Retention and degradation of 125I-insulin by perfused livers from diabetic rats.

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Research Article

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Retention and Degradation of ^{125}I-Insulin by Perfused Livers from Diabetic Rats

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ABSTRACT The retention and degradation of insulin by isolated perfused liver have been examined. Noncyclically perfused livers from streptozotocin-diabetic rats retained 25% and degraded 10% of ^{125}I-insulin administered as a 1-min pulse. On gel filtration (Sephadex G50F), the degradation products released into the vascular effluent eluted in the salt peak. During the 45-min interval after the end of the ^{125}I-insulin infusion, 0.19% of the total dose was excreted in the bile. 60–90% of this material consisted of iodinated, low-molecular-weight degradation products.

Inclusion of native insulin with the ^{125}I-insulin in the pulse depressed both the retention and degradation of iodinated material; however, this reflected increased retention and degradation of the total insulin dose (^{125}I-insulin plus native hormone). The log of the total amounts of insulin retained and degraded were linearly related to the log of the total amount of insulin infused at concentrations between 12.7 nM and 2.84 μM. Increasing the amount of native insulin in the infused pulse also depressed the total amount of iodinated material found in the bile and led to the appearance in the bile of intermediate-sized degradation products that did not simultaneously appear in the vascular effluent.

Addition of high concentrations of glucagon to the infused ^{125}I-insulin had no effect on the retention or degradation of the labeled hormone, or on the apparent size and amount of iodinated degradation products found in the vascular effluent or in the bile. Preinfusion of concanavalin A inhibited both ^{125}I-insulin retention and degradation. A greater depression by concanavalin A of degradation than binding was also observed with isolated hepatocytes.

In contrast to ^{125}I-insulin, the retention and degradation of two iodinated insulin analogues of relative low biological potency, proinsulin and desalanyl-desasparaginyl insulin, were small. The amount of radioactivity appearing in the bile after infusion of these analogues was almost negligible. However, degradation products of these analogues that appeared in the bile and in the vascular effluent were qualitatively similar to those found after the infusion of ^{125}I-insulin.

Our findings suggest that the rapid initial uptake of ^{125}I-insulin after its infusion into noncyclically perfused liver, as well as its subsequent degradation, behaves in a qualitatively similar fashion to the binding of ^{125}I-insulin and its degradation by isolated rat hepatocytes. This uptake and the subsequent phase of degradation may be attributable to binding of insulin at specific recognition sites, preliminary to its transfer to a degradative site(s) presumed to be located inside the cell.

INTRODUCTION

We have shown previously, using isolated rat hepatocytes, that the binding of ^{125}I-insulin to an insulin recognition site is coupled to the degradation of ^{125}I-insulin (1). Observations leading to this conclusion can be summarized as follows: (*a*) the velocity of insulin degradation is directly related to the amount of insulin bound at steady state at total insulin concentrations ranging from 70 pM to 1 μM; (*b*) at various concentrations, insulin and several analogues of insulin depressed both the degradation velocity of ^{125}I-insulin and the amount of ^{125}I-insulin bound to cells at steady state to the same extent, whereas peptide hormones unrelated to insulin had no effect; and (*c*) protease pretreatment of cells depressed the binding and degradation velocity of ^{125}I-insulin to the same extent.

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Other reports in the literature have suggested that insulin retention and degradation are related. Crofford et al. found that although extracts of fat cell plasma membranes both bound and degraded insulin, extracts of plasma membranes from trypsin-treated cells could neither bind nor degrade insulin (2). The work of Mortimore and Tietze with cyclically perfused rat livers suggested that insulin degradation required prior "capture" and transport to a presumed intracellular degrading site (3). Stoll et al. (4) and Rubenstein, et al. (5) have shown that proinsulin, which has relatively low biological potency (6, 7) and which binds poorly to rat liver plasma membranes (8), is extracted very slowly by the isolated perfused liver. In addition, Csorba, et al. (9) have shown that ^{125}I-desalanyl-desasparaginyl insulin, an analogue with very low biological potency (10–14), which inhibits the binding of ^{125}I-insulin to insulin receptors only at relatively high concentrations (10–15), is removed more slowly from the plasma of dogs than ^{125}I-insulin in a comparable dose.

There appear to be many similarities between isolated hepatocytes and intact liver with respect to insulin metabolism. Mortimore and Tietze (3), using ^{125}I-insulin, and Mondon et al. (16), using native insulin, have observed that the hormone is rapidly removed by cyclically perfused liver after its addition to the perfusate. Similarly, insulin binds relatively rapidly to isolated rat hepatocytes and, in agreement with observations on perfused livers (3), degradation products begin to appear linearly with time only after a 7–9-min interval (1). To compare more fully the interactions of insulin in intact liver with those observed in isolated hepatocytes, we have studied the uptake and degradation of a 1-min pulse of ^{125}I-insulin and of two iodinated insulin analogues in noncyclically perfused liver preparations.

METHODS

Materials

Porcine insulin (monocomponent) was obtained from the Novo Company, Copenhagen; bovine proinsulin was isolated from bovine pancreas according to the method of Steiner et al. (17); and desalanyl-desasparaginyl insulin was generously provided by Dr. F. H. Carpenter (10). Insulin and insulin analogues were iodinated as described previously (1) by a modification of the method of Freychet et al. (18). The iodinated proteins, containing 0.2–0.4 g·atom of ^{125}I/mol protein (sp act 70–140 μCi/μg protein), coeluted with the native protein on gel filtration (Sephadex G50F eluted with 6 M urea, 1 M acetic acid, and 0.15 M NaCl) and migrated with the native protein on polyacrylamide gel electrophoresis (8 M urea, pH 8.7, 10% acrylamide). [*Hydroxymethyl*-^{14}C]Inulin (sp act 2.2 μCi/mg) was obtained from Amersham/Searle Corp., Arlington Heights, Ill.; [3,0-*methyl*-^{3}H]D-glucose (sp act 1.2 Ci/mmol) from ICN Isotope and Nuclear Division, International Chemical and Nuclear Corp., Irvine, Calif.; and glucagon (monocomponent), from Novo Company, Copenhagen.

Rats

Diabetic rats were used to insure that minimal endogenous insulin would compete with the infused insulin or analogues for retention and degradation by the liver. Streptozotocin (65 mg/kg in 0.02 M sodium citrate buffer, pH 4.5) was administered intravenously to female Sprague-Dawley rats fasted for 24 h. The rats were housed for at least 2 wk to permit stabilization of the diabetic state. These rats, weighing 110–114 g, were used after an overnight fast (blood glucose concentration: over 400 mg/100 ml). Liver weights ranged from 5.5 to 7.0 g.

