Binding and Receptor-mediated Degradation of Insulin in Adipocytes

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Previous studies have shown that most of the degradation of insulin in a suspension of rat adipocytes (37°C, pH 7.4, 10 mg/ml of albumin) is accounted for by protease(s) unrelated to receptors (Gammeltoft, S., and Gliemann, J. (1973) Biochim. Biophys. Acta 320, 16-32). The aim of the present study was to investigate whether receptor-mediated insulin degradation also occurred.

Total insulin degradation in the adipocyte suspension was decreased to 4% in the presence of albumin, 50 mg/ml, as compared to 0.5 mg/ml, due to inhibition of protease(s) partially released into the incubation medium. The inhibition constant was about 3 mg/ml.

Receptor-mediated degradation was studied by incubation of cells in the presence of 50 mg/ml of albumin to steady state of insulin binding followed by a rapid wash, flotation through oil, and reincubation in nonradioactive medium. At least 90% of the radioactivity transferred with the cells was receptor-bound [125I]insulin and less than 5% was iodotyrosine. About half of the radioactivity dissociated from the cells as iodoinsulin and the other half as iodotyrosine as judged by gel filtration and paper chromatography. The same fraction of receptor-bound iodoinsulin was degraded at receptor occupancies ranging from 1% to 90%.

The following model is suggested. At steady state, half of the bound (iodo) insulin molecules are degraded whereas the other half dissociate in intact form. This occurs at any receptor occupancy. Any initially formed labeled peptide is rapidly degraded to iodotyrosine which is immediately released into the medium.

Until recently it was thought that binding of insulin to a particular group of receptors with a particular affinity was a bimolecular reversible reaction (for review see Ref. 1). The degradation of insulin which occurs at near-physiological temperatures in various preparations of cells and plasma membranes (e.g. liver plasma membranes (2), hepatocytes (3), and adipocytes (4)) was considered to be a process independent of the receptor binding of insulin. Previous work from our laboratory (4) showed that insulin was degraded in a suspension of adipocytes at 37°C, pH 7.4) with an apparent K_m of about 300 nM. The apparent dissociation constant for binding was about 3 nM. It can be calculated from the data that the rate of degradation of insulin was much higher than the total turnover on the receptors, indicating that most of the degradation was independent of receptor binding.

Terris and Steiner (5) found that the rate of degradation of insulin in a suspension of hepatocytes (30°C, pH 7.4) was proportional to the steady state occupancy. Furthermore, insulin bound to the hepatocytes was degraded more rapidly than an equivalent amount of insulin added to the suspension. Medium in which the hepatocytes had been incubated did not degrade insulin to any significant extent. It was concluded that a fraction of the receptor-bound insulin was degraded and that this process accounted for essentially all insulin degradation in that preparation of hepatocytes.

Our previous finding (4) that most of the insulin degradation in a suspension of rat adipocytes (37°C, pH 7.4) is unrelated to receptor binding did not exclude the possibility that some receptor-bound insulin is degraded. If so, the kinetic models had to be revised and the question of whether degradation of receptor-bound insulin was related to biological effects such as that on glucose transport had to be considered. The aim of the present study is to investigate whether a receptor-mediated insulin degradation occurs in addition to the receptor-independent degradation. In this context it is important to distinguish between [125I]-labeled insulin bound to receptors and radioactivity associated with the cells in some other way ("nonspecific" binding). It is also essential to minimize the insulin degradation which is not related to receptors. We have modified our previously published procedures in order to meet these requirements.

Some preliminary results have been reported elsewhere (6-8).

MATERIALS AND METHODS

Adipocytes were prepared from epididymal and perirenal fat from Wistar rats weighing 120 to 140 g as previously described (4). The incubation buffer contained (in millimolar concentrations) Na+, 140; K+, 4.7; Ca²⁺, 2.5; Mg²⁺, 1.25; Cl⁻, 142; HPO₄²⁻/H₂PO₄⁻, 2.5; SO₄²⁻, 1.25; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM; and bovine serum albumin (Miles) as indicated. pH was 7.45 at 37°C. Collagenase (type I) was from Worthington. Pig insulin (monocomponent) and guinea pig anti-pig insulin serum were from Novo.

