Lysosomal Association of Internalized

\(^{125}\)I-Insulin in Isolated Rat Hepatocytes

DIRECT DEMONSTRATION BY QUANTITATIVE ELECTRON MICROSCOPIC AUTORADIOGRAPHY

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ABSTRACT Quantitative electron microscopic autoradiographic studies in cultured human lymphocytes and isolated rat hepatocytes have demonstrated that labeled insulin initially localizes to the plasma membrane and is subsequently internalized to a limited region of the peripheral cytoplasm.

When 0.5 nm \(^{125}\)I-insulin is incubated with isolated rat hepatocytes, binding to the plasma membrane occurs at both \(20^\circ\)C and \(37^\circ\)C. Under steady-state binding conditions \(\approx 30-40\%\) of the labeled hormone is internalized to a distance of \(\approx 15\%\) of the radius of the cell. When the localization of the internalized labeled material is analyzed, by 2–5 min of incubation at \(37^\circ\)C there is a fivefold preferential association of autoradiographic grains with lysosomal structures, and by 30–60 min of incubation at \(37^\circ\)C there is a 10-fold preferential association. When the cell-associated radioactivity is extracted and filtered on Sephadex G-50 at each time point of incubation, radioactivity elutes predominantly in the position of \(^{125}\)I-insulin and is predominantly tri-chloracetic acid precipitable, bindable to t alc, and rebindable to liver membranes.

With increasing time of association at \(37^\circ\)C the initial rate and absolute amount of labeled material dissociable from the cell is reduced. With increasing time of dissociation both the cell-associated radioactivity and the radioactivity released into the incubation medium is progressively degraded.

These data demonstrate that in isolated rat hepatocytes labeled insulin initially localizes to the plasma membrane, is progressively internalized, and associates preferentially with lysosomal structures. These events may provide a mechanism that links cell surface binding to the degradation of insulin and to insulin-induced loss of its specific receptor.

INTRODUCTION

When insulin binds to specific surface receptors on target cells several diverse effects take place. First, a variety of biologic responses occur, the nature of which depend on the cell. Second, there must be a mechanism of terminating the stimulatory effect. Thus there is degradation or inactivation of the hormone. Finally, insulin exerts a regulatory effect on its specific receptor that may lead to either an alteration of the affinity (1) or of the concentration of the receptor (2).

The steady-state binding of insulin to its receptor has been visualized as an equilibrium between the receptor on the cell surface and its external environment (including a component of noncompetitive binding [non-specific binding], and degradation of the labeled ligand). When the binding process is directly visualized, as can be done by combining quantitative electron microscopic autoradiography with direct binding studies, there is an additional component of the process that is represented by an intracellular translocation of the labeled hormone. Our initial studies involved the cultured human lymphocyte of the IM-9 line (3). We demonstrated that labeled insulin initially localized to the plasma membrane but at \(37^\circ\)C a small component was translocated intracellularly. Further studies in isolated hepatocytes
have demonstrated that labeled insulin initially localizes to the plasma membrane in a similar fashion to the cultured human lymphocyte but as a function of time at 37°C there is a systematic and progressive translocation of the labeled hormone intracellularly (4, 5).

Because the liver is a primary target for the biologic, degradative, and receptor regulatory effects of insulin, we have studied the visual events of binding in detail. In the present study we have (a) characterized the isolated hepatocyte morphologically, (b) determined whether labeled insulin binds to specialized regions of the cell surface (i.e., coated segment), (c) defined the intracellular localization of the labeled material, (d) investigated the nature of the cell-associated and dissociated radioactivity, and (e) studied the dissociation of cell-bound radioactivity as a function of association time and temperature.

METHODS

Cells and reagents. Hepatocytes were isolated from normal 6- to 8-wk-old Wistar rats fed ad libitum by a modification of the method described by Seglen (6). The salient features of the method are: (a) nontraumatic cannulation of the portal vein, (b) immediate blanching of the liver upon perfusion of the wash solution; perfusion without recirculation (50 ml/min) of 200 ml of 10 mM Hepes buffer (pH 7.8) at 37°C, (c) perfusion with recirculation of buffer that contained from 0.25 to 0.50 mg/ml collagenase (Sigma Chemical Co., St. Louis, Mo. or Worthington Biochemical Corp., Freehold, N. J.) and 5–10 mM CaCl₂ for 6–10 min, (d) liver is teased apart and cell suspension filtered through three layers of cheese cloth followed by centrifugation at 50 g for 1 min and resuspension in Krebs-Ringer bicarbonate buffer (KRB)¹ repeated four times, and (e) the final cell pellet is resuspended in 40 ml of buffer in a Falcon cell culture flask (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and cells are gassed with 95% O₂ and 5% CO₂. The percentage of parenchymal cells (hepatocytes) in the purified cell suspension exceeds 98%; the viability of the cell suspension estimated by the trypan blue exclusion and by morphological criteria (peripheral refractoriness of intact cells under the light microscope) was 85–95%. ¹²⁵I-insulin was prepared at a specific activity of 250 μCi/μg by a modification of the chloramine T method (7). The labeled insulin was purified by filtration on Sephadex G-50 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) at 4°C before each experiment.