Perfusion

Set-up. Rats were anesthetized with Nembutal (pentobarbital, Abbott Laboratories, North Chicago, Ill. (0.1 ml of a 50 mg/ml solution) intraperitoneally, and their livers, maintained at 33–35°C, were noncyclically perfused *in situ* through the portal vein. Noncyclic perfusions were used to obtained greater sensitivity in detecting small changes in the amounts of final degradation products and to avoid recycling of iodinated degradation products and liver metabolites. The bile duct was cannulated to monitor bile flow and to collect bile. The liver outflow was collected from a catheter placed through the right atrium into the inferior vena cava above the diaphragm. Perfusate consisted of Krebs-Ringer bicarbonate buffer containing 2.54 mM $CaCl_2$, 300 mg/100 ml glucose, and 3.5% bovine serum albumin fraction V (Miles Laboratories, Inc., Miles Research Div., Elkhart, Ind.), equilibrated with 95% O_2 and 5% CO_2, pH 7.4, at 35°C. Flow rates of 10 ml/min [physiological: 1.25–2.00 ml/min/g liver (19)] were continuously maintained by pumping infusate contained in 100-ml syringes with an infusion pump (Model 975; Harvard Apparatus Co., Inc., Millis, Mass.). The syringes could be changed within 15 s without significant interruption of flow.

The livers appeared to be uniformly perfused, and bile flow was well maintained throughout the perfusion. Portal vein-inferior vena cava oxygen differences, measured on five livers at the end of the perfusions on separate days, showed extraction of 75% of the oxygen present in the influent; the Po_2 was lowered from 425 in the inflow to 100 mm Hg in the outflow, as measured with a blood-gas monitor (BMS 2, Radiometer Co., Copenhagen).

Protocol. The livers were initially perfused with supplemented buffer for 15–20 min. Then 10 ml Krebs-Ringer buffer, containing 2.5% bovine serum albumin and the appropriate labeled materials, was infused over 1 min. The liver was then flushed for 2 min with buffer alone, then for 43 min with buffer or, when appropriate, with buffer containing 1 μM native bovine insulin (Sigma Chemical Co., Inc., St. Louis, Mo.). In some experiments, 20 ml of Krebs-Ringer buffer containing 0.4–3.5 mg/ml of concanavalin A (Miles-Yeda Ltd., Rehovot, Israel) was infused over 2 min beginning 4 min before the ^{125}I-insulin infusion. The livers were then infused for 2 min with buffer alone to wash out excess concanavalin A before administration of the 1-min ^{125}I-insulin pulse. These procedures are summarized in Fig. 1. The beginning of the perfusion (time 0) was considered to be the end of the pulse of iodinated material. A 100-μl sample of ^{125}I-labeled insulin or analogue was incubated at 35°C in 1 ml of liver effluent for the duration of the infusion to control for degradation by the medium alone.

Sample collection and analysis. Radioactivity in the effluent was monitored by collecting 0.5 ml of liver effluent into

0.5 ml of 6 M urea, 3 M acetic acid, and 0.15 N NaCl at 1-min or 0.5-min intervals (as indicated in the figures) up to 5 min after the end of the ^{125}I-insulin infusion and at 5-min intervals thereafter, up to 45 min. These samples were frozen immediately and stored at −20°C for later analysis; insulin was stable under these conditions. At the termination of the perfusion (45 min after the end of the infusion of the iodinated material), the liver was immediately removed and cut into four or five sections, each of which was frozen between blocks of dry ice and stored at −20°C for later analysis.

The bile and each diluted sample taken throughout the perfusion were gel-filtrated over a 1 × 50 cm column of Sephadex G50F (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), eluted with 6 M urea, 1 M acetic acid, and 0.15 M NaCl at flow rates of 6–7 ml/h and with fraction volumes of 1.0–1.2 ml. The radioactivity that eluted in the internal volume was considered to comprise the final degradation products (see Fig. 3 and Results). The percentage of iodinated hormone degraded in any one sample was computed as the ratio of the radioactivity in the salt peak to the total radioactivity applied to the column, on a PDP8 computer (Digital Equipment Corp., Marlboro, Mass.).

To determine the amount of iodinated material retained by the liver at the end of the 45-min perfusion, the frozen liver was broken into small pieces and, by pressing between Lucite blocks, kept at −20°C over dry ice and then homogenized in 50 ml of 1.2% Triton × 100 containing 6 M urea, 3 M acetic acid, and 0.15 M NaCl with a Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.; setting 3). The radioactivity of a portion of this homogenate was measured for determination of total iodinated material remaining in the liver. Another portion was centrifuged at 100,000 *g* for 60 min at 4°C, and the pellet was washed several times until 90% of the total sample radioactivity had been taken up in the combined supernates of the first centrifugation and subsequent washes (usually four or five). The combined supernates were then analyzed by gel filtration for intact and degraded iodinated material, as described above.

Radioactivity of the iodinated compounds was measured in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with a counting efficiency of 50%; [*hydroxymethyl*-^{14}C]inulin and [3,0-*methyl*-^{3}H]D-glucose were measured in a Packard Tricarb spectrometer with counting efficiencies of 75% and 25%, respectively.

Quantitation of results. After correction for dilution, the radioactivity of 100-μl aliquots of the same effluent samples that had been gel-filtered were plotted against time to give a washout curve of total iodinated material eluting from the liver. The absolute amounts of degraded iodinated material in 100 μl of the instantaneous samples, determined by gel filtration of the sample (percent of total sample degraded × total amount of iodinated material in the 100-μl sample), were plotted similarly to obtain washout curves of degraded iodinated material. The total amount of radioactivity eluting from the liver between 2 and 45 min after the end of the pulse infusion of radioactivity was determined by estimation of the area under washout curves with trapezoidal approximations over the short time intervals between samples. If the washout curve is approximately linear with time over a short time interval, Δt, and the first sample during this time interval contains x_1 cpm, and the final sample x_2 cpm, then the radioactivity in the total washout over that time interval is: $0.5 \cdot \Delta t \cdot (x_1 + x_2)$. In our calculations, Δt was converted to the number of 100-μl samples in the washout during the corresponding Δt. Specifically, with a flow rate of 10 ml/min, and a sample size of 100 μl, a 5-min interval would be equivalent to (10 ml/60 s) × (1 sample/0.1 ml) × 300 s, or 500 samples. This number was then substituted for Δt in the expression above.

