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Receptor- and non-receptor-mediated uptake and degradation of insulin by hepatocytes

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Native insulin inhibits the binding and degradation of ^{125}I-labelled insulin in parallel. Half-maximal inhibition of degradation occurs with 10nM-insulin, a hormone concentration sufficient to saturate the insulin receptor. The proportion of bound hormone that is degraded increases as the insulin concentration is increased, suggesting that low-affinity uptake is functionally related to degradation. Since only a small fraction (approx. 10%) of the overall degradation occurs at the plasma membrane, or in the extracellular medium, translocation of bound hormone into the cell is the predominant mechanism mediating the degradation of insulin. In the presence of 0.6nM-insulin, a concentration at which most cell-associated hormone is receptor-bound, chloroquine increases the amount of ^{125}I-labelled insulin retained by hepatocytes. However, chloroquine increases the retention of degradation products of insulin in incubations containing sufficient hormone (6nM) to saturate the receptor and permit occupancy of low-affinity sites. Glucagon does not compete for the interaction of ^{125}I-labelled insulin (1nM) with the insulin receptor. In contrast, 20μM-glucagon inhibits 75% of the uptake of insulin (0.1μM) by low-affinity sites. A fraction of the cell-bound radioactivity is not intact insulin throughout a 90min association reaction at 37°C. During dissociation, fragments of ^{125}I-labelled insulin are released to the medium more rapidly than is intact hormone. The production and transient retention of degradation products of the hormone complicates the characterization of the insulin receptor by equilibrium or kinetic methods of assay. It is proposed that insulin degradation occurs by receptor- and non-receptor-mediated pathways. The latter may be related to the action of glutathione–insulin transhydrogenase, with which both insulin and glucagon interact.

The degradation of insulin by rat hepatocytes is an important mechanism by which hormone stimulation of the target cell is regulated. The binding of insulin to specific receptor sites on the surface of cells initiates a sequence of events during which the hormone and/or the hormone–receptor complex is internalized (Terris & Steiner, 1975; Gorden *et al.*, 1980; King & Cuatrecasas, 1981; Goldstein & Livingston, 1981). A portion of insulin degradation probably occurs in secondary lysosomes (Carpentier *et al.*, 1979), with the low-molecular-weight degradation products being rapidly released to the medium (Terris & Steiner, 1975). Liver cells contain enzymes, such as insulin proteinase (Mirsky, 1964; Burghen *et al.*, 1972) and glutathione–insulin transhydrogenase (thiol:protein-disulphide oxidoreductase, EC 1.8.4.2) (Katzen & Stetten, 1962; Varandani, 1972), which degrade insulin but are not lysosomal. The relationship between insulin degradation in lysosomes and that mediated by glutathione–insulin transhydrogenase or insulin proteinase has not been elucidated.

I previously demonstrated that there is a concentration-dependence related to the degradation of insulin by rat hepatocytes (Donner, 1980). As the concentration of insulin was increased, the fraction of bound, degraded hormone became greater. Degradation was observed at hormone concentrations that saturated the insulin receptor (Gammeltoft *et al.*, 1978; Donner, 1980). This led to the suggestion that not all of the insulin degradation was receptor-mediated. The goals of the present study were to characterize further the concentration-dependence of insulin degradation, to determine whether the concentration-dependence of degradation could be related to receptor- and non-receptor-mediated pathways, and to characterize the

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effects of degradation on the uptake and release of insulin by the hepatocyte.

Materials and methods

Materials

Male Sprague–Dawley rats (150–250g) were purchased from Charles River Laboratories and fed on Purina Laboratory Chow *ad libitum*. Glucagon (lot 258-D30-138-4), assayed by radioimmunoassay, contained 0.6 μunit of insulin/mg (0.0000025%, w/w), and was a gift from Eli Lilly. Pig zinc insulin (25.5 U.S.P. units/mg) was obtained from Eli Lilly. The sources of other materials were as previously described (Donner *et al.*, 1980).