Incubation conditions. Hepatocytes at a final concentration of 1 × 10⁶ cells/ml were incubated in duplicate in 0.5 ml of modified KRB (pH 7.7) that contained 25 mg/ml bovine serum albumin (fraction V) and 0.8 mg/ml of bacitracin with 0.5 mM (3 ng/ml) ¹²⁵I-insulin at 30° and 37° for varying periods of time. Bacitracin was added to prevent insulin degradation in the medium during exposure to hepatocytes (8). Identical incubations were carried out in the presence of 27 μM unlabeled insulin to determine nonspecific binding (i.e., cell-associated radioactivity in the presence of an excess of unlabeled hormone). At the times indicated, 1 ml of chilled buffer was added to each tube, immediately followed by centrifugation for 20 s at 50 g. The supernate was discarded and the cell pellet quickly resuspended in 1 ml of chilled buffer and centrifuged for 90 s at about 500 g in a Beckman plastic microtube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Cell pellets were further washed (without resuspension) in chilled buffer that contained 100 mg/ml of sucrose. At the end of the wash procedure, 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and the radioactivity in duplicate cell pellets were determined.

Analysis of cell-associated radioactivity. In a separate set of incubations at 20° and 37°C, cells were centrifuged and washed as described under incubation conditions. The washed pellet was then extracted with a mixture of 0.1% Triton X-100 (Rohm & Hass Co., Philadelphia, Pa.), 3 M acetic acid, and 6 M urea. The mixture was centrifuged in a Beckman microtome at 12,000 g for 5 min at room temperature and 95–98% of the total cellular radioactivity was found in the supernate. The supernate was applied to a Sephadex G-50 fine column (0.9 × 50 cm), which was equilibrated and eluted by the extraction mixture or by 1 M acetic acid. 1-ml samples were collected on an automated fraction collector. Precipitation by 10% trichloroacetic acid (TCA), adsorption to talc, and specific binding to rat liver membranes were carried out as described (9), except that the binding assay was conducted for 60 min at 30°C with plasma membranes at 0.2 mg protein/ml.

Analysis of dissociation of cell-associated radioactivity. A separate set of incubations were carried out exactly as described above except that ¹²⁵I-insulin concentrations were between 5 and 7 ng/ml. At the end of the association period 1 ml of chilled buffer was added to each tube and centrifuged at 50 g for 30 s. The supernate was aspirated, the cells resuspended, and the centrifugation repeated. The cell pellet was resuspended in 3 ml of chilled buffer and a 0.2-ml aliquot of this suspension was further diluted to 10 ml in the absence (dissociation by dilution alone) and in the presence of unlabeled insulin (1 μg/ml) in the dilution buffer (dissociation by dilution plus unlabeled insulin). Dissociation of hepatocyte-bound radioactivity was then allowed to proceed at 30°C. Experiments at 37°C were performed in the absence and in the presence of bacitracin (0.8 mg/ml) in incubation medium during incubation. At the longest exposure time points the cell suspension was filtered on cellulose acetate 1-μm Millipore filters (Millipore Corp., Bedford, Mass.) as described (7).

Properties of radioactivity dissociated from hepatocytes. In a separate set of incubations at 37°C, ¹²⁵I-insulin at 16–20 ng/ml was incubated with hepatocytes for 30 min at 37°C (as described under incubation conditions). At the end of incubation, cells were sedimented by centrifugation and the supernatant media were collected and stored at −20°C. The cell pellets were washed, quickly resuspended in 3 ml of insulin-free buffer, and dissociation of hepatocyte-bound radioactivity was allowed to proceed at 37°C. At the times indicated, cells were sedimented by centrifugation and the supernatant media collected as indicated above. The properties of cell-dissociated radioactivity were analyzed by gel filtration, TCA precipitation, t alc adsorption, and receptor binding to liver membranes (as described under analysis of cell-associated radioactivity). In a parallel set of incubations, the washed cell pellets were extracted and analyzed by gel filtration as described above. Bacitracin (0.8 mg/ml) was present in the incubation buffer throughout association and dissociation to prevent insulin degradation in the medium during exposure to hepatocytes (9).