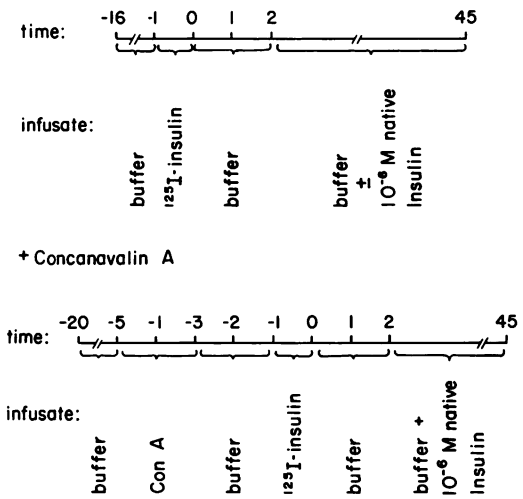

FIGURE 1 Schedule of infusion of experimental agents into noncyclically perfused rat liver. Upper part of the figure shows the protocol for all perfusions except those involving concanavalin A, which is shown in the lower part of the figure.

Thus, the total amount of iodinated material retained or degraded by the liver was considered to be the sum of the total, or degraded, iodinated material eluting from the liver in the 2–45-min interval after the end of the pulse infusion of iodinated material plus the amount of total or degraded iodinated material remaining in the liver at the end of the 45-min perfusion. The total dose was estimated by direct measurement of the infused material.

Binding of ^{125}I-labeled insulin, proinsulin, and desalanyl-desasparaginyl insulin to rat liver plasma membranes

Plasma membranes, isolated from streptozotocin-diabetic rats by the method of Neville (20), were used to determine the relative amounts of ^{125}I-labeled insulin and insulin analogues bound at equilibrium. These experiments are described in the legend of Fig. 9.

Binding and degradation of ^{125}I-insulin by hepatocytes in the presence of concanavalin A

Cells were isolated from normal rats, and the binding and degradation of ^{125}I-insulin in the presence of concanavalin A (0–30 μg/ml) were determined as described in the note to Table V.

RESULTS

More ^{125}I-insulin was retained by rat liver than [3-0-*methyl*-^{3}H]D-glucose or [*hydroxymethyl*-^{14}C]inulin (Fig. 2). Over 98% of the [3-0-*methyl*-^{3}H]D-glucose, which distributes in the total water space, and over 99% of the [*hydroxymethyl*-^{14}C]inulin, which distributes only in the extracellular space, eluted from the liver within 2 min after the end of the pulse infusion (see Table III).

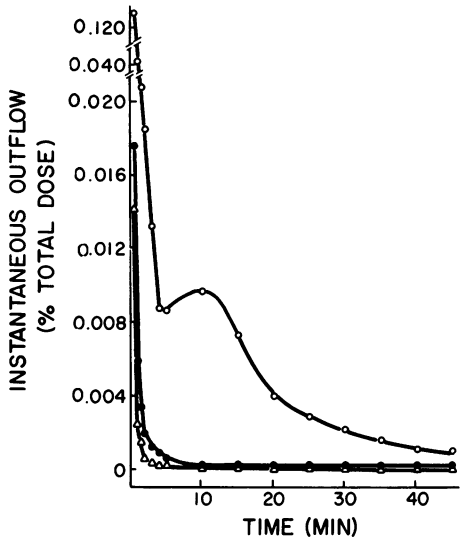

FIGURE 2 Washout curves of ^{125}I-insulin ($3 \times 10^7 - 1 \times 10^8$ cpm), [*hydroxymethyl*-^{14}C]inulin (10 μCi) and [3,0-*methyl*-^{3}H]D-glucose (0.2 mCi). The radioactivity in 100-μl aliquots has been corrected for dilution and is expressed as percentage of total dose. Time 0 represents the end of the pulse infusion of labeled material. ^{125}I-Insulin (average of five perfusions) O--O; [3,0-*methyl*-^{3}H]D-glucose (average of four perfusions) ●--●; and [*hydroxymethyl*-^{14}C]inulin (average of five perfusions) △--△.

Therefore, in subsequent determinations of the amount of ^{125}I-labeled insulin and insulin analogues retained, measurement of the outflow of iodinated material was begun not earlier than 2 min after the end of its pulse infusion.

Gel filtration patterns of liver effluent samples taken at 2, 10, and 20 min after the end of the pulse infusion of ^{125}I-insulin are shown in Fig. 3. Iodinated material eluted in the void volume, in the insulin peak, or in the internal volume with Na^{125}I, representing degradation products. As in our studies with hepatocytes (1), intermediate products of degradation, including the A chain of insulin, which contains the bulk of the ^{125}I-label (21, 22), were not apparent. Liver effluent, sampled before the infusion of iodinated insulin, degraded no ^{125}I-insulin during incubation at 37°C for 60 min. Therefore, the iodinated degradation products in the liver effluent were attributable to the degrading activity of the liver and not to that of the perfusate. The iodinated void volume material in the liver effluent was probably not significant, since aggregates comprised 2–3% of the total iodinated infusate in all perfusions and since the amount of aggregate retained by the livers constituted less than 0.1% of the total dose in all cases. This iodinated aggregate was not converted to iodinated A chain upon reduction with dithiothreitol at pH 8.5, and therefore was probably not the B-chain-rich, A-chain aggregate described by Varandani (23).

The washout of total iodinated material and iodinated degradation products after a 1-min pulse infusion of 10 ml of ^{125}I-insulin (approximately 20 nM) is shown in Fig. 4. Although the radioactivities of small instantaneous samples of liver washout, as recorded on the ordinate of Fig. 4, individually represent only small amounts of the total dose, their integration between 2 and 45 min indicates that the retained radioactivity eluting from the liver between 2 and 45 min after the end of the ^{125}I-insulin infusion represents about 20% of the total dose. Fig. 4 (lower curve) demonstrates the lag in the initial appearance of degradation products, whose outflow reached a maximum at 10 min after all infusions of ^{125}I-insulin. Approximately 5% of the total dose remained in the liver at the end of the perfusion.

