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Receptor-Linked Degradation of ^{125}I-Insulin Is Mediated by Internalization in Isolated Rat Hepatocytes

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When hepatocytes were freshly isolated from rat liver and incubated for various periods of time at 37 °C, the media from the incubation, when completely separated from the cells, actively degraded ^{125}I-insulin. This soluble protease activity was strongly inhibited by bacitracin but was unaffected by the lysosomatropic agent ammonium chloride (NH_4Cl). When hepatocytes were incubated with ^{125}I-insulin at 37°C in the presence or absence of 8 mM NH_4Cl the ligand initially bound to the plasma membrane and was subsequently internalized as a function of time. When hepatocytes were incubated at 37°C for 30 minutes with ^{125}I-insulin in the presence of bacitracin and NH_4Cl or bacitracin alone and the cells were washed, diluted, and the cell-bound radioactivity allowed to dissociate, the percent intact ^{125}I-insulin in the cell pellet and in the incubation media was greater in the presence of NH_4Cl at each time point of incubation. Under these same conditions a higher proportion of the cell-associated radioactivity was internalized and a higher proportion was associated with lysosomes.

The data suggest that receptor-mediated internalization is required for insulin degradation by the cell, and that this process, at least in part, involves lysosomal enzymes. Furthermore, the data demonstrate that internalization is not blocked by the presence of bacitracin or NH_4Cl in the incubation media, but that degradation is inhibited.

INTRODUCTION

The liver is the major organ involved in the degradation of insulin *in vivo*. Insulin degradation in the isolated perfused rat liver is consistent with a receptor-linked process [1,2] and in intact hepatocytes, *in vitro*, kinetic data suggest that insulin degradation is receptor-linked [3]. For the most part, however, studies of insulin degradation, *in vitro*, have not considered the intact cell but have been carried out in soluble components of cells [4] or membranes [5], or in homogenates [6-9]. While it is clear that potent insulin-degrading activity is present in these preparations, there is

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no evidence that this soluble degradative activity is receptor-linked. In fact, analogues of insulin with very low receptor binding affinity inhibit at least some forms of soluble degradation [5,10]; the process may be unrelated to receptor concentration [11], and degradation may occur when binding is blocked by insulin receptor antibodies [12]. Thus, in order to study true cell-related degradation it is necessary to minimize this soluble degradative activity with a protease inhibitor or by some other means.

We have shown that following initial binding to the plasma membrane, ^{125}I-insulin is internalized in isolated rat hepatocytes and is preferentially localized intracellularly to membrane-bounded structures with the morphologic characteristics of lysosomes [13,14]. On the basis of these morphologic studies we have proposed that receptor-linked insulin degradation in isolated hepatocytes is mediated by receptor-linked internalization in these cells [13,14].

In the present study we have used combined morphological and biochemical probes to investigate the degradation and compartmentalization of ^{125}I-insulin at physiological temperatures in freshly isolated rat hepatocytes.

MATERIALS AND METHODS

Cells and Reagents

Hepatocytes were isolated from six- to eight-week-old Wistar rats fed ad libitum by a modification of the method of Seglen [15] as previously described [14]. Buffers, ^{125}I-insulin, and other reagents were exactly as previously described [14].

Incubation Conditions

Hepatocytes at a final concentration of 1×10^6 cells/ml were incubated in duplicate in 0.5 ml of modified Krebs-Ringer Bicarbonate buffer (KRB) that contained 30 mg/ml bovine serum albumin (BSA, Fraction V), 0.5 nM (3 ng/ml) ^{125}I-insulin (specific activity = 250 μCi/μg) at 37°C for varying time periods. Where indicated, the buffer was supplemented with bacitracin, ammonium chloride, or both, and the pH was adjusted to 7.7.

At the end of appropriate periods of incubation, cells were separated from the incubation media. The cells were analyzed by quantitative electron-microscopic (EM) autoradiography, extracted for gel filtration, and the media were analyzed by gel filtration. These studies were carried out as previously described [13,14,16] (for specific variations, see legends to Figs. 1–6).

