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*J Clin Invest.* 1980;**65**(2):461-468. <https://doi.org/10.1172/JCI109689>.

### Research Article

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# Time-Course of Insulin Degradation in Perifused Isolated Rat Adipose Cells

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**ABSTRACT** Isolated fat cells from rat epididymal adipose tissue were preincubated with 50  $\mu$ U/ml (0.33 nM)  $^{125}$ I-insulin at 23°C to enhance binding while retarding degradation. The fat cells were then perifused at that temperature to remove unbound  $^{125}$ I-insulin, and fractions of perfusate were collected each minute. The temperature of the cells in the perfusion chamber was then rapidly increased to 37°C, and perfusion was continued. The fat cells degraded a portion of the bound  $^{125}$ I-insulin measured by loss of immunoprecipitability with excess antisera to insulin. The percentage of degraded  $^{125}$ I-insulin dissociating from the fat cells increased progressively with time at 37°C, and the rate of dissociation of  $^{125}$ I-insulin degradation products showed a first-order dependence on the amount of degraded  $^{125}$ I-insulin bound to the cells. To explain this first-order dependence it is necessary to postulate a "processing" step after binding and before degradation. The first-order rate constant at 37°C is  $0.023 \pm 0.004 \text{ min}^{-1}$ . Fast and slow dissociating components can be resolved from kinetic plots of the dissociation of undegraded  $^{125}$ I-insulin (immunoprecipitable) from the isolated fat cells. The antilipolytic activity of the  $^{125}$ I-insulin on epinephrine-stimulated lipolysis is evident over much of the time-course of dissociation. A model for the degradation of insulin bound to isolated fat cells is discussed.

## INTRODUCTION

Insulin stimulates a variety of metabolic processes such as glucose transport, phosphodiesterase activity, lipogenesis, protein synthesis as well as glycogen

synthesis, and it is also antilipolytic (1). Insulin forms a complex with receptors located on the plasma membrane (2), and Gliemann et al. (3) have presented evidence that at least one of these stimulated processes, lipid synthesis from glucose in isolated fat cells, is regulated solely by the extent of occupancy of the receptors. To explain the multiphasic nature of insulin binding to adipocytes and other cells, heterogeneous receptors (4), negative cooperativity (5), and a variety of other mechanisms (6–8) have been postulated to be involved. However, the binding phenomenon is still not well understood (9).

Roles for the degradation process in initiation (10, 11) and termination (12) of insulin action have been proposed. Terris and Steiner (13) have reported that insulin is bound by hepatocytes and then degraded. In the present paper, the time-course of dissociation of insulin degraded by perifused isolated fat cells is examined. The use of the perfusion method has advantages relative to incubation experiments, e.g., the cells remain viable longer, unbound insulin is continuously removed, and the time-course is easily followed (14, 15).

## METHODS

**Materials.** Reagent grade chemicals were used in all experiments. The perfusing buffer was composed of the following: 0.127 M NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, and 20 mM N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid. The final pH of the perfusing buffer was 7.4 at 23°C, and it contained 1.5 or 4.0% bovine serum albumin when indicated.

Bovine serum albumin, fraction V, lot R59811, was purchased from Reheis Chemical Co., Kankakee, Ill. The bovine serum albumin was prepared as previously described (14) by dialyzing 250 ml of a 40% solution against 4 liters of perfusing buffer at 5°C. The dialysis was changed after 1, 2, and 4 h of dialysis. The dialyzed bovine serum albumin was then diluted to 20% with perfusing buffer, divided into 10-ml aliquots, and frozen.

Crude bacterial collagenase (EC 3.4.24.3) type I, lot CLS,

Dr. Duckworth is the recipient of Research Career Development Award AM 00187-01 from the U. S. Public Health Service.

Received for publication 1 May 1979 and in revised form 17 September 1979.

4N111 was obtained from Worthington Biochemical Corp., Freehold, N. J., and stored at 5°C.

The  $^{125}\text{I}$ -insulin was purchased from Immuno Nuclear Corp., Stillwater, Minn., and further purified by chromatography on a  $0.9 \times 30\text{-cm}$  column of DEAE-cellulose (Whatman DE 52 Whatman, Inc., Clifton, N. J.). The  $^{125}\text{I}$ -insulin (specific radioactivity,  $100\text{--}160 \mu\text{Ci}/\mu\text{g}$ ) was washed and eluted from the column with a concave gradient of ammonium acetate, pH 9.0 (i.e., gradient mixer chamber 1 contained 125 ml of 0.1 M; chamber 2, 125 ml of 0.2 M; chamber 3, 125 ml of 0.3 M; chamber 4, 125 ml of 0.4 M; chamber 5, 125 ml of 0.5 M; and chamber 6 containing 1.0 M ammonium acetate). Fractions containing 4 ml of  $^{125}\text{I}$ -insulin were collected in tubes already containing 1 ml of 20% bovine serum albumin in perfusing buffer. The first of two major peaks of  $^{125}\text{I}$  counts was eluted between 200 and 220 ml of the ammonium acetate gradient. These fractions were then combined and dialyzed against 500 ml of perfusing buffer containing 4.0% bovine serum albumin with changes after 1, 2, and 4 h.