Preparation for electron microscopy and autoradiography.

¹Abbreviations used in this paper: EGF, epidermal growth factor; EM, electron microscopy; KRB, Krebs-Ringer bicarbonate buffer; LDL, low density lipoprotein; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; TCA, trichloroacetic acid.
RESULTS

General characteristics of the isolated hepatocytes. The hepatocytes used for these experiments are 22.0 ± 0.1 μm in diameter and are typical of other isolated hepatocytes that have been morphologically characterized (14, 15). The cells were well isolated and the surface was morphologically intact as seen by scanning electron microscopy (EM) (not shown) and in thin section by transmission EM (Fig. 1). In addition to their surface membrane structure, the intracellular organelles of these cells were well preserved (Fig. 1). Further evidence of functional integrity of these cells was demonstrated by their ability to bind labeled insulin and glucagon (15) and to respond metabolically to catecholamines (16), glucagon (17, 18), corticosteroids (19), and most importantly, insulin (20).

Relationship of grains to cellular structures. Grains that initially localize to the plasma membrane at 5 min of incubation at 20°C were frequently related to microvillous structures. Because other ligands, i.e., low density lipoprotein (LDL), have been shown (21–23) to localize to specialized structures of the membrane called coated invaginations, we examined photographs of grains that were either on the plasma membrane or ≥200 nm from the plasma membrane for localization to coated segments. Though coated invaginations are easily detected on the liver plasma membrane (24), no localization of autoradiographic grains to these structures were apparent in an examination of over 250 photomicrographs taken from 5 min of incubation at 20°C (Fig. 2).

To determine whether grains appearing beyond 300 nm of the plasma membrane have a preferential localization to cytoplasmic organelles, we determined by morphometric means the volume density of the glycogen, mitochondria, lysosomal structures, microbodies, SER, RER, lipid droplets, and nucleus as well as the percentage of grains related to these organelles within this same region of the cell (Figs. 1 and 3). To be certain that there was no major redistribution of organelles induced by labeled insulin these determinations were carried out in three experimental conditions (2–5 min and 30–60 min of incubation at 37°C, and 90–120 min at 20°C) where translocations of grains were observed. Morphometric data shown in Table I indicate that the volumetric density of most of the intracellular organelles studied at the cell periphery did not change significantly with time or temperature of incubation.

Note, however, that the volumetric density of glycogen was significantly higher after 90–120 min at 20°C than at 37°C. Values obtained were also compared to data published by Weibel et al. (25) and referred to the total hepatocyte studied in situ. To the extent that our data can be compared to data from the whole cell, there is the suggestion that lysosomes and SER were more fre-
FIGURE 1 General view (×9,000) of an isolated hepatocyte in thin section. The dashed line is drawn at 1.6 μm from the plasma membrane to illustrate the maximum penetration of radiation. Because the radius of the cell represents about 22 μm this distance represents no more than 15% of the cell radius. (For the assumption made in this figure, see Fig. 1 in reference 3).

quent at the hepatocyte periphery and that RER was less abundant in this region.

To determine if there was a preferential localization of autoradiographic grains to specific intracellular organelles, we calculated the ratio of the percentage of grains related to organelles over percentage of organelles within the periphery of the hepatocyte. After only 2–5 min of incubation at 37°C there was a fivefold increase in the ratio of grains associated with lysosomal structures and this increased to 10-fold by 30–60 min of incubation (Figs. 3 and 4). A significant preferential localization of grains to lysosomes was also observed at 90–120 min at 20°C. By contrast, there was no preferential localization to structures of similar size such as microbodies or other organelles such as mitochondria, SER, RER, or glycogen. Less than 1% of grains were associated with the nucleus and fat droplets.

We wished next to determine if a particular class of
lysosomal structure was preferentially labeled. We first measured (in random pictures unselected for autoradiographic grains) the mean diameter of all lysosomes seen in peripheral cytoplasm (Fig. 3). The range of values and distribution is shown in Fig. 5A. We next determined the mean diameter of all lysosomal structures that were previously scored by the probability circle (see above). Note that autoradiographic grains were related to a class of lysosomal structures of relatively uniform size (Fig. 5B).

Analysis of cell-bound radioactivity. It has been previously reported that 125I-insulin exposed to isolated hepatocytes (8) or purified liver membranes (9) is progressively degraded in a temperature-dependent fashion. At 37°C, this degradation is minimized when bacitracin is added to the incubation medium (Table II).\(^2\) The degradation of 125I-insulin during its exposure to hepatocytes can be accounted for largely by the release of degradative material(s) (presumably, proteolytic enzymes) into the medium. The nature of the radioactive products in the incubation media has been investigated (8) but the nature of the exclusively cell-associated radioactivity has not been studied.