Addition of native insulin to the 10-ml ^{125}I-insulin pulse depressed the amount of ^{125}I-insulin retained and degraded (Table I). However, the depression reflected increased retention and degradation of total insulin (iodinated plus native). To express the full range of insulin concentrations used, the data was plotted in log-log form. As seen in Fig. 5 (upper and middle), the log of the total amounts of insulin retained and degraded varied

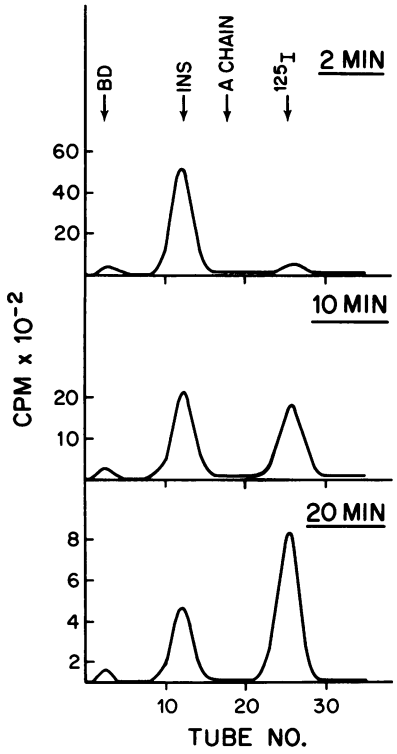

FIGURE 3 Gel filtration of instantaneous samples of liver effluent taken at 2, 10, and 20 min after pulse infusion of ^{125}I-insulin into noncylically perfused liver. Columns were calibrated with blue dextran (BD), indicating void volume, native insulin (INS), A chain of insulin, and ^{125}I, indicating internal volume.

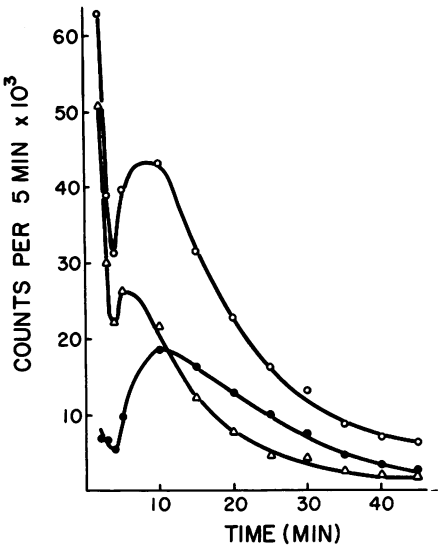

FIGURE 4 Washout curves of total liver effluent and of intact and degraded iodinated material after the 1-min infusion of 10 ml of ^{125}I-insulin (1.5×10^8 cpm or 15.5 nM) into the liver. The radioactivity in 100-μl aliquots from instantaneous samples of liver effluent has been corrected for dilution and is expressed as percent of total dose. Total radioactive material, ○--○; intact ^{125}I-insulin, △--△; degraded iodinated material, ●--●.

directly as the log of the total amount of insulin infused, with slopes of 0.798 and 0.583, respectively. Accordingly, the log of the total amount degraded also varied linearly as the log of the amount retained, with a slope of 0.711 (Fig. 5, lower). Regardless of the insulin concentration

TABLE I

Disposition of ^{125}I-Insulin in the Presence of Various Concentrations of Native Insulin

	Total dose	^{125}I-material retained	^{125}I-insulin degraded	
	M	*% total dose*	*% total dose*	*% of retained*
Exp. 1	1.27×10^{-8}	19.71	8.92	45.26
	1.90×10^{-8}	14.89	5.51	37.00
	1.16×10^{-7}	11.67	4.69	40.19
	8.52×10^{-7}	8.64	2.67	30.90
Exp. 2	1.50×10^{-8}	21.39	9.92	46.38
	3.67×10^{-8}	18.83	6.50	34.52
	2.88×10^{-7}	8.70	1.58	18.16
	2.83×10^{-6}	6.25	0.84	13.44

^{125}I-Insulin (12.7–15.0 nM) in the absence or presence of native insulin (6.3 nM–2.83 μM) was infused into the liver as a 1-min pulse. The livers were then perfused with buffer for 45 min. The amounts of ^{125}I-insulin retained and degraded by the liver were estimated as the sum of the radioactivity in the 2–45-min washout plus the amount remaining in the liver at 45 min, as described in Methods.

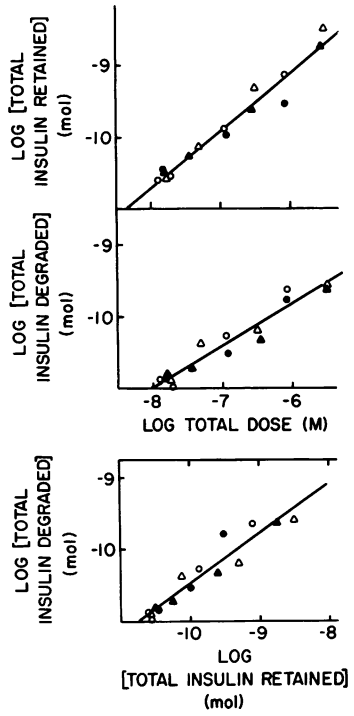

FIGURE 5 Dependence of amount of insulin retained and degraded on total dose (upper and middle panels), and of the amount of insulin degraded on the amount of insulin retained (lower panel). 10 ml of ^{125}I-insulin (12.7–17.0 nM) in the absence or presence of native insulin (6.0 nM–3.16 μM) were infused over a 1-min interval into noncyclically perfused liver. The percentage of ^{125}I-insulin retained and degraded over the 45-min interval, estimated as the sum of the 2–45-min washout + the amount remaining in the liver at 45 min, was multiplied by the total amount of insulin infused (^{125}I-insulin + native) to yield the total amount of insulin retained and degraded. The linear regression equations and correlation coefficients are: top, $y = 0.798x - 4.322$, $r = 0.981$; middle, $y = 0.583x - 6.329$, $r = 0.967$; lower, $y = 0.711x - 3.372$, $r = 0.949$, where y represents the ordinate, and x the abscissa. Each symbol represents a series of three or four perfusions performed on separate occasions.

in the infusate, the amount of insulin remaining in the liver at 45 min, determined as described in Methods, comprised 19.86±2.24% (mean±SE [24]) of five perfusions of the total retained iodinated material. As shown in Fig. 6, increasing the concentration of insulin in the infusate had no effect on the time interval that preceded the appearance of maximal amounts of degradation products in the washout curve.

Glucagon affected neither the retention nor the degradation of ^{125}I-insulin, even at high concentrations (Table II). The relative contributions of the 2–45 min outflow and liver radioactivity at 45 min to total retention were the same at each glucagon concentration as in the controls. The amount of ^{125}I-insulin remaining in the liver at the end of the perfusion was 19.70±4.21% (mean±

TABLE II

Disposition of ^{125}I-Insulin in the Presence of Glucagon

	Glucagon concentration	^{125}I-material retained	^{125}I-insulin degraded
	M	*% of total dose*	*% of total dose*
Exp. 1	0	22.24±3.80	10.88±2.17
	1.56×10^{-9}	17.25±2.00	7.57±1.68
	1.56×10^{-8}	27.21±3.15	14.13±3.11
	1.56×10^{-7}	21.31±4.48	10.21±2.18
	1.56×10^{-6}	23.58±3.80	11.29±2.04
Exp. 2	0	25.93	9.44
	1.56×10^{-6}	20.90±3.67	7.47±1.44

^{125}I-Insulin (exp. 1, 36.4 nM; exp. 2, 25.9 nM) containing glucagon was infused into the liver in a 10-ml volume over a 1-min interval. The livers were then noncyclically perfused with buffer for 45 min. The amounts of ^{125}I-insulin retained and degraded were estimated as the sum of the 2–45-min washout plus the amount remaining in the liver at 45 min. In most cases, the data is expressed as the average of two values ±one half the range.