Preparations of [125I]monoiodoinsulin with specific activities of about 200 Ci/g were used. Some were obtained from Drs. K. H. Jørgensen and U. D. Larsen, Novo Research Institute, and the iodine was predominantly located in tyrosine 19 of the A chain. Other preparations were obtained from Dr. J. C. Sodoyez or prepared in collaboration with Dr. S. Linde, Steno Memorial Hospital, following procedures similar to those described by Sodoyez et al. (9).

Before use the tracers were purified by gel filtration (Sephadex G-50 Fine, elution buffer with albumin 10 mg/ml, and the same ionic composition as the incubation buffer), and 98 to 99% of the radioactivity was then precipitable in 12% (w/v) trichloroacetic acid. The location of the label was studied according to previously published methods (10) and in the tracers prepared according to Sodoyez et al. about 80% was found in tyrosine A14. There were only minor differences in binding of these tracers to the adipocytes. Other radiochemicals were from the Radiochemical Centre, Amersham.

Bioassay of insulin was performed as described previously (11) with the following modifications. [U-¹⁴C]Glucose was used as a tracer when

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1 K. H. Jørgensen and U. D. Larsen, personal communication.
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125I-labeled insulin was assayed. The incubations were terminated by the addition of toluene-based scintillation fluid, and the samples were allowed to extract overnight. Half of the scintillation fluid (containing the 14C-lipids) was transferred to another vial and counted in a liquid scintillation counter set with a narrow 14C-window.

Incubation and separation of cells from the medium were routinely carried out as follows. A concentrated cell suspension was made up, and the packed cell volume was determined as described previously (12). The cells were diluted in buffer containing 5% (w/v) albumin to a packed cell volume of 5 to 7%. Since the mean diameter was about 60 μm (13) this corresponds to approximately 5 × 10⁶ cells/ml. Aliquots (400 μl each) were added to 14-ml round-bottom polypropylene tubes containing tracer and test substances in a volume of 100 μl. Incubations were carried out at 37°C with quite vigorous shaking (160 cycles/min) to keep the cells in a homogenous suspension. The incubations were terminated by the addition of 10 ml of chilled (10°C) 0.9% NaCl followed by 1.8 ml of silicone oil (density 0.99, viscosity 50 or 100 centistokes). The tubes were centrifuged for 40 s at about 1000 x g in a table top centrifuge. The cells concentrated on top of the oil as one or two "islets" which were recovered with one-eighth of a pipet cleaner bent to form a brushlet which could be held by a pair of forceps. The pipet cleaner with the adsorbed cells was either assayed directly for radioactivity in a γ-counter (background 4 cpm, efficiency 50%) or extracted for 1 h with shaking in 1 ml of acetic acid (3 M) with urea (6 M). The recovery of activity by this extraction procedure was at least 95%. In some experiments, the cells were sucked into a disposable polypropylene pipette tip and resuspended in new incubation medium. This treatment did not change the ability of the cells to bind labeled insulin.

Comments on Method—The [125I]monoidoinsulin tracer (100 pm) was not bound significantly to polypropylene or polystyrene tubes, even after incubation for 180 min in the absence of albumin. This was also true for pig insulin as measured by bioassay with fat cells.

The following factors were important for the formation of easily recoverable "islets" of concentrated cells. A tube should contain at least 20 μl of packed cells; lower amounts resulted in incomplete cell recovery, probably because a thin film of cells could not pass the water/oil interface. The 10 ml of saline should be added with a syringe with a large bore outlet in order to avoid formation of foam, and centrifugation should be started within about 30 s. When these precautions were taken into account, the recovery of labeled material was either estimated directly for radioactivity in a γ-counter (background 4 cpm, efficiency 50%) or extracted for 1 h with shaking in 3 M acetic acid (3 M) with urea (6 M). The recovery of activity by this extraction procedure was at least 95%. In some experiments, the cells were sucked into a disposable polypropylene pipette tip and resuspended in new incubation medium. This treatment did not change the ability of the cells to bind labeled insulin.