General methods

Insulin was iodinated by the method of Greenwood *et al.* (1963) and Lesniak *et al.* (1973) and separated from free ^{125}I on Sephadex G-50 (fine grade). ^{125}I-labelled insulin was immunocompetent and precipitable (>95%) in 5% (w/v) trichloroacetic acid. Hepatocytes, isolated by a modification of the method of Berry & Friend (1969), were over 90% viable when assayed by Trypan Blue exclusion, possessed a functional amino acid-transport system, synthesized protein and were hormonally responsive (Donner *et al.*, 1978). Incubations were conducted in Hanks' balanced salt solution containing 1% (w/v) albumin (fraction V). A standard micro-centrifuge method was used to assay uptake of ^{125}I-labelled insulin (Rodbell *et al.*, 1971). Partially purified liver plasma membranes were prepared by the procedure of Neville (1968) as described by Pohl *et al.* (1971).

Trichloroacetic acid precipitation of bound radioactive label

During the association of ^{125}I-labelled insulin with hepatocytes, the precipitability of bound radioactive label in 5% (w/v) trichloroacetic acid was assayed. Trichloroacetic acid (250 μl) was added to cell pellets recovered from the binding assay (Donner, 1980). The pellet was stirred with a fine wire rod until it was well mixed with the trichloroacetic acid solution. The tube was centrifuged at 1500 ***g*** for 1 min and the supernatant, containing non-precipitable radioactive label, was aspirated. The tip of the micro-centrifuge tube was cut off and placed in a glass tube for assay of ^{125}I. During dissociation, 1 ml of 5% (w/v) trichloroacetic acid was added to cell pellets recovered during assay (see the legend to Fig. 7). The tube was vigorously agitated to suspend the cells in the acid and then centrifuged (1500 ***g***, 3 min). The supernatant was siphoned from the resultant pellet, containing precipitated radioactive label, which was then assayed for ^{125}I in a gamma counter.

Trichloroacetic acid precipitation of applied radioactive label

Samples of incubation medium (containing free and bound radioactivity) were added to Hanks' balanced salt solution/1% albumin (1 ml). Trichloroacetic acid solution (10%, w/v; 1 ml) was added to produce a protein precipitate. The solution was centrifuged (3 min, 1500 ***g***) and the supernatant was recovered from above the pellet of ^{125}I-labelled protein. The supernatant and pellet were assayed for ^{125}I. The precipitability of radioactive label was the ratio of radioactivity (c.p.m.) in the precipitate to that in the precipitate and supernatant.

Data analysis

A PDP 11/70 computer (Digital Equipment Corp.) was used to fit hormone-binding data to appropriate mathematical expressions by using non-linear least-squares techniques of curve fitting (Bevington, 1969) as described previously (Donner *et al.*, 1980). In both kinetic and equilibrium binding assays each point is the mean of triplicate determinations and error bars represent standard deviations. Each experiment has been repeated at least three times with essentially identical results.

Results

In order to characterize the concentration-dependence of insulin degradation, the amount of trichloroacetic acid-soluble hormone fragments retained by hepatocytes was assayed as a function of the applied insulin concentration. As shown in the insert to Fig. 1, plotting such data on a log–log scale demonstrated the broad concentration range over which degradation and retention of the products of degradation occurred. This plot emphasizes that insulin degradation occurs at concentrations that saturated the high-affinity receptor (Gammeltoft *et al.*, 1978; Donner, 1980). However, it does not suggest whether any component of the degradation results from a saturable process. In order to explore the relationship between saturable insulin binding and degradation, the ability of native insulin to compete for each process was compared (Fig. 1). The course of competition for binding and degradation was similar, occurring between 0.1 nM and 0.1 μM applied insulin. Half-maximal binding and degradation occurred at 10 nM-insulin. At concentrations above 0.1 μM-insulin, both binding and degradation were apparently non-saturable.