**Preparation of isolated fat cells.** The cells were prepared from the epididymal adipose tissue of 150–200-g Holtzman rats as described previously (14). In this procedure 1.0 g of minced adipose tissue was incubated with 2.0 ml of collagenase solution for 45 min at 37°C by gentle shaking. The collagenase solution was prepared immediately before use by dissolving 5.0 mg of collagenase in 2.0 ml of perfusing buffer containing 4.0% bovine serum albumin. The cells were then centrifuged for 2 min at 200 g in plastic tubes and washed twice in perfusing buffer containing 4.0% bovine serum albumin. Finally, the cells were filtered through polyester silk. Aliquots of cells were then removed for cell counts and for incubation with  $^{125}\text{I}$ -insulin.

**Perfusion of isolated fat cells.** The perfusion chamber was a disposable 1-cm<sup>3</sup> Stylex tuberculin syringe (Pharmaseal, Glendale, Calif.) mounted in a water jacket. As previously described (14–17), the isolated fat cells float to the top of the chamber, i.e., syringe, during an experiment and a peristaltic pump is used to continuously pump perfusing buffer containing 1.5% bovine serum albumin down through the fat cells. During perfusion the cells were continuously stirred and bathed by the downward stream of fresh medium. The force of the downward flow of perfusing medium (1.0 ml/min) on the cells was balanced by the upward buoyant force exerted by the fat cells, and the stream did not carry the cells out of the perfusion chamber.

The perfusion chamber was thermostated as described previously (14) except that a PK circulating pump (Fisher Scientific Co., Pittsburgh, Pa.) was used to rapidly circulate water from a temperature bath. To monitor and record the temperature of the cells in the perfusion chamber, a system was developed by Kenmac Corp., Memphis, Tenn. In measuring the temperature the direct current output of a hypodermic thermistor (Cole-Parmer Instrument Co., Chicago, Ill.) was recorded by a strip-chart recorder. The thermistor was calibrated at 37°, 25°, and 0°C before use and was accurate to 0.5°C.

At the beginning of the experiment an aliquot of cells was incubated for 20 min at 23°C with 50  $\mu\text{U}/\text{ml}$   $^{125}\text{I}$ -insulin in perfusing buffer containing 4.0% bovine serum albumin. During this time 0.7–1.2 million cells were loaded into the perfusion chamber. For the next 20 min the fat cells were perfused with perfusing buffer containing 1.5% bovine serum albumin at 23°C to remove unbound  $^{125}\text{I}$ -insulin. Fractions of perfusate were collected at 1-min intervals and immediately cooled to 0°C. Perfusion was then stopped and the infranate containing no cells was carefully drained from the perfusion chamber into tube 21. The fat cells and a small amount of perfusing buffer remained in the perfusion

chamber. Perfusing buffer plus 1.5% bovine serum albumin at 37°C was then added to the cells in order to shorten the time required to bring them to 37°C. Perfusion was then reinitiated with perfusing buffer plus 1.5% bovine serum albumin at 37°C. In this procedure the perfusion chamber was reproducibly thermostated at 37°C from 25 min until the end of the experiment. Fractions of perfusate were again collected at 1-min intervals from 24 to 50 min or longer. As appropriately noted, in some experiments perfusion was continued at 23°C without changing the temperature, and fractions were sometimes taken at 2-min intervals. The samples were immediately placed on ice as they were collected.

**Determination of intact  $^{125}\text{I}$ -insulin by immunoprecipitation.** Degradation of insulin can be monitored by the loss of precipitability with excess antibody to insulin (11, 18, 19). Undegraded  $^{125}\text{I}$ -insulin in 0.8 ml of the perfusate fractions was routinely measured by the immunoprecipitation method of Desbuquois and Aurbach (20) using polyethylene glycol to aid precipitation of the insulin-antibody complex. The samples were spun at 1,000 g for 45 min at 5°C. The pellets and supernates were counted in a Packard Autogamma spectrometer, model 5230 (Packard Instrument Co., Inc., Downers Grove, Ill.). The purified  $^{125}\text{I}$ -insulin was 87–94% immunoprecipitable by this procedure. In all experiments the values reported for the percentage of undegraded  $^{125}\text{I}$ -insulin are corrected for <100% immunoprecipitability. This is done by dividing the experimental value by the fraction precipitated in the control experiment in which the  $^{125}\text{I}$ -insulin was not exposed to degradation (i.e., 0.87–0.94).