Because autoradiography measures total cell-associated radioactivity, it is essential to characterize the nature of the cell-associated radioactivity. At the end of the incubation periods, shown in Table III, cells were centrifuged, separated from the assay buffer, washed, and the pellet dissolved in a mixture of Triton, urea, and acetic acid. Under these conditions 95–98\% of the cell-associated radioactivity was extracted and applied to a Sephadex G-50 column. For incubations carried out at 20°C, 81–87\% of the radioactivity eluted in the position of 125I-insulin (Fig. 6, Table III). The radioactivity eluting in this peak was 94–97\% TCA precipitable, 87–92\% bindable to talc, and predominantly rebindable to liver membranes (Table III). The remainder of the radioactivity eluted after the iodide marker in the position of iodotyrosyl peptides that loosely adsorb to the

\(^2\) To exclude the possibility that results on internalization or morphometric analysis were caused by either bacitracin or some potential artifact of the collagenase isolated hepatocytes, two different kinds of experiments were carried out. First an experiment was carried out at 37°C at 30 min of incubation in bacitracin-free media. The results were essentially identical to the 30-min time point shown in (5) and to data in Fig. 4. Second, a similar type of study was performed in intact rat liver 10 min after the intraportal injection of 125I-insulin and again the results were very similar (Carpentier, J-L., P. Gorden, P. Barazzzone, P. Freychet, A. Le Cam, and L. Orci. 1979. Intracellular localization of 125I-insulin in hepatocytes from intact rat liver. Proc. Natl. Acad. Sci. U. S. A. In press.) Bergeron et al. (26) have reported in abstract form that 10 min after the injection of labeled insulin into the rat that autoradiographic grains are internalized and, although no quantitative data is presented, it is stated that grains overlie lysosomes, the Golgi apparatus, other organelles and plasmalemma.

![Figure 2](image-url) View (×52,000) of developed autoradiographic grain and coated invagination (see arrow). In an evaluation of 250 photomicrographs from 5 min of incubation at 20°C, we found only two grains associated with coated invaginations with techniques identical to ones used in previous studies that have shown significant associations in human fibroblasts (30, 47).
Although it has been clearly shown that degradation products of I25I-insulin do not bind to the insulin receptor (9), we wished to be certain that these products were not taken up by the cell in some other way during the course of our 37°C incubation. When I25I-insulin was preincubated with hepatocytes for 60 min at 37°C

![FIGURE 3 View (×27,000) of the periphery of isolated hepatocytes incubated for 60 min at 37°C with I25I-insulin. In these selected pictures, internalized autoradiographic grains are either over or close to lysosomal structures. We have considered as lysosomal structures membrane bounded intracytoplasmic organelles known to exhibit a lysosomal activity, i.e., dense bodies, multivesicular bodies, autophagosomes, glycogenosomes, etc. Most of the intracytoplasmic structures analyzed by morphometry and by the probability circle method are illustrated at the same magnification as that used for these quantitative determinations. m, mitochondria; Ly, lysosomes; Gly, glycogen.]

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Volumetric Densities of Intracellular Organelle at the Periphery of Isolated Hepatocytes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2–5 min 37°C (n = 48)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>23.3±1.2</td>
</tr>
<tr>
<td>Microbodies</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>SER</td>
<td>18.8±1.4</td>
</tr>
<tr>
<td>RER</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>Glycogen</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Lipid droplet</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>Remaining cytoplasm</td>
<td>45.4</td>
</tr>
</tbody>
</table>

n, number of pictures analyzed.
in the absence of bacitracin to allow for degradation and then incubated with fresh cells, binding was only one-fifth of that seen with both fresh tracer and fresh cells (Table V). Furthermore, when free $^{125}$I-insulin was incubated with fresh hepatocytes essentially none of the radioactivity associates with the cell (Table V).

Results represent the mean of duplicate measurements.

* $^{125}$I-insulin (3 ng/ml) was exposed to hepatocytes ($10^6$ cells/ml) for 60 min at 37°C in the absence or presence of bacitracin (0.8 mg/ml). Cells were then sedimented by centrifugation and the radioactivity in the supernate was analyzed for its ability to specifically rebind to fresh liver membranes, as indicated in Methods.