RESULTS

Degradation of Insulin by Protease and the Effect of Albumin—Fig. 1 shows the effect of bovine serum albumin on the degradation of [125I]iodoinsulin in a suspension of adipocytes. The values of the apparent Kₐ were less than 15% at the times when the incubations were terminated and was taken as a measure of the initial velocity of insulin degradation. In other experiments (not shown) it was found that albumin 0.5 mg/ml did not influence insulin degradation significantly. It is seen that albumin in higher concentrations inhibits the total insulin degradation and that half-maximal inhibition is obtained with about 3 mg/ml. The type of inhibition appears complex which is not surprising in view of the fact that the incubations were carried out with suspensions of cells and for markedly different times. The apparent Kₐ for pig insulin increased with increasing concentrations of albumin. In the experiment shown here, the apparent Kₐ was 64 nM in the presence of albumin 0.5 mg/ml and ranged in four other experiments from 55 to 90 nM. In the presence of albumin 10 mg/ml the apparent Kₐ was about 300 nM in agreement with previous results (4). It should be noted that even though the proteolytic activity was markedly inhibited it was not completely blocked by albumin 50 mg/ml (data not shown).

We have previously reported that some insulin degrading activity is released into the incubation medium (14). The characteristics of this protease activity have been further explored and the results are shown in the miniprint supplement. The main conclusions are as follows. Protease released to the medium accounts for approximately 50% of the total insulin degradation in a cell suspension containing albumin 0.5 mg/ml (Fig. 2), although some day to day variations were observed. The inhibition constant (Kᵢ) for pig insulin is about 80 nM, for hagfish insulin 8 nM, and for pig proinsulin 160 nM (Fig. 3). Bovine serum albumin (Kₐ, 3 mg/ml, highly purified human serum albumin, and egg albumin as well as other proteins and peptides (gelatine, Kᵢ, 0.2 mg/ml; adrenocorticotropic hormone, Kᵢ, 1 μM; glucagon, Kᵢ, 1.5 μM; bacitracin, Kᵢ, 20 μM) inhibit the degradation of [125I]-labeled insulin (Fig. 4).

Fig. 1. Inhibition of total insulin degradation by albumin. Aliquots (300 μl each) of cell suspension (1.7% packed cell volume) were incubated at 37°C with 50 pm [125I]iodoinsulin and pig insulin as indicated. The incubations were carried out for the following time period. Albumin, 0.5 mg/ml ( ), 10 min; albumin, 3 mg/ml ( ), 30 min; 10 mg/ml ( ), 70 min; and 50 mg/ml ( ), 130 min. The incubations were terminated by the addition of albumin to a final concentration of 50 mg/ml (when needed) and trichloroacetic acid to a final concentration of 12% w/v. The radioactivity in the soluble and precipitable fractions was determined in the samples with cells and in buffer incubated for the same time as the samples. The increase in solubility of the labeled material above that in buffer was calculated on triplicate samples.
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Uptake of Labeled Degradation Products—It is difficult to evaluate binding data in suspensions of cells with high protease activity because the concentration of free insulin decreases. Furthermore, as shown in Fig. 5, labeled degradation products are rapidly accumulated in adipocytes incubated with albumin 50 mg/ml and insulin 1 μM, i.e. a suspension which does not bind or degrade 125I-labeled insulin significantly. Fig. 6 shows the uptake of 125I activity in cells suspended to a packed cell volume of 10% in buffer with albumin 10 mg/ml. The radioactivity associated with the cells increased slowly in the presence of 100 nM insulin, i.e. a concentration more than 30-fold higher than the apparent Kd for insulin binding although not high enough to block protease-mediated degradation of 125I-labeled insulin. In the presence of tracer alone the cell-associated radioactivity first increased and then declined. After incubation for 60 min 65% of the radioactivity in the medium was soluble in trichloroacetic acid and more than half of the cell-associated radioactivity was non-insulin (data not shown).