Hepatocytes release into the medium enzymes that degrade insulin (Gammeltoft *et al.*, 1978). Thus it was necessary to ensure that a substantial fraction of the degradation in the cell suspensions used in this study was not extracellular. The production of trichloroacetic acid-soluble insulin fragments in a hepatocyte suspension was temperature-dependent

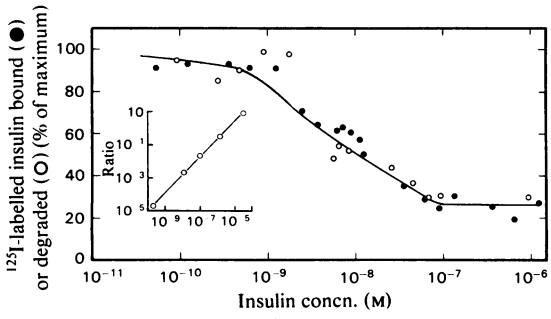

Fig. 1. *Degradation of insulin as a function of hormone concentration*

Hepatocytes (3×10^6/ml) were incubated with ^{125}I-labelled insulin (3 nM) and various concentrations of native insulin at 23°C. Competition between native and ^{125}I-labelled insulin for binding was assayed after 1 h (●). The difference between the total uptake and that precipitated by trichloroacetic acid assayed retained fragments of insulin. The ability of insulin to inhibit degradation of ^{125}I-labelled insulin is shown (○). Inset: the amount of ^{125}I-labelled insulin and native insulin degraded was assayed by the production of acid-soluble radioactivity that was retained. The ratio of insulin degraded to maximal degradation (5.2 pmol/10^6 cells) in the presence of 50 μM-insulin is plotted against hormone concentration.

(Fig. 2*a*). Degradation was not observed at 4°C, but increased markedly between 23° and 37°C. Native insulin decreased the rate of degradation of ^{125}I-labelled insulin, suggesting that the systems responsible for this were saturable. Since only a small fraction of the applied radioactivity was bound at any time (e.g. 1.5% after 60 min at 37°C), most of the insulin fragments assayed in this experiment were not cell-associated. Medium conditioned by hepatocytes for 2 h degraded ^{125}I-labelled insulin (Fig. 2*b*). The temperature-dependence and ability of native insulin to inhibit degradation of ^{125}I-labelled insulin by conditioned medium were similar to those of the whole cell suspension. After 60 min at 23°C, 16% of the degradation in the suspensions could have been extracellular, on the basis of the amount of acid-soluble fragments produced by conditioned medium relative to the whole cell suspension. Since the release of enzymes proceeds linearly with time (Gliemann & Sonne, 1978) and medium was conditioned for 2 h, the amount of degradation in the medium after 1 h at 23°C would maximally have been 8% of that in the suspension. This overestimates the ability of medium to degrade insulin,