**Assay of insulin-degrading activity in perfusate fractions.** Some of the perfusate fractions were tested for the presence of insulin-degrading activity by incubating 0.10 ml of the perfusate fraction with 0.10 ml of buffer containing bovine serum albumin and  $^{125}\text{I}$ -insulin for 2 h at 37°C. The final concentration of intact  $^{125}\text{I}$ -insulin added to the incubation mixture was 25  $\mu\text{U}/\text{ml}$  (0.17 nM), and the added counts were more than 50 times greater than the total counts in the perfusate fraction before the incubation. After incubation 0.8 ml of the perfusing buffer-bovine serum albumin was added and the percentage of intact  $^{125}\text{I}$ -insulin in each sample was determined by immunoprecipitation or by precipitation with an equal volume of 10% trichloroacetic acid followed by centrifugation at 800 g for 10 min at 5°C (21).

**Preparation of liver cell membranes.** Plasma membranes from liver cells were prepared from 100-g male Holtzman rats by the method of Neville (22).

**Determination of binding of  $^{125}\text{I}$ -insulin to plasma membranes from liver cells.** The binding was measured as described by Duckworth (19) in which  $\sim 0.05 \text{ nM}$   $^{125}\text{I}$ -insulin was incubated for 30 min at 30°C with 120  $\mu\text{g}$  of plasma membranes in a total volume of 0.30 ml. The incubation was done in the presence and absence of a final concentration of  $1 \times 10^{-6} \text{ M}$  unlabeled insulin to determine nonspecific binding (i.e., binding in the presence of a high concentration of unlabeled hormone). The Tris-Ringer medium (25 mM Tris, 120 mM NaCl, 2.5 mM KCl, 1.2 mM  $\text{MgSO}_4$ ) contained 0.5% bovine serum albumin, pH 7.4. Duplicate 100- $\mu\text{l}$  aliquots of the incubation mixture were spun at 17,000 g for 5 min at 4°C in microfuge tubes. The pellets were then counted to determine binding.

**Lipolysis assay.** Lipolysis was monitored by analysis of glycerol which was released into the perfusate fractions by the fat cells during stimulation by 0.50  $\mu\text{M}$  epinephrine. The glycerol concentration was determined by the method of Chernick (23) which measures the fluorescence of NADH produced by the coupled enzyme reactions catalyzed by glycerokinase and glycerol-3-phosphate dehydrogenase (14, 16).