Liver Membrane Assay

Insulin integrity following exposure to media was assessed by measuring the binding ability of ^{125}I-insulin to purified rat liver plasma membranes (step 15 of Neville) [17]. The binding assay was carried out as follows: aliquot samples from control and experimental media were incubated for 60 minutes at 37°C with liver membranes (0.2 mg protein/ml) in Krebs-Ringer phosphate buffer, pH 7.5, containing 2 percent BSA and 0.8 mg/ml bacitracin; non-specific binding was determined in parallel by measuring the binding of ^{125}I-insulin in the presence of 60 μg/ml unlabeled insulin. Membranes were separated from the incubation media as previously described [5].

RESULTS

Inhibition of Insulin Degradation in Hepatocytes by Bacitracin and Ammonium Chloride

In previous studies using purified liver membranes and isolated rat hepatocytes, it has been shown that a soluble insulin-degrading activity is released into the incubation medium [4,5]. This activity, as previously shown, was strongly inhibited by bacitracin which presumably acts as a nonspecific protease inhibitor (Fig. 1). Ammonium chloride, at the concentrations used for these experiments, had no effect on this soluble form of insulin degradation (Fig. 1) and for the purposes of these experiments we assume that any inhibitory effect of ammonium chloride is on a cell-related process that most likely involves lysosomal stabilization [18-20].

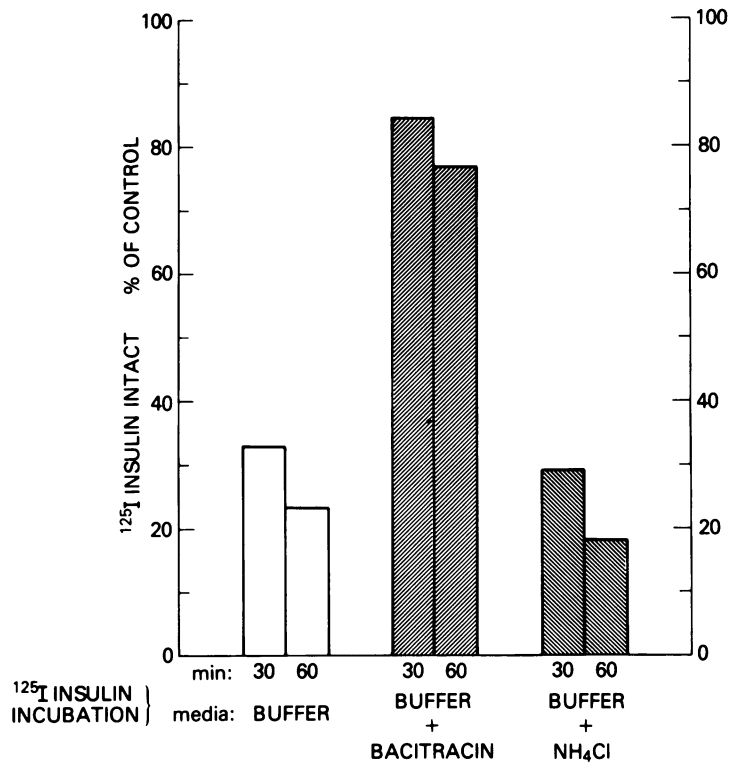

FIG. 1. Degradation of ^{125}I-insulin by Incubation Media from Hepatocytes.

Hepatocytes 1 × 10^6 cells/ml were first incubated (without ^{125}I-insulin) for 30 minutes at 37°C in KRB buffer containing 2.4 percent BSA or in the same buffer supplemented with 6.4 mM ammonium chloride; pH was adjusted to 7.5 for each medium. Media and cells were then separated by centrifugation for one minute at 50 × *g* and the insulin-degrading ability of each medium was studied by incubating aliquots from cell-free media with ^{125}I-insulin (1.7 ng/ml) for 30 and 60 minutes at 37°C. Controls consisted of the same ^{125}I-insulin preparation which was exposed for 30 and 60 minutes at 37°C to each type of medium but without prior incubation of media with hepatocytes. The data shown here are representative of three separate experiments. Insulin integrity following exposure to media was assessed by measuring the binding of ^{125}I-insulin to purified rat liver membranes [5].