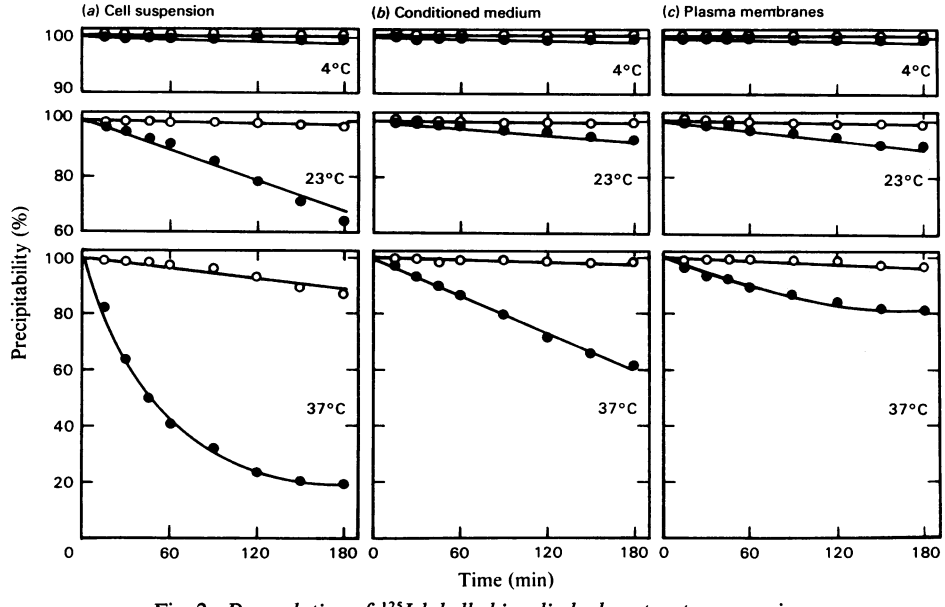

Fig. 2. *Degradation of ^{125}I-labelled insulin by hepatocyte suspensions*

(*a*) Hepatocytes (1×10^6/ml) were incubated with ^{125}I-labelled insulin (1 nM, ●) or ^{125}I-labelled insulin and native insulin (8 μM, ○) at 4°C (top), 23°C (middle) or 37°C (bottom). At appropriate times, 50 μl samples were withdrawn for assay of the fraction of radioactivity precipitated by trichloroacetic acid. (*b*) Hepatocytes (1×10^6/ml) from the suspension used above were incubated in Hanks' balanced salt solution/1% albumin for 2 h at 23°C. Cells were separated from the medium by centrifugation (1500 *g*, 3 min). Conditioned medium was added to ^{125}I-labelled insulin (1 nM, ●) or ^{125}I-labelled insulin and native insulin (8 μM, ○). Degradation of ^{125}I-labelled insulin was then assayed. (*c*) Partially purified liver plasma membranes (0.75 mg/ml) were added to incubations containing ^{125}I-labelled insulin (●) or ^{125}I-labelled insulin and native insulin (8 μM, ○). Degradation of ^{125}I-labelled insulin was then assayed.

since before 2h less enzyme would have been released in the cell suspension than was in the conditioned medium. Therefore extracellular degradation could have accounted for only a small fraction of that in the cell suspension.

Liver plasma membranes degrade ^{125}I-labelled insulin (Fig. 2*c*; Freychet *et al.*, 1972; Duckworth, 1979). The characteristics of the degradation were similar to that observed for cell suspensions. The yield of partially purified plasma membranes from the procedure of Neville (1968) is 1.0–2.0mg of membrane protein/g wet wt. of liver (Pohl *et al.*, 1971). There are approx. 20×10^7 cells per g of liver tissue (Seglen, 1973). The incubation described in Fig. 2 contained 4×10^6 cells. Therefore, the use of 0.75mg of membrane protein/ml (3mg of protein/incubation) greatly exaggerates the fraction of degradation at the membrane. This assumes that the degradation does not result from contamination of plasma membranes by lysosomal contaminants (Terris & Steiner, 1975). A small component of the degradation (about 10%) in hepatocyte suspensions occurs in the medium or at the plasma membrane.

The extent of insulin degradation that was lysosomal was examined by administration of chloroquine, which inhibits this component of proteolysis (de Duve *et al.*, 1974). Preincubation of hepatocytes with chloroquine increased the amount of radioactive label subsequently accumulated during an association reaction with 1nM-^{125}I-labelled insulin (Fig. 3*a*). A similar augmentation in the accumulation of radioactive label was observed in incubations containing 6nM-^{125}I-labelled insulin (results not shown). Radioactive label was extracted from hepatocytes, either with or without chloroquine pre-treatment, and passed over columns of Sephadex G-50 (fine grade). ^{125}I-labelled insulin was eluted with a peak at fraction 20, whereas fragments derived from ^{125}I-labelled insulin were eluted in later fractions. Chloroquine increased the amount of intact ^{125}I-labelled insulin retained by hepatocytes in incubations containing 0.5nM applied hormone (Fig. 3*b*). In incubations containing 6nM-^{125}I-labelled insulin, the increase of cell-associated radioactivity in the chloroquine-treated hepatocytes resulted from retention of hormone fragments (Fig. 3*c*).