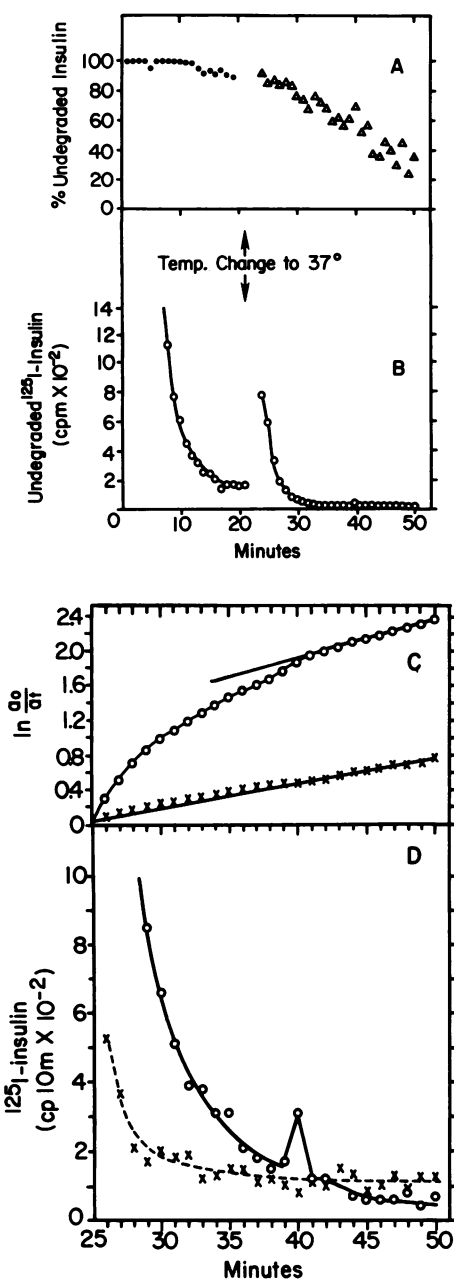


FIGURE 1 Plots of the time-courses of dissociation of undegraded (immunoprecipitable) and degraded  $^{125}\text{I}$ -insulin from perfused, isolated fat cells. The fat cells were preincubated with  $50 \mu\text{U/ml}$  ( $0.33 \text{ nM}$ )  $^{125}\text{I}$ -insulin at  $23^\circ\text{C}$  for 20 min, perfused with buffer containing 1.5% bovine serum albumin at a rate of  $1.0 \text{ ml/min}$  for 20 min more, and then perfused at  $37^\circ\text{C}$ . (A) A plot of the percentage of undegraded  $^{125}\text{I}$ -insulin in the perfusate fractions with time during perfusion at  $23^\circ\text{C}$  ( $\bullet$ ) and then at  $37^\circ\text{C}$  ( $\Delta$ ). (B) A plot of counts per minute in undegraded  $^{125}\text{I}$ -insulin in the perfusate vs. time. (Right, panel C) A plot of  $\ln$  (total counts per 10 min in undegraded  $^{125}\text{I}$ -insulin at 25 min/total counts per 10 min in undegraded  $^{125}\text{I}$ -insulin ( $\circ$ ) remaining with the fat cells at any given time) vs. time, and an analogous plot for degraded  $^{125}\text{I}$ -insulin ( $\times$ ). The counts due to either degraded or

Kinetic analysis of data. Rate constants for first-order reactions were determined from the slopes of plots of  $\ln a_0/a_t$ , in which  $a_t$  is the concentration of component  $a$  at time 0 or  $t$ , vs. time (24).

**Analysis of  $^{125}\text{I}$ -labeled material in isolated fat cells after perfusion.** Immediately after the last perfusion fraction was collected, the fat cells were removed from the perfusion chamber and frozen. The cells were extracted with 0.1% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) containing 3 M acetic acid and 6 M urea. The intact  $^{125}\text{I}$ -insulin and degradation products in the extract were then separated by the method of Terris and Steiner (13) by chromatography on a  $0.9 \times 60\text{-cm}$  column of G-50 Sephadex (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, N. J.) by elution with a solution containing 1 M acetic acid, 6 M urea, and 0.15 M NaCl. Fractions containing 1.0 ml of eluate were collected, counted, and compared with the position of elution of intact  $^{125}\text{I}$ -insulin.

## RESULTS

**Dissociation of degraded and undegraded  $^{125}\text{I}$ -insulin during perfusion of isolated fat cells.** Isolated fat cells were incubated with  $50 \mu\text{U/ml}$  ( $0.3 \text{ nM}$ )  $^{125}\text{I}$ -insulin, which is within the physiological concentration range of insulin, for 20 min at  $23^\circ\text{C}$ . The cells were then perfused, and the time-courses of dissociation of undegraded and degraded insulin from the cells were examined. Degradation of insulin was determined by the loss of immunoprecipitability in the presence of excess antiserum to insulin (11, 18–20). In panel B of Fig. 1 the immunoprecipitable  $^{125}\text{I}$ -insulin in the various 1.0-ml fractions of perfusate is plotted against the time after perfusion was initiated at  $23^\circ\text{C}$ . After 21 min the temperature of the fat cells in the perfusion chamber was increased, and after 25 min it had stabilized at  $37^\circ\text{C}$  as indicated by a hypodermic thermistor inserted into the top of the chamber in some experiments. During perfusion at  $23^\circ\text{C}$   $^{125}\text{I}$ -insulin was removed that was not bound by the cells. In some experiments the cells were washed by centrifugation and then loaded into the perfusion chamber in lieu of the 20-min perfusion at  $23^\circ\text{C}$ . This procedure required 16–18 min and caused no significant difference in the experimental results.

After removal of the unbound  $^{125}\text{I}$ -insulin by perfusion at  $23^\circ\text{C}$ , the temperature was increased to  $37^\circ\text{C}$

undegraded  $^{125}\text{I}$ -insulin bound to the cells at any time,  $t$ , were determined indirectly from the perfusate fractions by the immunoprecipitation method assuming that all of the degraded  $^{125}\text{I}$ -insulin was present shortly after the temperature change to  $37^\circ\text{C}$ . Rate constants were determined from the slopes of the linear portions of the plots. (D) A plot of counts per 10 min in undegraded or degraded  $^{125}\text{I}$ -insulin in the fractions collected during perfusion of isolated fat cells at  $37^\circ\text{C}$  vs. time. Control experiments were run that contained no cells. When compared with experiments in which fat cells were present, only 1–5% of the counts were observed in the controls after 32 min and only undegraded  $^{125}\text{I}$ -insulin was detected.