SD [24] of 10 perfusions) of the amount of ^{125}I-insulin retained.

^{125}I-labeled proinsulin and desalanyl-desasparaginyl insulin, analogues with low biological potency (6, 7, 10-13), are retained and degraded very little by perfused rat liver (Table III; Fig. 7). In the experiments with ^{125}I-labeled analogues and the ^{125}I-insulin controls, medium containing 1 μM native insulin was infused beginning at 2 min after the end of the infusion of iodinated hormone

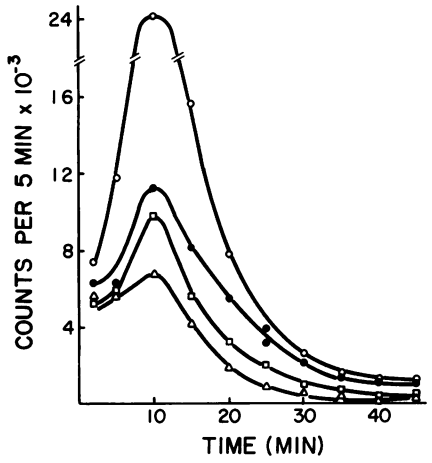

FIGURE 6 Washout of iodinated degradation products from liver between 2 and 45 min after the infusion of ^{125}I-insulin and different concentrations of native insulin. The 10-ml infusate contained 12.7 nM ^{125}I-insulin alone (○) or an identical concentration of ^{125}I-insulin plus 6.0 nM (●), 0.103 μM (□), or 0.84 μM (△) native insulin. Ordinate values represent the radioactivity of 100-μl samples of washout, after correction for dilution.

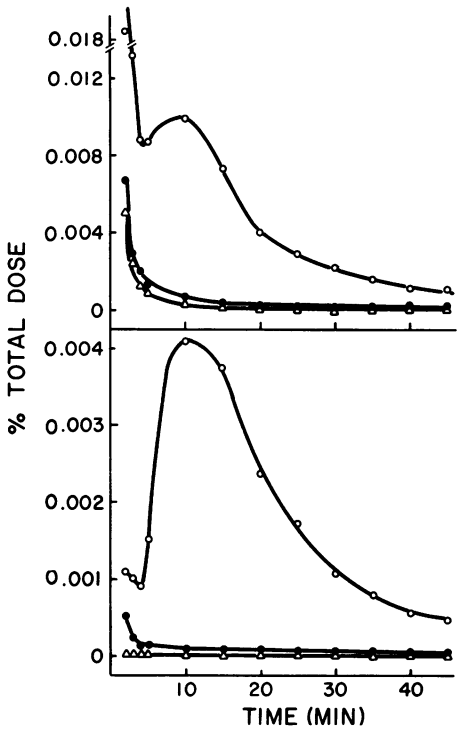

FIGURE 7 Washout curves of total (upper panel) and degraded (lower panel) iodinated material after the infusions of ^{125}I-labeled insulin, proinsulin, and desalanyl-desasparaginyl insulin. The concentrations of ^{125}I-insulin were between 7.9 and 14.0 nM; of ^{125}I-proinsulin, between 6.5 and 11 nM; of ^{125}I-desalanyl-desasparaginyl insulin, between 9.0 and 13 nM. The radioactivity in 100-μl aliquots of instantaneous effluent samples has been corrected for dilution and is expressed as percent of total dose. Each washout curve represents the average of several perfusion. ^{125}I-Insulin (five perfusions), ○--○; ^{125}I-proinsulin (six perfusions), ●--●; ^{125}I-desalanyl-desasparaginyl insulin (seven perfusions), △--△.

(see Fig. 1) to insure that no reassociation of dissociated material occurred. Since this chase was introduced at 2 min, it did not affect the determination of the amount retained (cf. Tables I and III). Fig. 7 shows the washout curves of total and degraded iodinated material with time. The retention of iodinated analogues was approximately 1/12 that of the ^{125}I-insulin controls, but is significantly greater than the retention of inulin (Table III). The amounts of both iodinated analogues degraded were also significantly lower than that of ^{125}I-insulin controls. However, expressed as percent of the amount retained, the difference between the degradation of iodinated analogues and of ^{125}I-insulin, though significant, was smaller. It is doubtful that the degradation of proinsulin is attributable to small amount of contaminating insulin, since its purity was found to be greater than 99% by gel filtration and polyacrylamide gel electrophoresis (8 M urea, pH 8.7, 10% acrylamide). Amino acid

analysis of bovine desalanyl-desasparaginyl insulin also suggested that it contained little if any contaminating insulin. Regardless of the possible slight contamination of this analogue by insulin, its very low retention must reflect a greatly reduced affinity of the retention site for desalanyl-desasparaginyl insulin.

After the infusion of ^{125}I-insulin at concentrations of 7.9 to 15.0 nM, 0.19±0.01% (mean±SD [24] of four perfusions) of the total dose was found in the bile during the 45-min perfusion. This was significantly higher than the amount of radioactivity excreted in the bile after infusion of [*hydroxymethyl*-^{14}C]inulin (0.00086± 0.00041% of the total dose; mean±SD [24] of five perfusions) or of [3-0-*methyl*-^{3}H]D-glucose (0.0040± 0.0012% of the total dose; mean±SD [24] of four perfusions). The biliary excretion of ^{125}I-insulin also greatly exceeded that of ^{125}I-proinsulin (0.021±0.0074% of the total dose; mean±SD [24] of six perfusions) or that of ^{125}I-desalanyl-desasparaginyl insulin (0.0092±0.0067% of the total dose; mean±SD [24] of seven perfusions). Iodinated material migrating after ^{125}I-labeled insulin and analogues on gel filtration comprised in all cases 60-90% of the total radioactivity in the bile, a considerably higher proportion of degradation products than in the total 2-45 min washout or within the liver at the end of the perfusion. No iodinated A chain was evident in these gel filtration profiles. Bile collected in separate experiments from streptozotocin-diabetic rats during a 45 min non-