Quantitative EM-Autoradiographic Studies of the Binding of ^{125}I-Insulin in Hepatocytes in the Presence of Ammonium Chloride

We have previously shown that when ^{125}I-insulin is incubated with isolated hepatocytes at 37°C in buffer supplemented with bacitracin, the ligand is initially bound to the plasma membrane and progressively internalized as a function of time [13]. To determine whether labeled insulin bound to the plasma membrane is internalized

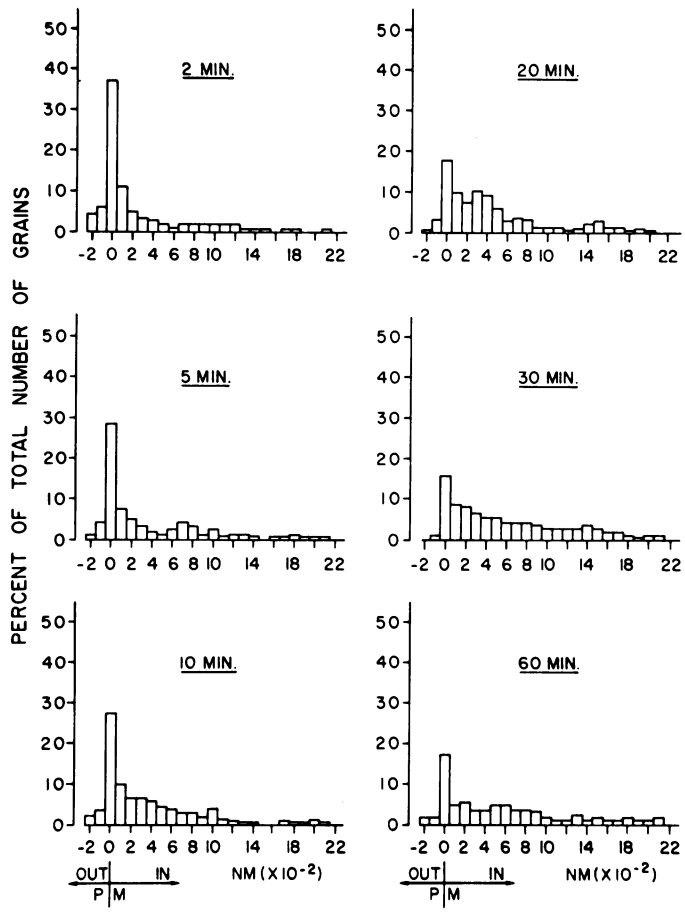

FIG. 2. Autoradiographic Grain Distribution Histograms of the Incubation of ^{125}I-insulin with Isolated Rat Hepatocytes. ^{125}I-insulin was incubated with hepatocytes 1 × 10^6 cells/ml in KRB buffer, pH 7.7, supplemented with 8 mM ammonium chloride but without bacitracin at 37°C for the time periods shown. At the end of the specified periods of incubation, cells were separated from the buffer by centrifugation, fixed with 4 percent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for four hours and transferred to 0.1 M phosphate buffer, pH 7.4, until processed. They were then processed for autoradiography and analyzed as shown. The percent of total number of grains was plotted as a function of the distance of the grain center from the plasma membrane. The progressive rightward shift of the histogram profile indicates internalization of the labeled insulin. In this experiment 100 to 150 photographs were made for each time point shown. The 0 point refers to the plasma membrane (PM).

by the hepatocyte in the presence of 8 mM ammonium chloride, ^{125}I-insulin was incubated with hepatocytes at 37°C in the presence and absence of ammonium chloride and the hepatocytes were fixed at appropriate times, processed and analyzed by quantitative EM-autoradiography. The initial binding and internalization of the ligand were qualitatively similar in the presence and absence of ammonium chloride (Figs. 2 and 3) and were also similar to studies carried out in the presence of bacitracin [13] (Table 1).