Native insulin competed for the uptake of ^{125}I-labelled insulin (2nM) by hepatocytes (Fig. 4). A decrease in the accumulation of radioactivity was first observed in the presence of 1nM native insulin at 15°C. This experiment was conducted at a lower temperature than others to minimize degradation which might complicate the interpretation of competition for insulin-binding sites. Essentially all of the saturable binding of radioactive label was inhibited by 1μM native hormone. High concentrations of glucagon (50μM) did not compete for the uptake of 2nM-^{125}I-labelled insulin by hepatocytes (results not

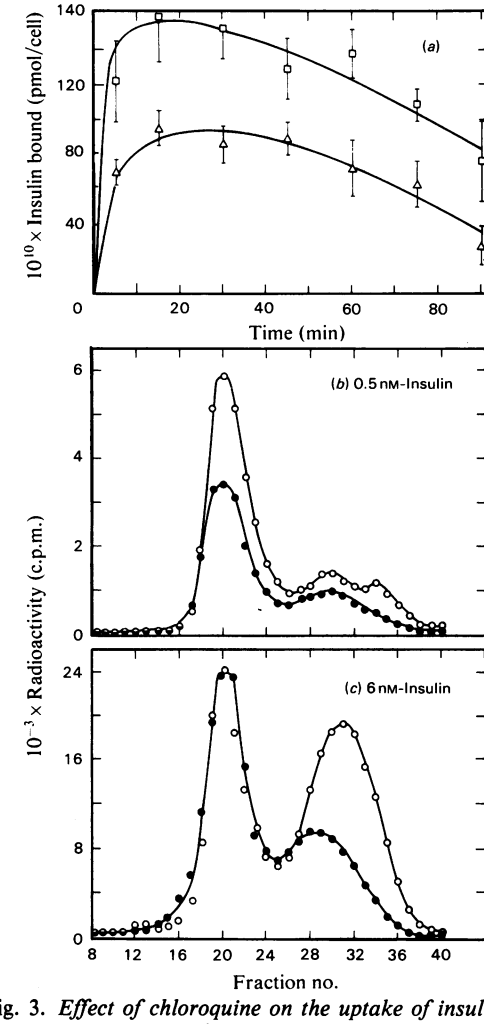

Fig. 3. *Effect of chloroquine on the uptake of insulin by hepatocytes*

(*a*) Hepatocytes (1×10^6/ml) were equilibrated in Hanks' balanced salt solution/1% albumin at 37°C. Half of the cell preparation served as a control, and the remainder was incubated with chloroquine (1mM) for 15min. Samples of each suspension were added to flasks containing ^{125}I-labelled insulin (1nM) with or without chloroquine. Specific binding was assayed with chloroquine-treated (□) or non-treated (△) cells. (*b*) and (*c*) Hepatocytes (10^6/ml) were incubated with (○) or without (●) chloroquine (1mM, 15min, 37°C). ^{125}I-labelled insulin (0.5nM, *b*; or 6nM, *c*) was applied to each suspension. Incubations were shaken for 30min, layered over 5ml of ice-cold Hanks' balanced salt solution/3% albumin and centrifuged (1500*g*, 3min) to remove unbound hormone. Retained radioactive label was extracted by shaking cell pellets in 0.1M-HCl (2ml, 4°C, 10min, three times). Over 80% of the bound radioactivity was recovered by such extraction. A 0.5ml sample of each extract was passed over a column (0.9cm × 27cm) of Sephadex G-50 (fine grade); 0.5ml fractions were collected.

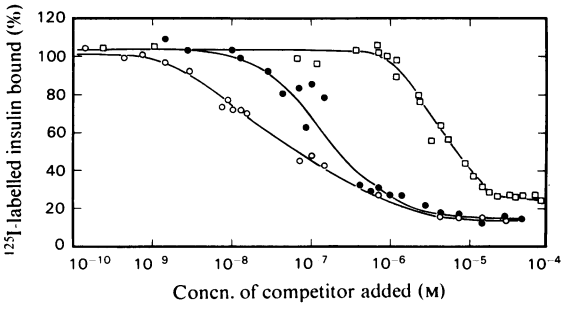

Fig. 4. *Competition for insulin uptake by hepatocytes* Hepatocytes (1×10^6/ml) were incubated with ^{125}I-labelled insulin (2 nM) and various concentrations of native insulin at 15°C. After 2 h, the uptake of ^{125}I-labelled insulin was assayed (○). In a similar experiment, a higher concentration of ^{125}I-labelled insulin (0.1 μM, sp. radioactivity 10 Ci/g) was added to hepatocytes and the uptake of radioactivity was assayed in the presence of insulin (●) or glucagon (□). Results are expressed as the percentage of ^{125}I-insulin bound in the presence of a competitor relative to control.