Lack of Effects of Albumin on Insulin Binding and Action—The finding that albumin inhibits total insulin degradation markedly prompted us to study whether it would also influence the binding and the effect on glucose metabolism. Table I shows that insulin binding is the same in cells incubated with albumin 50 mg/ml or 10 mg/ml, respectively, provided that the total insulin degradation is low. The maximal effect of insulin and the insulin dose-response relationship with respect to conversion of [3-3H]glucose to lipids was similar when dilute cell suspensions were incubated with albumin 0.5 mg/ml, 10 mg/ml, or 50 mg/ml, respectively (Fig. 7). It is concluded that high concentrations of albumin can prevent a large part of the protease-mediated insulin degradation without interfering with the binding of insulin or its effect on the conversion of labeled glucose to lipids.

Steady State of Binding—The following experiments were designed to study whether a constant binding of 125I-labeled insulin (i.e. a constant concentration of substrate for any receptor-mediated degradation) could be maintained for a prolonged period of time and whether the cell-associated radioactivity was in fact iodoinsulin. Fig. 8 shows that a steady state of binding of 50 PM labeled insulin (occupancy about 2%) was achieved at about 40 min and maintained for more than 2 h. The same level of binding was obtained when tracer was

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**Table I**

Effect of bovine serum albumin on binding and degradation of 125I-labeled insulin

<table>
<thead>
<tr>
<th>Albumin</th>
<th>% bound (1% cells)</th>
<th>% degraded (10% cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% w/v</td>
<td>0.162 ± 0.021</td>
<td>37.2 ± 3.1</td>
</tr>
<tr>
<td>5% w/v</td>
<td>0.151 ± 0.011</td>
<td>7.1 ± 1.0</td>
</tr>
</tbody>
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**Fig. 8.** The steady state of binding. Cells (4% v/v) were incubated in buffer with albumin 50 mg/ml. 125I-labeled insulin (50 μM) was added at time zero (Δ--Δ) or at time 90 min (X--X). Some samples contained in addition 0.8 mM (●) or 1 μM (○) insulin. Some samples were first incubated with tracer alone (35 to 120 min); then insulin was added to a final concentration of 1 μM, and the incubation was continued for 60 min (▲).

**Fig. 9.** Elution profile of extracted radioactivity on Sephadex G-50 fine. Cells (3% v/v) were incubated for 30 (X--X), 60 (○--○), or 180 min (●--●), respectively, in 1.2 ml of buffer with albumin, 50 mg/ml. Seven hundred microliters of the infranatant buffer was removed, saline and oil (10°C) added, the tubes centrifuged, and the cells were placed in extraction fluid (acetic acid 3 M, urea 6 M) and whirl mixed immediately. The gel filtrations were carried out at 4°C in 0.5 M acetic acid with albumin 1 mg/ml. The upper panel shows the elution of the extracts on a column (1.5 X 90 cm). The lower panel shows the elution profile of the 125I-labeled insulin preparation (●--●) and of unlabeled iodoypyrine (○--○). Kav = 0 indicates the void volume and Kav = 1 the total column volume (“salt peak”).
added at time 90 min. A steady state was also maintained for at least 2 h with a total insulin concentration of 0.8 nM ( occupancy about 20%). Addition of 1 μM insulin to samples containing tracer alone caused a decrease in the counts after 90 min to the level of the binding obtained when 1 μM insulin was present from the beginning of the incubation. Fig. 9 (upper panel) shows the gel filtration profile of material extracted from cells incubated with 100 pm 125I-labeled insulin. Ninety-five per cent of the radioactivity was eluted in the same way as the 125I-insulin preparation (lower panel), whereas about 5% was eluted with the same Kav as iodotyrosine. The radioactive material from the main peak was incubated with dilutions of anti-insulin serum and as shown in Fig. 10 the same binding curve was obtained with this material and with 125I-labeled insulin. In some experiments samples obtained after incubation with tracer plus insulin 1 μM were extracted and the radioactivity emerged nearly exclusively in the insulin peak (data not shown). It is concluded that under incubation conditions as described about 95% of the bound radioactivity throughout a 180-min incubation period represents iodoinsulin which is either not modified or only slightly modified.