causing an increase in the rates of dissociation and degradation of intact insulin (19, 25, 26). It is evident from panel A of Fig. 1 that the percentage of  $^{125}\text{I}$  label dissociating from the isolated fat cells as degraded insulin increased progressively with time so that by 50 min  $\sim 70\%$  was in a degraded form. It should be noted that before exposure to fat cells the purified  $^{125}\text{I}$ -insulin used in these experiments was 87–94% immunoprecipitable with antiinsulin antiserum by the procedure of Desbuquois and Aurbach (20). This was corrected to 100% in Figs. 1 and 3 as described in Methods. The  $^{125}\text{I}$ -insulin was 94–96% precipitable with an equal volume of 10% trichloroacetic acid, and its antilipolytic effect on fat cells is evident in Fig. 2. The specific and nonspecific  $^{125}\text{I}$ -insulin binding to liver plasma membranes was 14 and 18%, respectively, which is comparable to the respective values of 8.8–20% and 10.8–26% reported by others in similar determinations (27–29). The amounts of undegraded and degraded insulin appearing in the perfusate fractions from 25 to 50 min is plotted as a function of time in Fig. 1 D. Because some  $^{125}\text{I}$  counts were still associated with the fat cells when

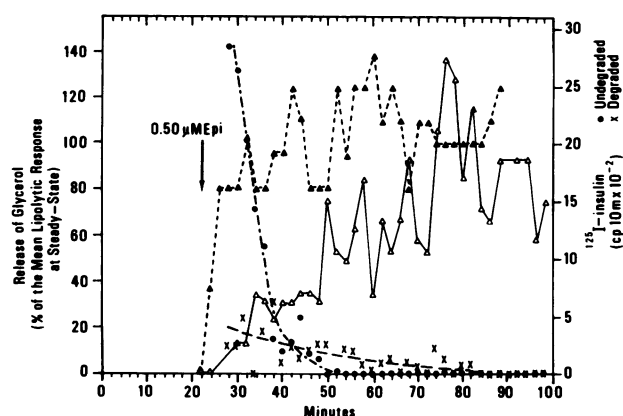


FIGURE 2 Plots of the time-courses of dissociation of  $^{125}\text{I}$ -insulin (undegraded or degraded) and glycerol output from perfused, isolated fat cells during epinephrine-stimulated lipolysis. The fat cells were preincubated for 20 min at  $23^\circ\text{C}$  in perfusing buffer containing 1.5% bovine serum albumin with or without  $50\ \mu\text{U/ml}$  ( $0.33\ \text{nM}$ )  $^{125}\text{I}$ -insulin. The cells were then perfused at  $23^\circ\text{C}$  with the buffer-serum albumin at a rate of  $1.0\ \text{ml/min}$  for 20 min more. Next, the cells were perfused at  $37^\circ\text{C}$  with buffer-serum albumin containing  $0.50\ \mu\text{M}$  epinephrine bitartrate (arrow). The counts per 10 min for immunoprecipitable i.e., undegraded (●) and degraded (×)  $^{125}\text{I}$ -insulin in the perfusate fractions are plotted vs. time. The time-courses for glycerol released are plotted for cells preincubated with  $^{125}\text{I}$ -insulin (Δ) or no insulin (▲). The concentration of glycerol in the perfusate fractions that is released per  $1.0$  million cells as a result of stimulation by epinephrine is determined by subtracting that resulting from basal lipolysis and is expressed as a percentage of the mean steady-state glycerol concentration obtained at 74–88 min as a result of stimulated lipolysis. The steady-state glycerol concentrations were  $10$  and  $7\ \mu\text{M}$  with and without insulin, respectively.

perfusion was terminated, the cells were immediately frozen. The cells were later thawed and extracted with a solution of 0.1% Triton X-100, 3 M acetic acid, and 6 M urea. When the extract was then chromatographed on G-50 Sephadex (see Methods), none of the  $^{125}\text{I}$  counts was eluted in a manner that corresponded to undegraded  $^{125}\text{I}$ -insulin.

In several experiments, aliquots of perfusate fractions collected at various times were tested for insulin-degrading activity. Results of a typical experiment given in Table I show that the degrading activity released at  $37^\circ\text{C}$  by the cells into the perfusate was negligible. Therefore, the  $^{125}\text{I}$ -insulin was degraded while the insulin was bound to the fat cells. Kinetic plots of the degraded and undegraded insulin that dissociated from the adipocytes are shown in panel C of Fig. 1. The plotted points were obtained indirectly by the immunoprecipitation assay of degraded and undegraded  $^{125}\text{I}$ -insulin in the various perfusate fractions. By assuming that all of the degraded  $^{125}\text{I}$ -insulin associated with the cells was present shortly after the temperature change to  $37^\circ\text{C}$ , the rate of dissociation of the degraded  $^{125}\text{I}$ -insulin can be shown to display a first-order dependence on bound degraded  $^{125}\text{I}$ -insulin (Fig. 1C, lower curve). The rate constant obtained from the plot is  $0.023 \pm 0.004\ \text{min}^{-1}$  (mean  $\pm$  SEM from five experiments).