TABLE III

Disposition of ^{125}I-Labeled Insulin and Analogues by Noncyclically Perfused Liver

Infusate	^{125}I-labeled material retained	^{125}I-labeled material degraded	
	% total dose	*% total dose*	*% of retained*
A. [3,0-*methyl*-^{3}H] D-glucose (4)	1.99±0.35		
B. [*Hydroxymethyl*-^{14}C] inulin (5)	0.86±0.17		
P (A–B)	<0.01		
C. ^{125}I-insulin (5)	24.58±2.26	9.11±1.26	37.40±4.68
D. ^{125}I-proinsulin (6)	2.00±0.28	0.37±0.06	18.48±1.13
P (C–D)	<0.005	<0.005	<0.005
E. ^{125}I-desalanyl-desasparaginyl insulin (7)	1.79±0.39	0.48±0.10	27.13±2.00
P (B–E)	<0.025		
P (C–E)	<0.005	<0.005	<0.05
P (D–E)	NS	NS	<0.025

^{125}I-labeled insulin or analogue (approximately 100 nM) was infused in a 10-ml volume as a 1-min pulse into noncyclically perfused liver. The pulse was followed by a 2-min perfusion with buffer alone and then by a 43-min perfusion with buffer containing 1 μM native insulin. The amount of iodinated material retained and degraded were estimated as described in the legends to Tables I and II and in Methods. The results of experiments with [3,0-*methyl*-^{3}H] D-glucose and [*hydroxymethyl*-^{14}C] inulin are included for comparison. The data is expressed as the mean±SE (24). The numbers in parentheses indicate the number of perfusions. Probabilities of significant differences were estimated by Student's *t* test for unpaired means (24).

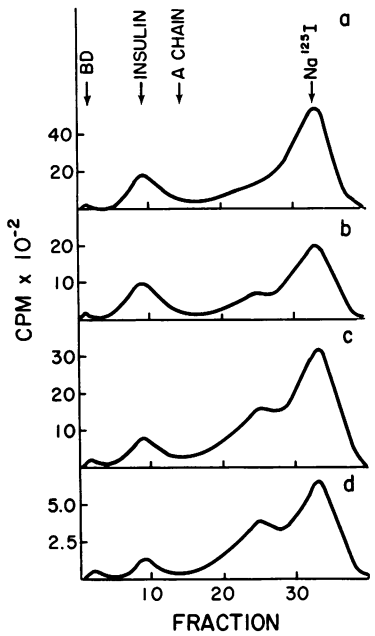

FIGURE 8 Gel filtration patterns of bile collected over the 45-min interval after pulse infusions of ^{125}I-insulin and increasing concentrations of native insulin. The 10-ml infusate contained 12.7 nM ^{125}I-insulin (*a*) or the same concentration of ^{125}I-insulin plus 6.0 nM (*b*), 0.103 μM (*c*), or 0.84 μM (*d*) native insulin. The variable range of ordinate values reflects the analysis of variable portions of the bile collected.

cyclic perfusion did not degrade added ^{125}I-insulin during a 45 min incubation at 37°C, indicating that degradation occurs before the appearance of iodinated material in the bile. Increasing the amount of native insulin in the 10-ml infusate depressed the amount of iodinated material in the bile (Fig. 8). Furthermore, with increasing doses of insulin, degradation products that eluted before the salt peak on gel filtration appeared in the bile in increasing amounts (Fig. 8). These degradation products did not appear in the vascular outflow of the same livers. Glucagon had no effect on the amount or on the nature of the iodinated degradation products in the bile.

As shown in Fig. 9, the relative amounts of the labeled insulin and insulin analogues bound to plasma membranes, displaced by high concentrations of native insulin, were similar to the relative amounts of iodinated material retained by perfused liver (Table III). At equivalent concentrations, ^{125}I-insulin was bound more than either of the iodinated analogues; ^{125}I-proinsulin was bound slightly more than ^{125}I-desalanyl-desasparaginyl insulin after 30 min at 35°C.

Concanavalin A has been reported to depress the binding of ^{125}I-insulin to fat cells (29, 30). Preinfusion of 20 ml of concanavalin A at concentrations between 0.4 and 3.5 mg/ml depressed the amount of ^{125}I-insulin retained by the livers, but depressed the amount degraded

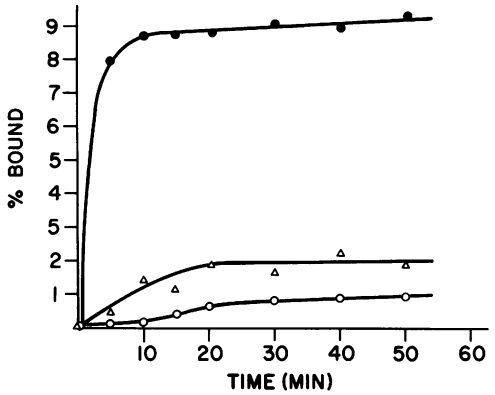

FIGURE 9 Time-course of binding of ^{125}I-insulin, ^{125}I-proinsulin, and ^{125}I-desalanyl-desasparaginyl insulin to plasma membranes isolated from streptozotocin-diabetic rats. Aliquots (30 μl) of the same solutions of iodinated proteins used in the perfusions (final concentration: 1.25–3.75 nM) were added to 250 μl of plasma membrane suspension in Krebs-Ringer phosphate buffer containing 2.5% bovine serum albumin and 130–150 μg protein, as estimated by the method of Lowry et al. (25), with bovine serum albumin as a protein standard. Native insulin at final concentrations of μM was added simultaneously to one half of the incubation mixtures. After the appropriate period of incubation at 30°C, 100-μl aliquots were layered over 250 μl of 10% sucrose in Krebs-Ringer phosphate buffer and centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 10,000 *g* for 5 min at 4°C. The radioactivity of the pellet was measured, and the amount of iodinated material specifically bound to the plasma membranes was calculated as the difference between the amount bound in the presence and in the absence of native insulin (11, 26–28). Each experiment was performed in duplicate. Nonsaturable binding did not exceed 3% of the total radioactivity. ^{125}I-insulin, ●--●; ^{125}I-proinsulin, △--△; ^{125}I-desalanyl-desasparaginyl insulin, ○--○.

to a far greater extent (see Table IV, last column). Similar results were obtained with isolated hepatocytes (Table V). It should be emphasized that over 90% of the ^{125}I-insulin bound to cells at this low concentration represents saturable binding (1).

