflects a temperature-dependent membrane phase transition (55). Third, the difference between the apparent affinity of the over-all degradation process for insulin which we report (Fig. 3, Table I) and the \( K_m \) values reported for degradation by isolated plasma membranes (20) and by various isolated insulin-digesting enzymes (17, 48, 54) suggests that an insulin-digesting step, per se, is not rate-limiting in the over-all degradation process. And fourth, the results of the protease experiments (Table III) suggest, but

6 Susan Terris and Donald F. Steiner, manuscript in preparation.
7 Susan Terris and Donald F. Steiner, unpublished observation.
do not directly prove, that an insulin-degrading enzyme is not exposed to proteases at the cell surface. If it were, degradation velocity would probably be more depressed than insulin binding.

Our data do not suggest a specific mechanism whereby insulin binding is related to its degradation, nor do they suggest a particular mode of enzymatic degradation. Barnett and Ball (56) found that insulin stimulates pinocytosis in fat cells. A similar endocytotic mechanism, associated with lysosomal degradation, might account for the dependence of degradation velocity on the total amount of insulin bound by isolated hepatocytes. However, Crofford et al. have observed that, in contrast to extracts of untreated fat cell plasma membranes which both bind and degrade insulin, plasma membrane extracts from trypsin-treated fat cells neither bind nor degrade insulin (19). This finding may indicate that the binding and degradation of insulin are related on a molecular level. The absence of measurable levels of intermediate products of degradation, including iodinated A chains, suggests that degradation, once begun, proceeds rapidly and that degradation intermediates exist only transiently and at low levels. We find the same gel filtration profiles in the vascular outflow of noncyclically perfused livers following a pulse infusion of $^{125}$I-insulin (44), and Izzo et al. have made similar observations on the in vivo degradation of $^{125}$I-insulin by the liver (57). Our finding that degradation velocity is not depressed even at high total insulin concentrations (Fig. 6, right) suggests that the capacity of the hepatocyte for insulin degradation greatly exceeds its binding capacity. In accordance with this possibility, several workers have reported that liver homogenates degrade insulin to a greater extent than do either liver slices or perfused liver (13, 14, 58).

Our findings differ from those of Gammeltoft and Gliemann (33) and of Freychet et al. (20). Gammeltoft and Gliemann (33) reported that the $K_d$ of degradation for insulin differed from the apparent $K_d$ of binding for insulin in isolated fat cells. Although this may be indicative of significant differences in tissue binding mechanisms, there are also important methodological differences in that we have considered total bound insulin while they considered only that portion of the bound insulin that was inhibited by the presence of $10^{-6}$ M native insulin. In addition, they assumed that degradation of insulin by intact cells proceeds in accordance with Michaelis-Menten kinetics, i.e., that an insulin-degrading step is rate limiting for the overall degradation process in intact cells and also that the substrate (insulin) concentration exceeds, by at least a factor of $10^4$, the concentration of the enzyme(s) (Ref. 59, p. 31). It is possible that these assumptions are not applicable to degradation of insulin by intact cells.

Freychet et al. (20) have suggested that insulin degradation and binding by isolated rat liver plasma membranes are unrelated because, whereas the insulin binding site has a low affinity for desalanyl-desasparaginyl insulin and a high affinity for insulin, degradation of both iodinated proteins by plasma membranes was comparable. In addition, desalanyl-desasparaginyl insulin inhibited the degradation of $^{125}$I-insulin as well as did native insulin (cf. our Table I). Several methodological differences may account for our different results. First, Freychet et al. also measured only a portion of the total binding of $^{125}$I-insulin and $^{129}$I-desalanyl-desasparaginyl insulin. Second, they did not directly assess the degradation of $^{125}$I-insulin. Third, there may be functional differences between the intact cell and isolated plasma membranes with respect to insulin degradation. Separate binding and degradation sites occurring in different cell compartments could have different specificities, which become manifest only in broken cell preparations which expose the degrading enzyme(s) directly to unbound substrate. Furthermore, the enzyme(s) whose degrading activity was measured may or may not be that normally involved in insulin degradation. The fact that Freychet et al. observed that degrading activity declined with increasing specific activity of insulin binding, an observation which may be compatible with solubilization of a physiological insulin-degrading enzyme loosely associated with the plasma membrane, is also consistent with the removal of proteases adsorbed during plasma membrane isolation. More work will be necessary to resolve these differences.

The data presented here indicate that the binding of insulin to cells is necessary for its subsequent degradation. However, not all the insulin bound is degraded (Fig. 5, lower). If our estimate of the specific rate constant of dissociation of bound insulin ($k_{-1}$), a value which is in general agreement with those reported in the literature (3, 27, 28, 33, 60), is approximately correct, a rough estimate of the relative amount of bound insulin which is subsequently degraded can be made. Assuming, in accordance with the considerations discussed above, that bound insulin is either released intact or degraded to small degradation products, the fraction of bound insulin which is degraded should be given by the expression: $k_{-1}/k_{-1} + k_{20}$, or 43.8%. Indeed, we have found that about 40% of the total amount of the insulin retained following pulse infusion of $^{125}$I-insulin into noncyclically perfused liver is degraded (44). Although more work is necessary to define in detail the relation between insulin binding and degradation, two immediate implications are clear. First, at physiological insulin concentrations, the specificity of the over-all degradation process in hepatocytes may be governed primarily by the specificity of binding. And, second, as suggested by many workers (19, 61–63), the functional, possibly molecular, link between insulin binding and degradation may perform a regulatory function in terminating the insulin signal.

Acknowledgments—The authors would like to thank Mr. Alan C. Ferber for computer analyses and for his useful advice throughout this study, Drs. Howard S. Tager and John Westley for their critical review of the manuscript, and Adelaide Jaffe and Sandy Stein for their secretarial assistance.

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