Degradation of Bound Insulin—Fig. 11 shows results of experiments in which the cells were first incubated with tracer for 45 min, washed, centrifuged through oil, and ejected into a large volume of medium containing insulin 1 μM. The label is released into the medium in two components, one soluble in trichloroacetic acid (55% of the total radioactivity) and one for 45 min, washed, centrifuged through oil, and ejected into a large volume of medium containing insulin 1 μM. The label is released into the medium in two components, one soluble in trichloroacetic acid (55% of the total radioactivity) and one

![Graph](image)

Fig. 11. Release of trichloroacetic acid-precipitable and -soluble material. Cells were incubated for 45 min with 125I-labeled insulin, 100 to 200 pmol/liter. The infranatant medium was removed after a slight centrifugation, the cells were washed once, concentrated and centrifuged through oil in a thin polyethylene tube. The tube was cut through the oil, the part containing the cells mounted on a syringe and the cells ejected into the washout medium. Samples (300 μl each) were added to microfuge tubes and centrifuged. The first sample was taken after about 30 s, and the following samples were corrected for radioactivity in that medium. △, total radioactivity; ○, activity precipitable in trichloroacetic acid; □, activity soluble in trichloroacetic acid. The curves represent the mean of nine experiments.
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precipitable in trichloroacetic acid. It appears that the half-
time for release of precipitable material is slightly shorter
than that for soluble material. It was noted in several experi-
ments that with a given batch of cells the same ratio of
precipitable/soluble material was obtained with tracer pre-
dominantly labeled in the A14 and with tracer labeled in the
A19 residue.

In order to study the nature of the released radioactive
material, cells from the top of the oil layer in a 14-ml tube
were resuspended in 1 ml of buffer with albumin 50 mg/ml
and incubated for 60 min. The suspension was centrifuged
and the clear medium applied to gel filtration. As shown in Fig. 12
the radioactivity appeared in two major peaks representing
the trichloroacetic acid-precipitable and -soluble material.
The first peak co-eluted with iodoinsulin, and the radioactivity
was bound to anti-insulin in the same proportion as

$^{[125I]}$-labeled insulin (Fig. 13'). The other peak co-eluted with
monoiodotyrosine and, as shown in Fig. 14, the radioactive
material moved together with iodotyrosine on paper chroma-
tography in two solvent systems. It is concluded that about
half of the cell-associated radioactivity (bound iodoinsulin) is
released from the cells as iodotyrosine and the other half as
iodoinsulin.

Degradation of Bound Insulin as a Function of Occupancy—Fig. 15 (upper panel) shows the amount of radioac-
tivity released from the cells in trichloroacetic acid-precipita-
ble and -soluble form as a function of the insulin concentra-
tion in the association phase. The sum of the two fractions reflects
the amount of tracer bound to the cells at steady state. In the
presence of 2 μM insulin nearly all radioactivity was released in
the trichloroacetic acid-precipitable form both without
incubation and after 45 min at 37°C. It should be noted that
binding of tracer plus unlabeled insulin was practically iden-
tical in the concentration range 0.25 to 5.0 pM (data not
shown). The percentage of the radioactivity released in soluble
form was independent of the occupancy when correction for
binding of tracer in the presence of insulin 1 μM was carried
out (numbers in parentheses). The degradation of receptor-
bound insulin is therefore proportional to the receptor occupa-
cy. Fig. 15 (lower panel) shows that this is also the case in
the very low concentration range where the ratio bound-free
is nearly independent of the total insulin concentration. In
addition it is seen that the dose-response relationship of the
tracer with respect to the conversion of glucose to lipids lies in
this range.