We conclude from these experiments that ammonium chloride does not inhibit either binding or internalization of the labeled ligand in the hepatocyte.

Though it has been shown for other ligands or other cell preparations that cell-associated radioactivity is increased in the presence of lysosomal stabilizers [20,21], we have been unable to consistently show this phenomenon in the present experiments. We attribute this to variation in soluble degradative activity in different preparations of isolated hepatocytes.

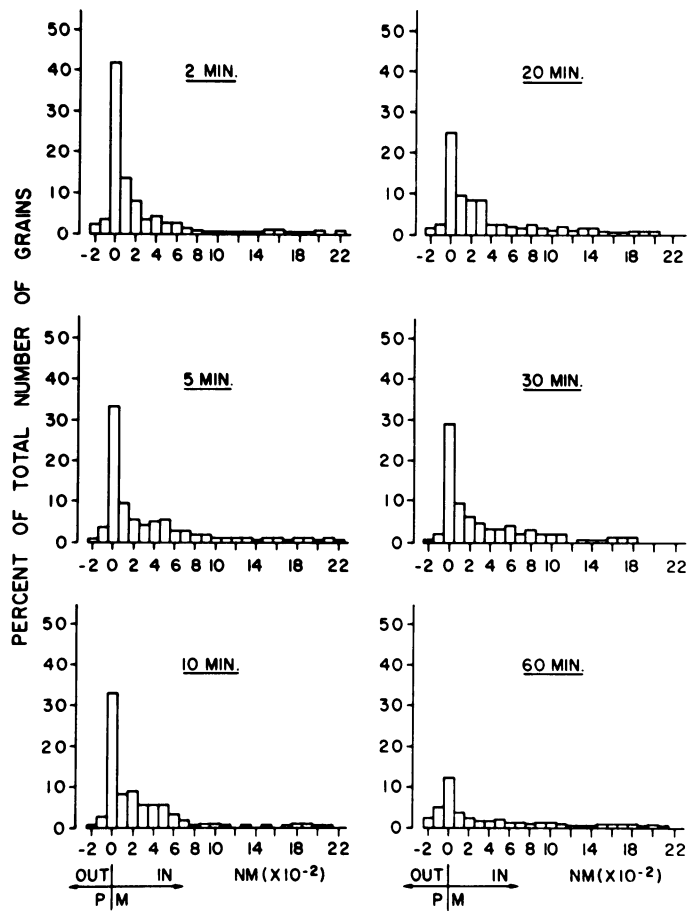

FIG. 3. Autoradiographic Grain Distribution Histograms of the Incubation of ^{125}I-insulin with Isolated Rat Hepatocytes. Exactly the same format as shown in Fig. 2 except that buffer was neither supplemented with ammonium chloride nor bacitracin. The concentration of ^{125}I-insulin bound at each time point shown was very similar to the concentration bound in the experiment shown in Fig. 2.

TABLE 1
Concentration of ^{125}I-Insulin Cell Associated and Internalized in Isolated Rat Hepatocytes

Condition		Time (Minutes) 2	5	10	20	30	60
Buffer	Cell-associated (pg/ml)[a]	68.4	119.1	143.7	134.7	118.8	81.0
	Internalized (pg/ml)[b]	11.0	34.9	45.0	49.0	40.8	41.1
Buffer + Bacitracin	Cell-associated (pg/ml)	63.6	121.2	152.7	178.2	184.2	136.2
	Internalized (pg/ml)	15.8	31.0	53.8	78.8	88.1	69.6
Buffer + Bacitracin + NH_4Cl	Cell-associated (pg/ml)	66.6	133.2	184.2	200.4	208.8	187.8
	Internalized (pg/ml)	13.9	50.5	59.7	91.2	106.5	98.8

[a]Concentrations ^{125}I-insulin cell associated = concentration of ^{125}I-insulin in incubation media × % ^{125}I-insulin cell-associated

[b]Concentration ^{125}I-insulin internalized = concentration of cell associated ^{125}I-insulin × % ^{125}I-insulin internalized

Effect of Ammonium Chloride on the Dissociation and Degradation of ^{125}I-Insulin Associated with Hepatocytes

When ^{125}I-insulin was incubated with hepatocytes for 30 minutes at 37°C, the cells were washed and the cell-bound radioactivity was allowed to dissociate. At each time studied there was less dissociation of labeled material from the cell in the presence of ammonium chloride (Tables 2 and 3).