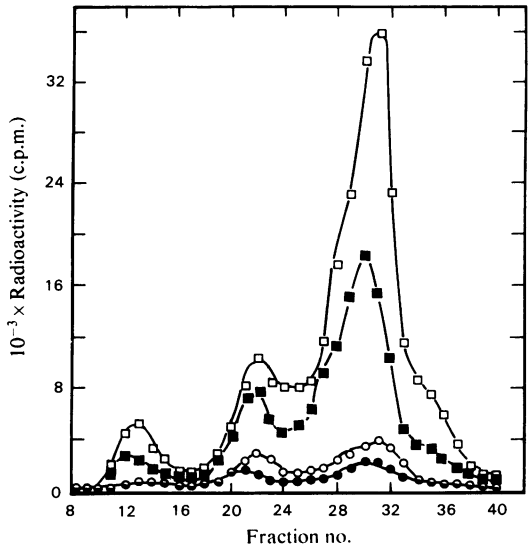

Fig. 5. *Recovery of radioactivity from hepatocytes* Hepatocytes (10^7/ml) were incubated with ^{125}I-labelled insulin for 0.5 h at 37°C. The suspension was layered over 10 ml of ice-cold Hanks' balanced salt solution/1% albumin and centrifuged (1500 *g*, 3 min). The supernatant was aspirated and the cell pellet washed without resuspension in ice-cold medium (1 ml). The pellet was resuspended in 0.1 M-HCl (1 ml, 4°C) and vigorously shaken for 10 min. Over 80% of the bound radioactivity was extracted when this process was sequentially repeated four times. ^{125}I-labelled insulin was not degraded by such treatment. The suspension was centrifuged (1500 *g*, 5 min) and neutralized. Extracts were frozen at −20°C before being passed over columns of Sephadex G-50 (fine grade) as described (Donner, 1980); 0.5 ml fractions were collected. Elution profiles of radioactivity from incubations containing 0.2 nM (●), 0.6 nM (○), 4.6 nM (■) and 7.5 nM (□) ^{125}I-labelled insulin are shown.

shown). In order to study the uptake of higher concentrations of insulin, a sample of iodinated insulin of low (approx. 10 Ci/g) specific radioactivity was prepared. The ability of native insulin to compete for the uptake of 0.1 μM-^{125}I-labelled insulin was determined. As expected, higher concentrations of native insulin were required to compete for the uptake of the radioactivity, and the competition curve was shifted to the right. Glucagon was found to compete for low-affinity insulin uptake. Competition became apparent in the presence of about 1 μM-glucagon. Some 75% of the insulin uptake by this low-affinity process was inhibited by 10 μM-glucagon. The ability of glucagon to compete for low-affinity insulin uptake did not result from contamination of the glucagon by insulin, as indicated by the inability of the same sample of glucagon to compete for high-affinity insulin binding determined at a lower (2 nM) ^{125}I-labelled insulin concentration. If the effect observed was due to the presence of insulin, more effective competition would have been expected against a low than a high concentration of labelled insulin. The sample of glucagon used contained 0.0000025% insulin. The concentration of glucagon (10 μM) that competed for 60% of the low-affinity insulin uptake could thus have contained less than 0.3 pM-insulin. This would have been insufficient to compete for either the high- or low-affinity uptake of insulin.

The concentration-dependence of insulin degradation was characterized at 37°C to add to my previous observations made at 23°C (Donner, 1980). ^{125}I-labelled insulin was incubated with hepatocytes for 30 min at 37°C and the bound radioactive label was extracted and fractionated by column chromatography (Fig. 5). In this experiment, a larger number of cells was used than in other studies, so that a sufficient amount of radioactive label could be recovered for characterization at the lowest hormone concentration studied. Radioactive label was eluted in the void volume of gel columns and in the volumes of insulin (fraction 22), iodotyrosine (fraction 31) and at fraction 26. The ratio of intact to degraded or processed insulin was 1.0:2.3,