TABLE I  
Insulin-Degrading Activity in the Perfusate Fractions

Time of collection of perfusate*	Percent degraded of $^{125}\text{I}$ -insulin in perfusate which dissociated from perfused fat cells	Percent degraded in 2 h of $^{125}\text{I}$ -insulin added to the perfusate fraction for the incubation assay of insulin-degrading activity†§
min		
0 <sup>  </sup>	—	$5.4 \pm 0.3$
25	15	$5.6 \pm 0.1$
31	24	$5.3 \pm 0.2$
41	58	$5.6 \pm 0.1$
51	73	$5.5 \pm 0.1$
56	74	$5.2 \pm 0.1$
Isolated fat cells¶	—	$21.0 \pm 0.1$

\* The isolated fat cells were perfused as described in the legend of Fig. 1.

† In the assay of insulin degrading activity  $^{125}\text{I}$ -insulin was added to give a final concentration of  $0.17\ \text{nM}$ , and the perfusate fractions were incubated for 2 h at  $37^\circ\text{C}$ . The percent degradation was determined by trichloroacetic acid precipitation. The  $^{125}\text{I}$ -insulin counts added for the assay were  $>50$  times greater than the total counts in the aliquot of perfusate before the assay.

§ Average of two values  $\pm$  one-half the range.

<sup>||</sup> Perfusing buffer that was not exposed to isolated fat cells.

¶  $180,000$  cells/ $0.20\ \text{ml}$  during incubation.

The first-order plot (Fig. 1 C, upper curve) of the dissociation of undegraded insulin from isolated fat cells at 37°C was obtained in a manner similar to that for dissociation of degraded insulin. The plot appears biphasic, and only a portion of the faster phase can be accounted for by the washout of unbound insulin that accumulated while changing the temperature. Because the  $t_{1/2}$  for the washout of the chamber is 1.2 min (14), after 32 min (7 min of perfusion after changing the temperature) <2% of the unbound  $^{125}\text{I}$ -insulin accumulated during the temperature-changing procedure would have remained in the chamber. So, at 37°C there are at least two processes for dissociation of undegraded insulin from the cells, i.e., one process with a dissociation rate constant,  $k_d$ , of  $0.071 \pm 0.018 \text{ min}^{-1}$  (mean  $\pm$  SEM from four experiments) and one with a larger rate constant.

The glycerol release is compared in Fig. 2 for perfusion experiments in which lipolysis was stimulated by  $0.50 \mu\text{M}$  epinephrine in fat cells that had been preincubated either with or without  $50 \mu\text{U/ml}$   $^{125}\text{I}$ -insulin. Some significant antilipolytic activity is still evident in the time period 54–74 min ( $P < 0.001$ ). Thus, the regulatory effect of the labeled hormone is observable for much of the time during which dissociation of degraded and undegraded  $^{125}\text{I}$ -insulin is monitored (also plotted in Fig. 2).

*The time-course of dissociation of degraded and undegraded  $^{125}\text{I}$ -insulin at 23°C.* In some experiments perfusion was continued at 23°C without a change to 37°C. A plot of the appearance of undegraded  $^{125}\text{I}$ -insulin in the perfusate fractions vs. time is shown in the lower curve of Fig. 3. A higher percentage of undegraded  $^{125}\text{I}$ -insulin dissociating from the isolated fat cells at 23°C compared with that at 37°C is evident from the upper curve in Fig. 3.

## DISCUSSION

Knowledge of the fate of insulin in the cell is essential in understanding the mechanism of its regulatory action. It is, therefore, appropriate to examine the interrelationship of the time-courses of the dissociation and degradation of insulin bound to perfused isolated fat cells. In these experiments  $^{125}\text{I}$ -insulin was incubated with the isolated fat cells at a lower temperature to enhance binding and minimize degradation (19, 25, 26). Unbound  $^{125}\text{I}$ -insulin was then removed from the cells during perfusion at the lower temperature. Next, the temperature was increased and dissociation of undegraded and degraded  $^{125}\text{I}$ -insulin from the cells was observed during perfusion of the cells at 37°C.