Under these same conditions the proportion of intact labeled insulin associated with the cell progressively declined as a function of dissociation time, and degraded products progressively increased in a reciprocal fashion (Fig. 4). The cell-related degradation process was markedly inhibited by the presence of ammonium chloride in the incubation media. The proportion of degraded products appearing in the incubation media was inhibited by ammonium chloride at each time of dissociation.

Quantitative EM-Autoradiographic Studies During Dissociation of ^{125}I-Insulin from Hepatocytes

Hepatocytes were incubated with ^{125}I-insulin in the presence of ammonium chloride and bacitracin in the incubation media for 30 minutes at 37°C; the cells were

TABLE 2
Dissociation (by Dilution) of ^{125}I-Insulin from Isolated Hepatocytes Incubated at 37°C
Percentage of Initial Binding (mean ± SEM of four experiments)

Time of Dissociation (Minutes)	Condition: Buffer + Bacitracin	Buffer + Bacitracin + NH_4Cl	p
5	81.0 ± 2.7	78.2 ± 3.4	N.S.
10	69.5 ± 1.9	69.2 ± 4.9	N.S.
20	54.5 ± 1.3	61.5 ± 3.7	N.S.
30	43.0 ± 1.5	52.7 ± 4.4	0.05
60	23.3 ± 1.8	38.0 ± 3.7	<0.01
90	15.0 ± 0.9	31.2 ± 3.9	<0.005

TABLE 3
Dissociation (by Dilution) of ^{125}I-Insulin from Isolated Hepatocytes Incubated at 37°C
Percentage of Initial Binding (mean ± SEM of four experiments)

Time of Dissociation (Minutes)	Condition		p
	Buffer	Buffer + NH_4Cl	
5	58.1 ± 5.7	58.4 ± 5.5	N.S.
10	44.7 ± 3.5	52.7 ± 6.0	N.S.
20	29.7 ± 1.1	48.0 ± 4.1	<0.005
30	23.7 ± 1.1	39.7 ± 1.9	<0.0005
60	13.7 ± 2.3	28.0 ± 4.2	<0.02
90	8.2 ± 0.5	21.2 ± 2.0	<0.0005