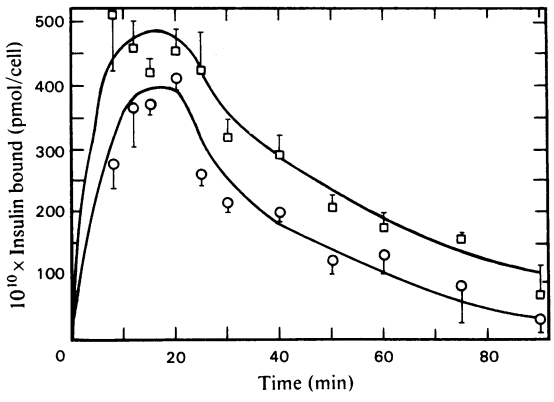

Fig. 6. *Degradation of insulin by hepatocytes during association*

Hepatocytes (3×10^5/ml) were incubated with ^{125}I-labelled insulin (1 nM) or ^{125}I-labelled insulin and native insulin (1.7 μM) at 37°C. At appropriate times, specific binding (□) or specifically bound material that was precipitated by trichloroacetic acid (○) was assayed. Error bars represent standard deviations of triplicate determinations.

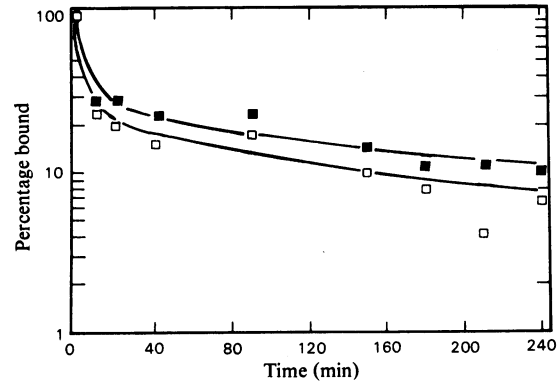

Fig. 7. *Degradation of insulin by hepatocytes during dissociation*

Hepatocytes were incubated with ^{125}I-labelled insulin (1 nM) or ^{125}I-labelled insulin and native insulin (1.7 μM) for 30 min at 37°C. Specific binding was assayed and dissociation was initiated by dilution of the incubation into 1 litre of Hanks' balanced salt solution/1% albumin (a 1:200 dilution). At timed intervals, 10 ml samples of the dissociation mixture were placed in glass test tubes (16 mm × 100 mm) and centrifuged (1500 ***g***, 3 min). The supernatants were aspirated and the cell pellets were assayed for ^{125}I-labelled insulin. Assay just before dilution was considered to represent the zero-time point. The amount of specific binding and specifically bound material that was precipitated by trichloroacetic acid before dilution were 165×10^{-10} and 137×10^{-10} pmol/cell respectively. Results are expressed as the percentage of the specifically bound radioactivity (□) or the specifically bound radioactivity precipitated by trichloroacetic acid (■) remaining bound at any time.

1.0:2.0, 1.0:3.9 and 1.0:4.0 at 0.2, 0.6, 4.6 and 7.5 nM hormone respectively. Therefore the presence of sufficient hormone to saturate the high-affinity receptor ($K_D = 0.6$ nM; Gammeltoft *et al.*, 1978; Donner, 1980) increased both the amount and proportion of bound insulin that was degraded.

As a control, cells were incubated with ^{125}I-labelled insulin for 4 h at 4°C. Characterization of radioactivity extracted from such cells by gel filtration revealed that hormone had been taken up and degraded (results not shown). However, the amount of degradation was much less than was observed at either 23°C or 37°C; therefore assay of the unbound radioactivity (Fig. 2) did not suggest any substantial degree of hormone turnover.

The consequences of the retention of fragments of ^{125}I-labelled insulin on the kinetic characterization of hormone binding was examined. The amount of bound radioactivity that was precipitated in trichloroacetic acid was assayed during the association of ^{125}I-labelled insulin with hepatocytes at 37°C (Fig. 6). A fraction of the bound radioactivity was not intact insulin throughout a 90 min association reaction. The decrease in the specific binding after 20 min probably resulted from the degradation of ^{125}I-labelled insulin in the incubation (Fig. 2). Assay of [^{3}H]inulin trapping (Donner *et al.*, 1976) demonstrated that less than 3% of the radioactive label in cell pellets recovered from the binding assay was extracellular. Thus the isolation of insulin fragments in cell pellets did not result from extracellular trapping of radioactivity.