<table>
<thead>
<tr>
<th>Bacitracin</th>
<th>Cells plus medium*</th>
<th>Cell-free medium†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>+</td>
<td>64</td>
<td>77</td>
</tr>
</tbody>
</table>

Because glutaraldehyde fixation involves cross-linking of proteins, it is likely that low molecular weight substances such as iodide or small iodo-peptides are not fixed to the cell. We found that a small component of radioactivity was lost from the cell pellet before processing and that an additional component was removed by osmic acid treatment and the successive alcohol washes during the processing phase (Table V). The radioactive material lost from the cell pellet was over 85% unbound by talc. Essentially all of the trivial amount of iodide associated with the cell was lost in the processing step (Table V).

Taken together, these studies suggest that most of the radioactivity that was recorded in the emulsion of our autoradiograms was derived from material with a molecular size similar to native insulin.

**Analysis of dissociation of cell-bound radioactivity.**

$^{125}$I-insulin progressively associated with hepatocytes as a function of time at both 20°C and 37°C. At any given time point a proportion of the cell-bound radioactivity was related to the plasma membrane and the remainder had shifted intracellularly and was preferentially associated with lysosomes. To determine the proportion of the cell-associated radioactivity that was dissociated from the cell after varying periods of association at 20°C and 37°C, cells were incubated with $^{125}$I-insulin for times shown in Fig. 7. At the end of
the association periods, cells were centrifuged, washed, and resuspended in a large volume of buffer without (dilution alone) or with 1 μg/ml of unlabeled insulin (dilution plus unlabeled insulin) and incubated at 30°C for the time periods shown (Fig. 7).

For the 20°C association, 90% or more of the labeled material was dissociated from the cell regardless of the time of association. There was, however, a small decrease in the total amount of labeled material dissociable at the 90- and 120-min association periods. For all periods of association the rate of dissociation was increased to a small extent in the presence of unlabeled insulin. The 37°C association differed in two ways. The initial rate of dissociation (dissociation after 15 min) and the total amount dissociated (dissociation after 120 min) decreased as a function of association time. The small degree of accelerated dissociation in the presence of unlabeled insulin was similar at both the 20° and 37°C association temperature (Fig. 7). When bacitracin was omitted from the incubation medium, binding fell rapidly after 30 min but the dissociation characteristics were similar to the experiment that included bacitracin (Fig. 7).

Properties of radioactivity dissociated from hepatocytes. Because most of the degradation of 125I-insulin exposed to hepatocytes appears to be related to the release of degradative material(s) into the incubation medium, it was essential to minimize this effect when investigating the properties of the radioactivity released from the cell. In the presence of bacitracin, the degradation of 125I-insulin after a 60-min exposure to the medium at 37°C did not exceed 23% (Table II). Experiments designed to analyze the properties of the radioactivity released from the cell were performed at 37°C with bacitracin present throughout both the association and dissociation periods. Tables IV and VI indicate the properties of the radioactivity released from hepatocytes at varying times over a 60-min period of dissociation after a 30-min association at 37°C. By a variety of criteria (gel filtration profile, TCA precipitation, tacle adsorption, and receptor binding) there was a progressive loss of integrity of 125I-insulin released from the cells. Whereas at zero dissociation time (that is, after 30-min exposure to hepatocytes) 76% of the radioactivity in the medium represented intact 125I-insulin by the most sensitive analysis, i.e., its ability to specifically rebind to liver membrane receptors (9), the proportion of intact insulin in the radioactivity subsequently released over 15-, 30-, and 60-min dissociation periods decreased to 49, 41, and 32%, respectively (Table VI). This extent of degradation largely exceeds that observed when 125I-insulin was exposed for 60 min to a cell-free medium (77% insulin remaining intact, see Table II) and presumably reflects, partly at least, a degradative process (or processes) related to the association of 125I-insulin with the hepatocyte. Furthermore, because this degradation was observed in a fresh medium (i.e., not previously incubated with hepatocytes) and because, in these experiments, the dilution factor reduced the cell concentration fourfold over that present in the association period, it is likely that most of the degradation observed in the dissociation medium was contributed by a cell-related degradative process (or processes). This conclusion is also supported by the observation that, as dissociation proceeded, an increasing proportion of the cell-associated radioactivity appeared to consist of degraded insulin, as denoted by the gel filtration profiles of cell extracts at the latest time points of dissociation (Table IV).

DISCUSSION

We have drawn the following conclusions from the present study: (a) In isolated rat hepatocytes considered to be morphologically and functionally intact, 125I-insulin initially localized to noncoated regions of the plasma membrane and was internalized to a limited area of the cell peripheral cytoplasm (10–15% of the

* The standard conditions are as described in Methods and the medium contains bacitracin, however, in the absence of bacitracin the characteristics are essentially identical.
1 Percentage of elution from Sephadex G-50 for peaks shown in Fig. 6.
2 Additional experiments demonstrate that lyophilized extracts of peak II radioactivity was from 60 to 100% bindable to liver membranes as compared to fresh 125I-insulin exposed to the same solvent and lyophilization conditions.