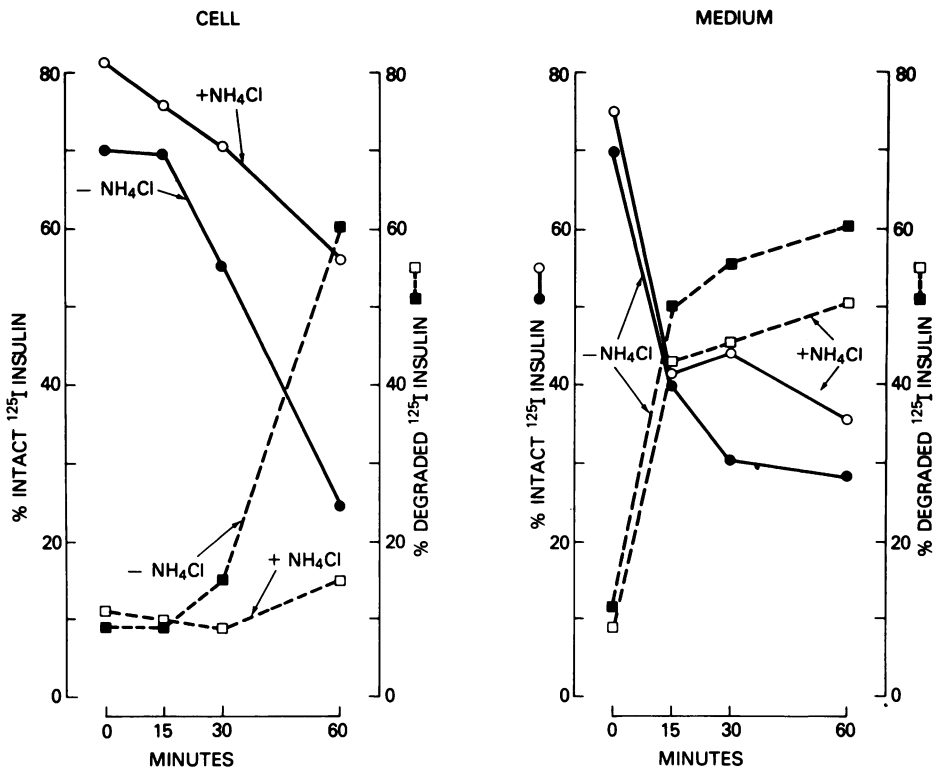

FIG. 4. Analysis of Radioactivity as a Function of Dissociation Time. ^{125}I-insulin was incubated with hepatocytes for 30 minutes at 37°C in KRB-albumin buffer (pH 7.7) and 0.8 mg/ml of bacitracin. At the end of the association period a large excess of unlabeled insulin was added to the media to prevent rebinding of the labeled insulin, and dissociation was allowed to proceed. At the end of the specified periods of dissociation, the cells were separated from the media by centrifugation and extracted as previously described [14]. The cell extract and the media extract were then filtered on G-50 Sephadex as described [14]. Percent intact insulin refers to the percent of the labeled insulin eluting in the region of the insulin marker, and the percent degraded refers to the proportion eluting as low molecular weight peptides. An additional peak corresponding to void volume (not shown) represented 5 to 15 percent of the eluted radioactivity and was the same in both the ammonium chloride and control incubations. The percent intact insulin as shown here, and the percent of the labeled material rebindable to fresh cells, were in good agreement (data not shown). The experiment shown here is representative of three separate incubations and the elution profiles of the individual column runs differed by less than 15 percent. The experiment shown here is representative of three separate incubations.

washed, resuspended, and further incubated. At appropriate times, cells were fixed and processed for EM-autoradiography. In experiments where ammonium chloride was omitted there was insufficient autoradiographic reaction for analysis.

Under the conditions of this experiment the percent ligand internalized increased as a function of dissociation time and reached up to 69 percent of the cell-associated grains (Figs. 5 and 6). Furthermore, as dissociation proceeded, a higher proportion of grains was associated with lysosomes (Fig. 6).

DISCUSSION

Soluble fractions have been the most frequently utilized preparation to study insulin degradation in liver [4-9]. Both protease and transhydrogenase activity have been demonstrated in liver homogenates, purified liver membranes, and freshly isolated cells. The mechanisms, however, whereby these reactions occur physiologically in the intact cell are unclear. These and other studies demonstrate that binding of the ligand is not a prerequisite for this type of degradation to occur.