The perfusion method is particularly suited to experiments that are designed to study the time-course of dissociation because (a) it is possible to monitor cell-bound  $^{125}\text{I}$ -insulin or its derivatives

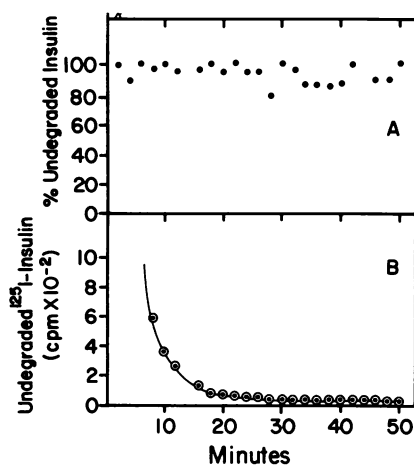


FIGURE 3 Plots of the time-courses of dissociation of undegraded (immunoprecipitable) and degraded  $^{125}\text{I}$ -insulin from perfused isolated fat cells at 23°C. The fat cells were preincubated with  $50 \mu\text{U/ml}$  ( $0.33 \text{ nM}$ )  $^{125}\text{I}$ -insulin at 23°C for 20 min and then perfused at 23°C with buffer containing 1.5% bovine serum albumin at a rate of 1.0 ml/min. (A) A plot of the percentage of undegraded  $^{125}\text{I}$ -insulin in the perfusate fractions with time during perfusion (●). (B) A plot of counts per minute in undegraded  $^{125}\text{I}$ -insulin in the perfusate vs. time (○). Control experiments were run that contained no fat cells. Only undegraded  $^{125}\text{I}$ -insulin was detected in the controls, and when compared with experiments in which cells were present, <9% of the counts were observed after 12 min. After 32 min, no counts were detected in the perfusate from control experiments.

minute by minute as they dissociate from the cells, and (b) kinetic complications as a result of the rebinding of the  $^{125}\text{I}$ -labeled material are circumvented. The negligible release of insulin-degrading activity into the perfusate by these cell preparations further simplifies interpretation of the experimental results (Table I). The results plotted in Fig. 1, therefore, indicate that the fat cells degrade a portion of the bound insulin, and both degraded and undegraded insulin subsequently dissociate from the cells. Using a different experimental approach, Terris and Steiner (13) concluded that insulin is bound and then degraded by hepatocytes. However, in contrast to their results obtained with hepatocytes, the experiments with perfused isolated fat cells indicated that the rate of appearance of insulin degradation products in the perfusate does not show a first-order dependence on the concentration of bound insulin (plot not shown). Instead, the rate is dependent on the concentration of degraded insulin bound to the cells with a first-order rate constant of  $0.023 \pm 0.004 \text{ min}^{-1}$  (mean  $\pm$  SEM from five experiments) at 37°C.

The rate constant of  $0.071 \pm 0.018 \text{ min}^{-1}$  (mean  $\pm$  SEM from four experiments) obtained at 37°C for the dissociation of undegraded  $^{125}\text{I}$ -insulin from the fat cells, agrees well with the value  $0.070 \text{ min}^{-1}$  reported by

Gammeltoft and Gliemann (30). The multiphasic nature of dissociation of undegraded insulin is consistent with results reported by Kahn et al. (4) with liver plasma membranes and by Olefsky and Chang (31) who used centrifugal methods for separating the unbound insulin from adipocytes. Heterogeneity in the receptor site population (4) and, more recently, negative cooperativity, which results in an increase in the rate of dissociation with an increase in bound insulin (5), have been postulated to explain the multiphasic character of dissociation. However, the negative cooperativity hypothesis has been questioned on the bases of methodology and alternative interpretation of results (6–9). It should be noted that in perfusion experiments there is a negligible accumulation of unbound insulin in the medium that surrounds the fat cells. Therefore, a potential effect of unbound insulin on the rate of dissociation of bound insulin from isolated fat cells is not permitted to occur in these experiments. Such an effect of unbound insulin has been reported by Pollet et al. (9) with human lymphocytes under different experimental conditions.

The experimental results are interpretable with the model shown in Fig. 4 in which insulin ( $I$ )<sup>1</sup> binds to a receptor ( $R$ ) on the exterior surface of the plasma membrane and forms a reversible complex ( $I \cdot R$ ). The rate of dissociation of degraded insulin ( $dI$ ) from the isolated fat cells shows a first-order dependence on the concentration of bound degraded insulin. To explain this dependence, formation of another type of the insulin-receptor complex ( $I \cdot R$ ) is postulated that is in a temperature-dependent equilibrium with  $I \cdot R$ . When the temperature is changed,  $k_{-2}$ , which is very small at 23°C, becomes much larger at 37°C. Such a change in the conformation of a hormone-receptor complex has previously been postulated by Williams and Lefkowitz (32) for the epinephrine complex with the  $\beta$ -adrenergic receptor of frog erythrocytes. Therefore, after the temperature change to 37°C an inconsequential amount of the insulin-receptor complex,  $I \cdot R$ , would be expected to convert to  $I \cdot R'$  or  $[I]$  through  $I \cdot R$  in these experiments, i.e.,  $k_{-2} \gg k_3, k_5$ . Because the isolated fat cells were perfused with medium containing no insulin and the unbound insulin ( $I$ ) is carried away in the perfusate as it dissociates from the cells, the  $k_{-1}$  step (in Fig. 4) is irreversible and  $I \cdot R$  dissipates. Under physiological conditions,  $I \cdot R$  would presumably attain a steady state with extracellular circulating insulin and would achieve a relatively small but significant flux to  $I \cdot R'$  and  $[I]$  through  $I \cdot R$ .