DISCUSSION

Our findings suggest that the rapid early uptake of ^{125}I-insulin by perfused liver reflects the binding of the hormone to sites on the plasma membrane of hepatocytes. The depression of hepatic uptake of ^{125}I-insulin by native insulin (Table I) and by concanavalin A (Table IV) but not by glucagon (Table II), as well as the diminished uptake of ^{125}I-labeled insulin analogues with low biological potency (6, 7, 10-13) suggests that the retention of these iodinated hormones by perfused liver is similar to the binding of these hormones to rat liver plasma membranes (Fig. 9, ref. 18, 27, 31-33), isolated hepatocytes (Table V, ref. 1), and fat cells (29, 30). Although our data do not preclude the possibility that retention of ^{125}I-insulin by the liver reflects in part the association of ^{125}I-insulin with reticuloendothelial cells, the facts that about 80% of the liver consists of hepatocytes (34) and that both isolated hepatocytes (1) and rat liver plasma membranes (11, 18, 27, 31-33) are known to bind insulin suggest that hepatocytes play a significant role.

A variety of experimental manipulations affect both the retention and the degradation of ^{125}I-labeled hormones similarly. The amount of ^{125}I-insulin degraded is depressed by increasing doses of native insulin (Table I); glucagon has no effect on the degradation of ^{125}I-insulin (Table II), although the liver is one of the major sites of glucagon degradation (35); and ^{125}I-proinsulin and ^{125}I-desalanyl-desasparaginyl insulin are degraded to a very small extent (Fig. 7, Table III), although ^{125}I-desalanyl-desasparaginyl insulin is degraded as well as ^{125}I-insulin by an isolated insulin-degrading enzyme (36) and by plasma membranes isolated from rat liver (27). Consistent with our observations on isolated hepatocytes (Table V), concanavalin A inhibits the degradation more than the retention of ^{125}I-insulin in perfused liver (Table IV).

Although the small amounts (2-3% of the total dose) of iodinated material that eluted in the salt peak contaminated the infusates, the calculated amount of this in the total effluent from 2-45 min, if it is distributed as [3,0-*methyl*-^{3}H]D-glucose (the worst case), was one order of magnitude lower than the amount of degradation products actually seen in the washout after the infusions of ^{125}I-proinsulin and ^{125}I-desalanyl-desasparaginyl

TABLE IV

Effect of Concanavalin A on the Disposition of ^{125}I-Insulin by Noncyclically Perfused Liver

Concanavalin A concentration	^{125}I-labeled material retained	^{125}I-labeled degraded	
mg/ml	*% of total dose*	*% of total dose*	*% of retained*
A. 0 (5)	24.58±2.26	9.11±1.26	37.40±4.68
B. 0.4 (2)	13.20±3.85	2.34±0.38	18.46±2.44
P (A–B)	<0.025	<0.025	<0.05
C. 1.35 (2)	7.15±1.17	0.95±0.14	13.26±0.29
P (A–C)	<0.005	<0.01	<0.025
D. 2.00 (3)	7.50±0.43	0.81±0.07	10.78±0.91
P (A–D)	<0.005	<0.005	<0.005
E. 3.50 (2)	4.57±1.29	0.54±0.12	12.01±0.69
P (A–E)	<0.005	<0.005	<0.025

^{125}I-Insulin (100 nM) was infused as a 1-min pulse after a 2-min preinfusion of 20 ml of concanavalin A. The rest of the perfusion was performed as outlined in Fig. 1. The retention and degradation of ^{125}I-insulin was measured as described in Methods. The data is expressed as the mean±SE (24). Probabilities of significant differences were estimated by Student's *t* test for unpaired means (24). The numbers in parentheses indicate the number of perfusions.

insulin, and was three to four orders of magnitude lower than that seen after the infusions of ^{125}I-insulin. Therefore, the degraded material occurring in the effluent after the infusions of ^{125}I-labeled insulin or analogues was derived primarily from the ^{125}I-labeled hormones retained by the livers and not from the small amounts of internal volume material initially infused.

Many reports indicate that insulin, iodinated at low levels, is a valid tracer for native insulin. ^{125}I-insulin is as potent as native insulin in stimulating glucose oxidation in fat cells (18, 28). Insulin iodinated at low levels is degraded significantly more rapidly both in vitro (37) and in vivo (38) than insulin iodinated at high levels. Brush has reported that ^{125}I-insulin is degraded to the same extent as native insulin by insulin-specific protease (36). For these reasons, we have estimated the behavior of native insulin on the basis of the behavior of ^{125}I-insulin (Fig. 5). That these plots are linear, in spite of the different proportions of native and iodinated material used, strongly suggests that ^{125}I-insulin and native insulin are retained and degraded similarly by perfused liver.

On log-log plots, the amounts of total insulin retained and degraded by perfused liver are linearly dependent upon the total amount of insulin (^{125}I-insulin plus native hormone) infused (Fig. 5, upper and middle panels). However, since the slope of a plot of log insulin degraded versus log insulin retained is less than one (Fig. 5, lower panel), the total amount of insulin degraded increases more slowly than the total amount retained. There are three possible explanations for this. First, the perfused diabetic liver possesses a supranormal capacity for the uptake of insulin (39), whereas homogenates of diabetic livers possess a subnormal capacity for insulin degradation (40, 41). Thus, if retained insulin represents the true substrate for insulin-degrading enzymes, gradual saturation of degrading enzymes may occur. This is consistent with the appearance of increased amounts of iodinated degradation intermediates in the bile with increasing insulin dose (Fig. 8). Second, the affinity of sites of insulin retention may decrease with increasing insulin retention, similar to the findings of de Meyts et al. with lymphocytes (42). Third, a small component of insulin uptake may be attributable to retention by nonparenchymal cells that degrade insulin with a lower efficiency than the majority of retention sites. We doubt that this component represents the binding of insulin either to saturable or to nonsaturable insulin-binding sites, since insulin bound to both groups of sites appears to be degraded with the same efficiency (1). It is also unlikely that this small component of insulin retention results from the trapping of insulin, since retention of [*hydroxymethyl*-^{14}C]inulin and [3,0-*methyl*-^{3}H]D-glucose was negligible (Fig. 2, Table III).

TABLE V

Effect of Concanavalin A on the Binding and the Degradation Velocity of ^{125}I-Insulin

Dose	^{125}I-Insulin bound	Degradation velocity
μg/ml	*% of control*	*% of control*
10	78.6	25
20	49.3	15.9
30	38.8	13.1

Hepatocytes (1×10^6 cells/ml) were preincubated with concanavalin A in 25 ml Erlenmeyer flasks under an atmosphere of 95% O_2 and 5% CO_2 in a shaking metabolic incubator at 88 oscillations/min. ^{125}I-Insulin (50 μl; final concentration, 1 nM) was then added, and the binding and degradation velocity were measured as described previously (1). Briefly, the total amount of ^{125}I-insulin bound at steady state was estimated as the radioactivity associated with the cell pellet obtained by centrifugation of the cell suspension which had been diluted 15-fold with ice-cold buffer. The degradation of ^{125}I-insulin was determined by gel filtration of extracts of cell suspension taken at regular intervals during the 1-h incubation period. Throughout the incubation, cell viability remained above 96%, as estimated by vital dye exclusion.