<table>
<thead>
<tr>
<th>Table III</th>
<th>Characteristics of Cell-Associated Radioactivity at 20°C under Standard Conditions of Incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Sephadex 1 TCA Talc</td>
<td>Sephadex 1 TCA Talc</td>
</tr>
<tr>
<td>Peak I</td>
<td>0.3</td>
</tr>
<tr>
<td>Peak II</td>
<td>80.6</td>
</tr>
<tr>
<td>Peak III</td>
<td>7.8</td>
</tr>
</tbody>
</table>

TABLE III

Characteristics of Cell-Associated Radioactivity at 20°C under Standard Conditions of Incubation*
and degraded materials but the relative proportion of the latter (presumably idotyrosyl peptides for the most part) increased as dissociation proceeded.

These data confirm previous studies demonstrating that the initial interaction of insulin with the cell is binding to a specific plasma membrane receptor (27) (references 1–12 in reference 3). It is clear, however, that steady-state binding involves additional processes. Beginning as early as 2 min of incubation at 37°C there is internalization of the labeled material and this process continues as a constant function of binding (5).

The most plausible mechanism to explain the internalization of labeled insulin is adsorptive pinocytosis. This process involves invagination of the plasma membrane, fusion of the neck of the invagination, and pinching off the membrane-bound vesicle (28). If this is the case it suggests that the hormone-receptor complex is being internalized. Because the label is exclusively on the hormone we cannot be certain that the receptor is internalized, but it is not apparent how the receptor would be released from the proposed pinocytic vesicle. The membrane-bounded vesicle, once released from the plasma membrane would be expected to associate with lysosomal structures and this is, in fact, what we see. Whereas, we cannot quantitatively relate developed grains to pinocytic vesicles at this stage of our study, possibly because these vesicles are very small and the process is very rapid, we can demonstrate a quantitatively increasing association of developed grains with lysosomal structures.

The process described in these studies is remarkably similar to the binding, pinocytosis, and lysosomal association of 125I-LDL to human fibroblasts (21–23), with the exception that the initial binding step does not occur in coated segments or invaginations of the membrane. Binding to coated segments of the membrane appears most important to fibroblasts because it is not observed for LDL binding to mononuclear leukocytes (29). In addition we have found a similar series of events involved in the binding of epidermal growth factor to human fibroblasts (30). The functional significance of this process for the lipoprotein system appears to be to make free cholesterol available to the cell for the regulation of key enzymes involved in cholesterol synthesis. The functional significance of this process for insulin and epidermal growth factor is less clear.

Mortimore and Tietze (31) demonstrated that labeled insulin is “trapped” by the cyclically perfused liver and, that after a brief delay period at 37°C perfusions, degradation products are released into the perfusion media. When the temperature was lowered, the trapping function continued but degradation largely ceased. These studies were confirmed by Terris and Steiner in the noncyclically perfused liver (32) and extended to isolated hepatocytes (33). It was shown that in the isolated hepatocyte, the degradation of labeled insulin

cell radius. (b) The intracellular labeled material preferentially associates with lysosomal structures of a uniform size and lysosomal association progressively increased with time at 37°C. (c) Autoradiographic grains predominantly represented radiation from intact 125I-insulin. (d) Both the initial rate and absolute magnitude of dissociation of 125I-insulin was decreased as a function of increasing time of association at 37°C and to a lesser extent at 20°C. At 37°C, the radioactivity released from the cell consisted of both intact 125I-insulin

**FIGURE 6** Sephadex G-50 (fine) gel filtration elution profile of cell associated radioactivity. Hepatocytes were incubated with 125I-insulin in KRB in the presence and absence of 0.8 mg/ml of bacitracin. At appropriate times of incubation cells were separated from the incubation media by centrifugation and washed with buffer. The cell pellet was then extracted with a mixture of 0.1% Triton X-100, 4 M acetic acid, and 6 M urea resulting in the extraction of 95–98% of the total radioactivity. The extract was then applied to a column developed in and eluted by 1 M acetic acid (a column developed in and eluted by the extracting solvent gives essentially the same elution profile). Total recovery of radioactivity from the column was 80–85% of that applied. The elution volume of the void, 125I-insulin and 125I are shown at the top and the slashes designate the fractions (1 ml each) pooled for peaks I, II, and III. Peak I (void) refers to fractions 9–13, peak II (intact 125I-insulin) to fractions 18–24, and peak III to fractions 34–40; when discrete small peaks were observed beyond fraction 40, they were included in peak III. The free Na125I peak eluted at fraction 31. The percentage radioactivity eluting in each peak is given in Table III for the 20°C data and in reference 5 for the 37°C data. After elution, appropriate fractions were lyophilized and further evaluated by TCA precipitation, tlc binding, and re-binding to liver membranes as previously described (Table III) (5).
was proportional to the binding of insulin to the cell and that the process was analogue specific. It was also shown that there was a short lag phase between initial binding and the release of degradation products.