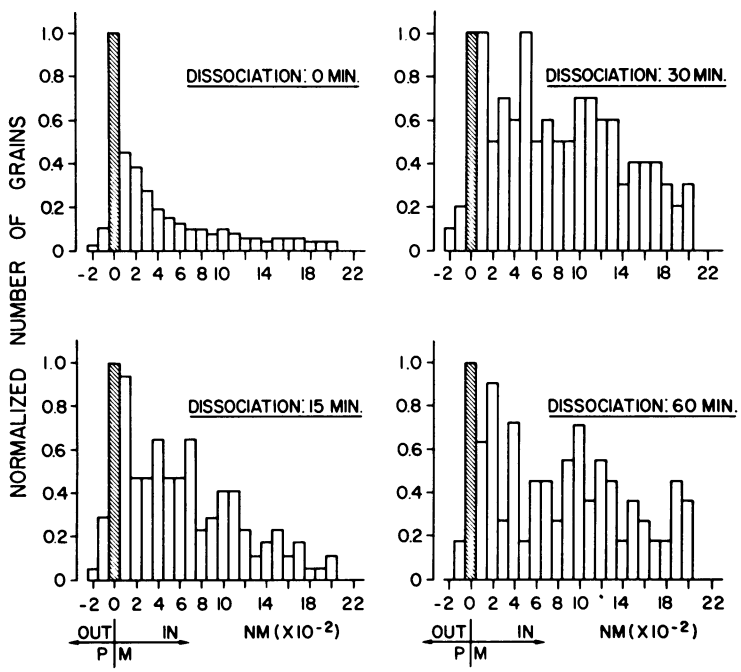

FIG. 5. Autoradiographic Grain Distribution Histograms of ^{125}I-insulin Incubated at 37°C for 30 minutes in KRB-Albumin Buffer (pH 7.7), in 0.8 mg/ml Bacitracin and 8 mM Ammonium Chloride. At the end of the 30-minute association period, cells were diluted 100-fold and the radioactivity was allowed to dissociate. The cell pellet was then fixed and processed for autoradiography at each time shown. The normalized number of grains is plotted as a function of the distance of the grain center from the plasma membrane, expressed in nanometers (NM). The shaded bar represents the plasma membrane (PM). For the percent grains internalized at each time point of dissociation, see Fig. 6. For the purpose of this analysis, grains appearing ±250 nm of the plasma membrane were considered to be associated with the plasma membrane and grains appearing intracellularly beyond 250 nm were considered to be internalized. In this experiment 100 to 150 photographs were made for each time point shown. The 0 point refers to the plasma membrane (PM).

It was initially suggested by Mortimore and Tietze that a "trapping" step preceded insulin degradation in the isolated perfused liver [1] and this was confirmed by Terris and Steiner [2]. Terris and Steiner further showed in the isolated hepatocyte that the degradation of insulin was linked to the binding of the ligand [3]. We have demonstrated in isolated hepatocytes that the binding of insulin to cell surface receptors is followed rapidly by the internalization of the ligand and that the internalized ligand preferentially associates with lysosome-like structures [13,14].

In the present study we have shown that NH_4Cl, an agent presumed to impair lysosomal function, inhibits insulin degradation by intact hepatocytes but has no effect on soluble degrading activity. At least one mechanism by which agents such as NH_4Cl are thought to exert their effect is by increasing intralysosomal pH; this should decrease the activity of proteolytic enzymes with an acid pH optimum. Recent studies by McKanna et al. suggest that the epidermal growth factor hormone receptor complex remains intact in the lysosomal membrane in the presence of amines, while in the absence of these agents the ligand appears in the lumen of the lysosome presumably accessible to lysosomal enzymes [22]. Our data are most consistent with a similar process for insulin associated with hepatocyte lysosomes. Fluorescent probes of EGF and insulin suggested that both bacitracin and primary amines inhibited the internalization of insulin and EGF [23,24]. If this were true, it would suggest a different mechanism to explain our results. However, the work presented here, as well as our previous studies [13,14,25] demonstrate that neither bacitracin nor the amines inhibit internalization of EGF or insulin and this has been more recently confirmed by Haigler et al. [26]. These potentially conflicting results demonstrate the importance of direct morphologic studies using different types of probes. These data do not exclude the possibility that the lysosomotropic agents have other effects such as inhibiting the egress of the ligand from the lysosome to another degradative site or other functions yet uncharacterized.