Three aspects of our data support the suggestion of Mortimore and Tietze that early "capture" and degradation of insulin by the liver occur in different compartments (3). First, the 10-min lag between the end of the exposure of the liver to maximum ^{125}I-insulin concentrations and the peak appearance of degradation products in the perfusate, is unaffected by increasing insulin concentrations (Fig. 6). This finding, compatible with our observations in intact hepatocytes (1) but contrasting with studies on broken-cell preparations (38, 43-46) and isolated enzymes (36, 47-49), suggests that the rate-limiting step in overall insulin degradation in this system is not a purely enzymatic one. Second, that the labeled components of the bile contained a disproportionately large amount of degradation products, including intermediates of degradation not seen in the vascular effluent (Fig. 8), suggests that these products are excreted directly into the bile from sites of degradation within the hepatocyte. Similarly, Boynes et al. (50) have reported that less than 40% of the radioactive material appearing in the bile of rabbits injected in vivo with ^{125}I-insulin was precipitable with trichloroacetic acid and less than 10% was immunoreactive. Third, concanavalin A, shown to inhibit phagocytosis by polymorphonuclear leukocytes (51) and to restrict the mobility of IgG receptors on the plasma membranes of lymphocytes (52, 53), depressed the degradation of ^{125}I-insulin to a greater extent than it did the binding of the hormone. This finding raises the possibility that mobility of membrane proteins may be required for insulin degradation.

Many workers have reported insulin-degrading ac-

tivity in association with rat liver plasma membranes (27, 54, 55), but the orientation of this insulin-degrading activity within the membrane is not known, i.e., whether it is oriented externally or internally. Moreover, the possibility has not been rigorously excluded that the membrane-associated degrading activity is a contaminant, arising either via adsorption of tissue proteases during isolation or via contamination of the plasma membrane fraction with lysosomes, microsomes, or cytosol, all fractions known to contain insulin-degrading enzymes (36, 44, 49, 56). Clearly, the demonstration that a particular subcellular fraction can degrade insulin in vitro does not constitute proof that it does so within the intact tissue under physiological conditions.

Our results compare favorably with other reports of insulin metabolism in intact liver. Madison and Kaplan reported that approximately 50% of the ^{125}I-insulin infused into the portal vein of humans at laparotomy was retained during one transhepatic circulation (57) and that hepatic uptake of infused ^{125}I-insulin was depressed by glucose-stimulated release of endogenous insulin (58). Although these results are similar to ours, they are difficult to interpret quantitatively since the portal vein concentrations of endogenous insulin were not measured, and since the precise relative timing of infused ^{125}I-insulin and endogenous insulin could not be experimentally controlled. Although rapid early uptake of insulin has been noted in both cyclic (3, 16) and noncyclic (16) perfusions, it has not been quantitatively related to the total dose. Compatible with our findings are reports that clearance of proinsulin by cyclically perfused liver is immeasurably small (4, 5). However, in general, it is difficult to compare our findings with those based on cyclic liver perfusions since, in many of these experiments, measurement of insulin uptake was begun at 5-6 min after the addition of insulin to the perfusate and since the parameters measured differ in important respects from those that we studied (4, 5, 40, 59, 60).

We have measured the final rather than the intermediate or total degradation products to assess the *overall* process of insulin degradation by isolated hepatocytes (1) and perfused liver. The findings that both intracellular (61-63) and extracellular (64, 65) proteins are degraded rapidly and completely to dipeptides or to amino acids, respectively, support this approach. The lower affinity of the degradative process than of the binding process for insulin in hepatocytes reported by others could reflect in part the use of different criteria to assess degradation (66, 67). Le Cam et al. (66), who did not directly rule out leakage of insulin-degrading activity from cells, recently reported that significant amounts of A chain are generated during insulin degradation by hepatocytes. This finding contrasts with our findings, both with perfused liver and with hepatocytes. Although our method of measuring degradation products was sufficiently sensitive to measure the amounts of A chain that those authors report, we observed A chain intermediates during insulin degradation by hepatocytes only when degradation by the control medium was significant. Our inability to observe A chain intermediates does not mean that insulin degradation does not proceed via an initial reductive cleavage, but only that A chains, if produced, are rapidly degraded.

Using isolated hepatocytes, we have shown that the degradation velocity of insulin is first-order with respect to the amount of total insulin bound at steady state, and that this reaction has an apparent rate constant (k_{ap}) of 0.030 ± 0.011 min^{-1} at 30°C (1). Furthermore, under conditions similar to those obtaining in these perfusions, namely, short binding times (5-10 min) and insulin concentrations of about 5 nM, the rate of dissociation of intact insulin from hepatocytes is first-order with respect to the amount of insulin bound and has an apparent rate constant (k_{-1}) of 0.0385 ± 0.0075 min^{-1} (1). If the events underlying the binding and degradation of insulin by isolated hepatocytes are similar to those underlying the retention and degradation of insulin by perfused liver, these rate constants would predict that approximately $[k_{ap}/(k_{-1} + k_{ap})]$ or 43.8% of the material retained by the liver would be degraded. Indeed, approxiamtely 45% of the ^{125}I-insulin retained by the liver was degraded (Tables I and III).

We have not attempted to measure an apparent dissociation constant for retention of insulin by the liver because measurement of the iodinated material in the liver washout after a 1-min pulse required relatively high portal vein insulin concentrations and because it is difficult to define rigorously parameters in perfused liver that are the true counterparts of binding of insulin to plasma membranes or to isolated cells. Nevertheless, the site(s) appears to show selectivity for insulin. Furthermore, the dependence of degradation of insulin and its analogues upon their prior retention by the liver is suggested by the parallel changes in their retention and degradation under different conditions. These properties are strikingly similar to those observed with isolated hepatocytes, in which the binding sites to which degradation is coupled have been shown by several methods to include the recognition sites generally believed to be important in the action of insulin (1). Thus our findings with perfused liver are compatible with the hypothesis that early retention of ^{125}I-insulin by perfused rat liver is attributable largely to insulin recognition sites in the hepatocyte plasma membrane, and that insulin retained at this site is systematically degraded.

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