The present study provides a direct mechanism for the findings in the perfused liver (31, 32) and in isolated hepatocytes (33). We find a receptor-linked internalization process that ultimately involves lysosomes, structures that are intimately associated with autophagic and heterophagic functions. When we estimate our cell-associated degradative products, we find that under steady-state binding conditions at 37°C and 20°C most of the labeled material is intact 125I-insulin both by gel filtration analysis and functional criteria (receptor binding). The remainder consists of a void volume fraction (mainly seen at 37°C, the nature of which is unclear) and small iodotyrosyl peptides. Kahn and Baird (34) report similar findings in adipocytes. This is also similar to the findings of Terris and Steiner (33), with the notable exception that their analysis of degradation was performed on whole cell suspensions (cells plus incubation media). In contrast to our data, they found no degradative activity in a cell-free medium previously exposed to hepatocytes. In the present study and in previous studies with liver membranes and isolated hepatocytes, degradative processes occurred in the incubation medium that were not influenced when the binding process was blocked (8, 9, 35). This form of degradation has a different specificity, Km, and pH optimum than the binding process and is, therefore, as previously reported separable from binding (9). Bacitracin inhibits degradation in the media but does not appear to significantly affect the cell-associated degradation. The fact that only small amounts of degradative products are associated with the cell under steady-state conditions suggests that these products are rapidly

### Table IV

**Sephadex G-50 Elution Profiles of Control 125I-Insulin Radioactivity Released in the Medium by Hepatocytes at Various Times of Dissociation after 30-min Association at 37°C, and Cell Extracts at the Corresponding Times**

<table>
<thead>
<tr>
<th>Time of dissociation</th>
<th>Control 125I-insulin</th>
<th>Radioactivity released in the medium</th>
<th>Cell extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I</td>
<td>Peak II</td>
<td>Peak III</td>
</tr>
<tr>
<td>min</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>7.5</td>
<td>77</td>
<td>9.2</td>
</tr>
<tr>
<td>15</td>
<td>8.9</td>
<td>77</td>
<td>9.0</td>
</tr>
<tr>
<td>30</td>
<td>11.3</td>
<td>77</td>
<td>10.2</td>
</tr>
<tr>
<td>60</td>
<td>9.0</td>
<td>70</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Experimental conditions were as described in Methods (under properties of radioactivity dissociated from hepatocytes). Bacitracin (0.8 mg/ml) was present throughout association and dissociation. Values represent the percentage of radioactivity eluting from Sephadex G-50 (fine) for peaks shown in Fig. 6. Control 125I-insulin was incubated under identical conditions, except that cells were omitted.

### Table V

**Cell-Associated Radioactivity before and after Processing for EM**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Percentage of cell-associated radioactivity at 60 min of incubation</th>
<th>Calculated initial radioactivity in cell pellet*</th>
<th>Radioactivity in cell pellet before processing for EM</th>
<th>Radioactivity in cell pellet after processing for EM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-insulin incubated with hepatocytes for 60 min</td>
<td>3.0</td>
<td>100</td>
<td>93</td>
<td>61</td>
</tr>
<tr>
<td>125I-insulin preincubated with buffer + hepatocytes for 60 min and incubated with fresh hepatocytes for 60 min</td>
<td>0.6</td>
<td>100</td>
<td>91</td>
<td>64</td>
</tr>
<tr>
<td>125I incubated with hepatocytes for 60 min</td>
<td>0.1</td>
<td>100</td>
<td>54</td>
<td>24</td>
</tr>
</tbody>
</table>

* Radioactivity in pellet plus supernatant buffer after 8 d.
† Processing involves removing buffer, washing pellet, osmification, and successive alcohol washes. The values should be considered as approximate upper limits because it is difficult to be sure that small fragments are not lost from the pellet after osmification because of the brittle nature of the pellet.
‡ These samples were incubated for only 30 min.
released by the cell. Our data clearly indicate that after \(^{125}\text{I}\)-insulin association with hepatocytes, dissociation results in the release of radioactive materials, which, as dissociation proceeds, consists of an increasing proportion of low molecular weight insulin fragments. These products are not capable of re-binding to insulin receptors of fresh hepatocytes or liver plasma membranes. This degradation is not inhibited by bacitracin, which, under similar conditions, largely prevents the degradation of insulin by a cell-free medium. Furthermore, bacitracin does not appear to substantially affect the rate and extent of dissociation of radioactivity from the cell. Under the same conditions, the remaining cell-associated radioactivity exhibits, as dissociation proceeds, an increasing proportion of low molecular weight fragments. Taken together the data strongly suggest that the cell-associated insulin is being progressively degraded into fragments that are released into the medium.