**DISCUSSION**

The Receptor-independent Degradation—The apparent
$K_m$ values of 80 nM for pig insulin and 160 nM for pig proinsulin
are in agreement with the values reported for a protease with
specificity for insulin extracted from muscle and liver (15, 16),
and the receptor-independent degradation of insulin in adi-
pocytes may therefore be accounted for by a similar protease.
We have previously found that insulin-degrading activity may
be released from isolated adipocytes and epididymal fat pads
(14). Hammond and Jarett (17) found that treatment of fat
cells with trypsin (150 to 250 μg/ml for 15 min) markedly
inhibit the insulin degradation by adipocytes, and we have
observed that degradation is abolished after treatment of the cells
with trypsin 1 mg/ml for 15 min, i.e. under conditions
where basal glucose transport is nearly normal and the effect
of insulin is abolished (18). Crofford et al. (19) showed that
adipocyte plasma membranes degrade insulin and that this
activity is abolished in membranes prepared from trypsin-
treated cells. These results suggest that the enzyme system is
loosely bound to the adipocyte plasma membrane.

It is important to minimize insulin degradation in order to
avoid a marked decrease in the concentration of free insulin
in the medium. The finding that labeled degradation products
are taken up by the cells shows that control of the degradation
is even more critical since the accumulation of degradation
products may be misinterpreted as related to receptor binding.
Moreover, the formation of labeled degradation products is
inhibited by unlabeled insulin according to the apparent
$K_m$ of 70 to about 400 nM (depending on the albumin concen-
tration) showing a decrease in the radioactivity associated with
the cells as a function of the insulin concentration in this high
range. In the analysis of “steady state” binding curves this
may be misinterpreted as evidence for low affinity receptors
or negative homotropic cooperativity.

Fig. 15. Degradation of receptor-bound insulin as a function of the
receptor occupancy. **Upper panel**, cells were incubated for 40 min
with $^{[125I]}$ labeled insulin, 200 pmol/liter, and insulin as indicated. They
were recovered after the addition of 10 ml of 9% NaCl and oil
followed by centrifugation and reincubated for 60 min in buffer with
insulin, 2 μM/liter. The cells were removed by centrifugation, trich-
loroacetic acid (final concentration, 12% w/v) was added to the
medium, and the soluble (C—O) and precipitable (■—■) fractions
were assayed for radioactivity. C and ■ indicate the medium from
cells recovered above the oil immediately after the addition of
tracer followed by reincubation for 60 min. The symbols represent
the mean of four replicates ± S.D. when this exceeds the size of the
symbol. The numbers in parentheses indicate the percentage of
released radioactive material soluble in trichloroacetic acid after
subtraction of the radioactivity in the medium of samples containing
insulin, 2 μM/liter, in the association phase. **Lower panel**, the design
was similar to that described above, and the numbers in parentheses
(with S.D., n = 5) indicate the percentage of released radioactive
material soluble in trichloroacetic acid when the cells had been
incubated with 30 pmol/liter and 60 pmol/liter of $^{[125I]}$-labeled insulin,
respectively. The effect of the labeled insulin preparation on the
conversion of $[14C]$glucose to lipids was measured as described under
"Materials and Methods."

$^3$ O. Sonne and J. Gliemann, unpublished observation.
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Binding Characteristics when the Total Degradation Is Low—Under these conditions a steady state of binding was maintained for at least 140 min, and 95% of the cell-associated radioactivity was eluted in the same way as the 125I-labeled insulin preparation whereas 5% co-eluted with iodothyrosine. This may represent iodothyrosine formed as the result of receptor-mediated degradation of iodoinsulin and accumulated in the suspended cells. However, it cannot be excluded that this small peak is an artifact in the sense that some receptor-bound iodoinsulin was degraded in the oil phase (even though this was chilled) after separation of the cells from the medium.