According to the model in Fig. 4, as  $I \cdot R$  is formed a portion of it is predetermined for degradation by

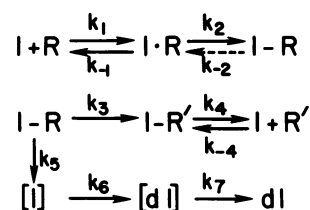


FIGURE 4 A model explaining the kinetics of degradation of  $I$  and dissociation of  $dI$ .  $R$  and  $R'$  are receptors for insulin in the isolated fat cell.

reaction 5 which produces  $[I]$ .  $[I]$  designates either a compartmentalized or bound form of insulin which is subsequently degraded to  $[dI]$ , degraded insulin, which is still associated with the cell. In step 7 the  $[dI]$  dissociates from the cell. Because of reactions 2 and 5 the rate of appearance of degraded insulin in the perfusate shows a first-order dependence on either  $[I]$  or  $[dI]$ , i.e., ultimately determined as  $dI$ . The rate constant of  $0.023 \pm 0.004 \text{ min}^{-1}$  determined for dissociation of  $dI$  would then correspond to either  $k_6$  or  $k_7$ , i.e., the smallest  $k$ . Although dissociation of degraded insulin from isolated fat cells is a single first-order process, dissociation of undegraded insulin at 37°C is multiphasic and changes to a slower process which is evident between 40 and 50 min (Fig. 1 C), i.e.,  $k_4 < k_{-1}$ . The principal features of the model in Fig. 4 are that insulin bound by the fat cell can (a) dissociate as undegraded insulin from the  $I \cdot R$  complex, (b) dissociate more slowly as undegraded insulin from  $I \cdot R'$ , or (c) be channeled toward degradation by step 5 and finally dissociate from the cell as degraded insulin. The model readily accounts for the observed increase with time in the percentage of degraded insulin dissociating from isolated fat cells which has been previously reported by our laboratory (33) and by Olefsky and Chang (31).

Another model can also be considered in which insulin that will be degraded is bound irreversibly to the cell at a different binding site from insulin that eventually dissociates from the cell as undegraded insulin. The irreversibility of the binding serves to make the degradation pathway independent of the dissociation kinetics of undegraded insulin. The data presented in this paper cannot be used to distinguish between this model and the one in Fig. 4. It should also be pointed out that the data on dissociation of undegraded insulin are insufficient to rule out a complex dissociation process involving negative cooperativity or a combination of negative cooperativity with multiple sites (31).

The model presented in Fig. 4 is the simplest general model compatible with the experimental results obtained with perfused isolated fat cells. In this postulated mechanism, the insulin bound to the fat cell is processed (reactions 2 and 5) and then degraded.

<sup>1</sup> Abbreviations used in this paper:  $dI$ , degraded insulin;  $I$ , insulin;  $R$ , receptor.

This is similar to the mechanisms proposed by Goldstein and Brown (34) for degradation of the low density lipoprotein-cholesterol complex, by Carpenter and Cohen (35) for degradation of epidermal growth factor, by Neumann and Bernhardt (36) for dissipation of acetylcholine, and by Knight and Klee (37) for degradation of enkephaline. Processing may be translocation across the plasma membrane or within the plane of the membrane. If processing for insulin is translocation of the insulin-receptor complex across the plasma membrane of the fat cell, it would be consistent with evidence reported by Kahn and Baird (38). They found a progressive decrease, with time, in the ability of excess insulin, trypsin, and mild acid treatment to enhance the dissociation of  $^{125}\text{I}$ -insulin bound to adipocytes. They have interpreted their results as indicating a time-dependent compartmentalization of the bound  $^{125}\text{I}$ -insulin. Schlessinger et al. (39) have reported internalization of fluorescent analogues of insulin in fibroblasts. Carpentier et al. (40) have interpreted their autoradiographic data from experiments using  $^{125}\text{I}$ -insulin to mean penetration of some labeled material into lymphocytes. Goldfine and Smith (41) and Bergeron et al. (42) have concluded that insulin exerts a direct effect inside the cell, whereas Kono et al. (43) have suggested that an energy- and temperature-dependent translocation of insulin within the plane of the membrane is important in the stimulation of sugar transport by insulin.

#### ACKNOWLEDGMENTS

We are grateful for the skilled technical assistance of Ms. Janice New.

This work was supported by grant AM 18022 from the National Institutes of Health, a grant from the Juvenile Diabetes Foundation, and grants MRIS 1942 and 8036 from the Veterans Administration.

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