Although insulin degradation has been studied extensively, information about a possible lysosomal action on degradation is limited (36, 37). Recent studies have emphasized that lysosomal degradation may be important for epidermal growth factor (EGF) (38). Thus, Carpenter and Cohen (38) have suggested that agents (i.e., chloroquine and ammonium chloride), thought to inhibit lysosomal function (39), inhibited the intracellular degradation of EGF. In preliminary studies we find that ammonium chloride inhibits the cellular degradation of labeled insulin in intact hepatocytes but in contrast, has no effect on the degradation of insulin by a cell-free medium previously incubated with hepatocytes or liver membranes.\(^3\)

Thus EGF in fibroblasts and insulin in hepatocytes appear to be involved in similar processes, i.e., specific binding, internalization, and lysosomal association, and in both systems binding appears to be enhanced by factors that impair lysosomal function. These data indicate a potentially significant role for lysosomes in the intracellular degradation of polypeptide hormones and growth factors but it is as yet unclear how important this function is compared to other enzymatic forms of insulin degradation.

In vivo in the rodent (40) and in man (41) hyperinsulinemia is associated with a decreased insulin receptor concentration and in vitro insulin induces loss of its receptor in cultured human lymphocytes (2)\(^4\) and in primary cultures of rodent hepatocytes (42). The mechanism of receptor loss is unknown and it is apparent that a complete understanding of the process must include events involved from receptor synthesis to insertion in the membrane as well as events involved in receptor loss. Present data, however, suggest that the major initial event is accelerated receptor loss.


(2). If, as we suggest, the process of internalization of labeled insulin occurs by adsorptive pinocytosis then the receptor is likely to be internalized. The receptor along with its internalized membrane could then be degraded by lysosomes or the membrane vesicle could be recycled back to the plasma membrane (28).

We find by freeze-fracture technique that insulin-induced receptor loss, in cultured human lymphocytes, is associated with increased membrane microinvergations and increased bulk pinocytosis as demonstrated by horseradish peroxidase uptake.2 Blackard et al. (42) have recently shown that lower concentrations of insulin will induce receptor loss in cultured rat hepatocytes (42) than in cultured human lymphocytes. Our data demonstrate that internalization of insulin at steady state is much greater in the rat hepatocyte (5) than in the cultured human lymphocyte (3). Ligand induced pinocytosis could explain the temperature dependence, concentration dependence, and exquisite specificity of the process of insulin-induced receptor loss.

Insulin's major effect in liver is the regulation of intracellular enzymes. Out data provide no specific information by which this process may come about. The internalization process is rapid, however, and the time-course of glycogen synthase activation in isolated hepatocytes (43) corresponds to the time-course of binding and internalization that we have described.

On the basis of their studies of the binding of insulin to isolated nuclei (44) and the subcellular localization of insulin to nuclei (45), Goldfine et al. (46) have suggested that insulin is internalized by the cell and recently they report that internalized insulin localizes to endoplasmic reticulum and nuclear membrane. Our studies differ in several ways for unexplained reasons: we have not found labeled insulin preferentially localized to either nuclei or endoplasmic reticulum in either cultured lymphocytes or isolated hepatocytes. Furthermore, we find, with the methods of this study, that labeled EGF (30) and LDL (47) localize intracellularly in lysosomal structures in human fibroblasts. There is considerable morphologic and biochemical evidence that LDL localizes in lysosomes (21, 22) and also morphologic evidence that EGF and insulin are handled by a similar process in the fibroblast (48).

It has been demonstrated that Golgi elements have specific binding sites for insulin (49). Whereas we find that labeled insulin preferentially localizes to structures morphologically distinguished as lysosomes, these structures are concentrated in Golgi-rich areas of the cell. We do not find, however, in the intact cell that labeled insulin localizes to morphologically detectable Golgi structures.

The process of binding, endocytosis, and lysosomal association may be a general process related to a number of polypeptide hormones and growth factors. The role of this process, however, is unknown and at the present time we can only speculate on how this process may link several of the diverse functions of insulin and related products.

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