Kahn and Baird (20) recently reported that a steady state of binding was not obtained at 37°C. The binding of 125I-labeled insulin showed a maximum at 10 to 15 min and declined to about 70% of this value at 90 min. When binding was maximal about 35% of the cell-associated radioactivity was eluted after the total column volume on Sephadex G-50. The binding of 125I-labeled insulin in the presence of 1 μM insulin increased slowly with time. Finally, Kahn and Baird (20) noted a time-dependent increase in radioactivity eluting in the void volume whereas we found no change in this fraction even after incubation for 180 min. The discrepancies between our results and those of Kahn and Baird are probably due to differences in incubation and cell separation techniques.

Degradation of Receptor-bound Insulin—The findings suggest the following model for the steady state interaction of (iodo)insulin with adipocytes. The bound insulin is either released to the medium or degraded. At 37°C, pH 7.4, the rate constants for these processes are approximately equal and independent of the receptor occupancy. Hypothetical labeled peptides (containing residue A14 and/or A19), which may be produced initially, are rapidly degraded, and iodothyrosine is therefore the only labeled product which can be identified. The size of the labeled iodothyrosine pool in the cells is 5% (or less) of the cell-associated radioactivity.

The molecular mechanism of receptor-mediated insulin degradation is not known. It is possible that the receptor and the degrading enzyme are subunits of the same macromolecule in the plasma membrane; the fact that temperatures below 20°C markedly depress degradation of receptor-bound insulin (5, 7, 8, 21) might in that case be explained by an obligatory, temperature-sensitive transformation of the macromolecule. It is also possible that the receptor transfers some insulin molecules to a degrading site in the membrane. There is some evidence that insulin bound to adipocytes is compartmentalized at physiological temperature. Thus, Kono et al. (22) found that when adipocytes were incubated with labeled insulin for 5 min at 15°C and homogenized, essentially all bound insulin was recovered in the plasma membrane fraction in sucrose gradients whereas about one-third of the radioactivity was recovered in an unidentified fraction when the incubation was carried out at 37°C. Kahn and Baird (20) observed that insulin bound to adipocytes became progressively less available for removal by trypsin or dilute acid (pH 6) during incubation at 37°C. This phenomenon was also temperature-dependent.

It seems less likely that the receptor-insulin complex is internalized in a way similar to that reported for epidermal growth factor (23) and low density lipoproteins (24). A steady state of binding of epidermal growth factor to fibroblasts is not obtained because the internalized receptor is degraded by lysosomal enzymes (23), and this is in marked contrast to prolonged steady state obtained in the present study even with a receptor occupancy of about 20%. Furthermore, NH4Cl (10 mM) or chloroquin (10 μg/ml), which is known to block degradation by lysozymes in hepatocytes (25), increase the binding of epidermal growth factor markedly whereas we have observed no change in the steady state binding to fat cells incubated under conditions where total insulin degradation is small. In the case of low density lipoproteins a steady state can be obtained, apparently because the internalized receptor is effectively recycled and only a small fraction degraded (24). However, in the steady state only 15 to 20% of the receptors are on the cell surface.

Irrespective of the mechanism, the finding that receptor-bound insulin is degraded raises the possibility that this process is related to one or more of the insulin effects in adipocytes, for instance that on hexose transport. The results also imply that binding data cannot be adequately described by equations derived from the law of mass action. The steady state binding will increase if the rate of degradation of receptor-bound insulin decreases, and this is at least in part the explanation for the increase in the steady state binding at lower temperature (7). It seems possible that receptor-mediated degradation is changed under other conditions which are known to influence binding, e.g., variations in pH of the incubation medium or variations in the metabolic state of the donor animal. Studies designed to answer these questions are in progress.

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