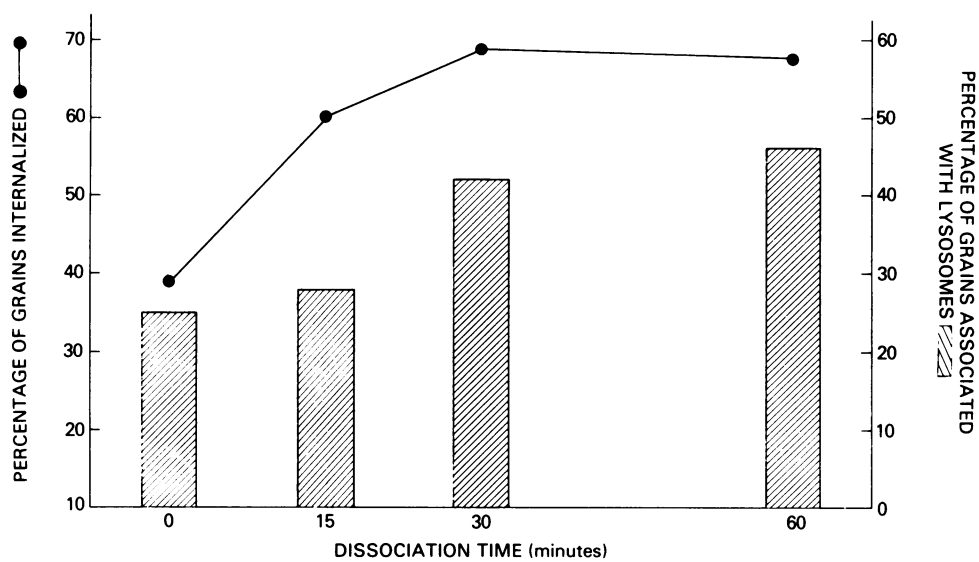

FIG. 6. Internalization and Lysosomal Association of ^{125}I-insulin in Isolated Hepatocytes as a Function of Incubation Time. These data were derived from Fig. 5. The percentage of grains associated with lysosomes was determined by a probability circle method as previously described [14]. For further details and representative photographs of cells see [14,39,40].

Kahn and Baird [27] and Olefsky et al. [28] have shown in adipocytes that, at 37°C, the rate or amount of labeled material dissociated is inversely proportional to association time; we have shown the same for insulin binding to hepatocytes [14]. In both the adipocyte [27] and the hepatocyte [14], as a function of dissociation time, a higher proportion of labeled material released by the cell is degraded. Though little work has been done to implicate the lysosome in insulin degradation [29,30], following the demonstration that internalized insulin associates with lysosomes [14], at least four other groups have confirmed that lysosomotropic agents inhibit cell-related insulin degradation [21,31-33].

The results of these studies on insulin degradation are remarkably similar to the binding, internalization, and lysosomal degradation of epidermal growth factor by human fibroblasts [20,25,34], to human choriogonadotropin degradation by gonadotropin binding cells [35-37], and to low-density lipoprotein degradation by human fibroblasts [38]. Thus, the process described here for insulin degradation appears to be a general mechanism applicable to many diverse ligands that bind to specific cell-surface receptors.

If receptor-linked insulin degradation is a physiologic process, receptor-linked internalization provides a simple mechanism for this process to occur. There are a number of implications of this process that have not generally been considered. The internalization of the ligand appears to occur by way of endocytosis. If this is true, then the ligand is internalized into a membrane-bounded endocytotic vesicle which in turn fuses with membrane-bounded lysosomes. The ligand may then be degraded in the intact cell incubated at physiologic pH by a lysosomal process. The morphological data demonstrating internalization and lysosomal association, coupled with the biochemical data demonstrating inhibition of degradation by lysosomatropic agents, is consistent with this process.

It is, therefore, possible that the physiologic mechanism of degradation of polypeptide hormones and growth factors occurs by binding to specific cell surface receptors, which induces endocytosis of the ligand into a membrane-bounded compartment. The ultimate degradative process need not be solely lysosomal; the endocytotic vesicle could provide a mechanism to concentrate non-lysosomal proteolytic enzymes.

Thus, it seems clear that lysosomal processes are involved in the degradation of polypeptide hormones and growth factors, but it is as yet unclear how important these processes are quantitatively relative to non-lysosomal proteases.

ACKNOWLEDGEMENT

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