The retention of ^{125}I-labelled insulin and fragments of ^{125}I-labelled insulin was assayed during dissociation at 37°C. Radioactive label that precipitated in trichloroacetic acid dissociated more slowly than did acid-soluble radioactivity (Fig. 7). Therefore hormone fragments were released from hepatocytes more rapidly than was ^{125}I-labelled insulin. A fraction of radioactivity released from hepatocytes was acid-soluble during the entire dissociation, suggesting that bound ^{125}I-labelled insulin was continually degraded. At lower temperatures than 37°C (results not shown), a greater difference between curves assaying specific binding and acid-precipitable binding was observed. This probably was due to slower release of hormone fragments from the cell with decreasing temperature.

Discussion

The interaction of insulin with specific receptors results in profound changes in the function of target

cells (Kahn, 1976; Czech, 1980). Equally as important as mechanisms leading to the onset of insulin action are those processes responsible for the termination of cellular response to this hormone. Down-regulation of receptor number (Kahn, 1976), changes of receptor affinity (Olefsky *et al.*, 1979; Donner & Corin, 1980; Huber *et al.*, 1980; McCaleb & Donner, 1981; Corin & Donner, 1982) and degradation of the hormone regulate insulin action. The experiments presented above confirm several previously reported characteristics of the degradation of insulin by liver cells. Hepatocytes degrade physiological or supraphysiological amounts of insulin (Fig. 1). A small fraction of the degradation by cultured (Duckworth *et al.*, 1981) or freshly isolated (Terris & Steiner, 1975; Gammeltoft *et al.*, 1978; Fig. 2) hepatocytes is extracellular. Also, less degradation occurs at the plasma membrane than within the hepatocyte (Terris & Steiner, 1975; Fig. 1). Therefore saturable, adsorptive uptake requiring translocation of bound hormone into the cell is the predominant mechanism mediating the degradation of insulin (Terris & Steiner, 1975). Fluid-phase endocytosis of medium into the hepatocyte (Ose *et al.*, 1980) may account for the apparently non-saturable component of insulin degradation (Fig. 1).

Chloroquine inhibits the degradation of insulin by hepatocytes (Duckworth *et al.*, 1981; Draznin *et al.*, 1981) and adipocytes (Marshall & Olefsky, 1979; Hammons & Jarett, 1980), possibly by raising lysosomal pH (de Duve *et al.*, 1974). The amount of intact insulin retained by hepatocytes incubated with hormone concentrations near or below the dissociation constant of the insulin receptor ($K_D \approx 0.6$ nM; Gammeltoft *et al.*, 1978; Donner, 1980) was increased by chloroquine (Fig. 3*b*). An internalization pathway in which receptor-bound insulin is transferred into secondary lysosomes (Carpentier *et al.*, 1979) probably functions in the hepatocyte as in the adipocyte (Marshall & Olefsky, 1979). During the degradation of receptor-bound insulin, ultrastructural studies localize radioactivity to lysosomes (Carpentier *et al.*, 1979), whereas cellular fractionation demonstrates that most internalized insulin is degraded in the cytosol (Brush & Jering, 1979; Papachristodoulou *et al.*, 1981). The low concentrations of insulin bound to receptors and translocated to the cytosol could interact with insulin proteinase (Brush & Jering, 1979), an enzyme with a K_m of about 10 nM (Duckworth & Kitabchi, 1974). The isolation of high-molecular-weight transients derived from receptor-bound insulin is also consistent with the intermediate action of insulin proteinase (Fig. 5; Duckworth *et al.*, 1981).

The course of the competition between insulin and ^{125}I-labelled insulin for binding and degradation requires that a further degree of complexity must be incorporated into the sequence outlined above. The insulin receptor in hepatic (Donner & Corin, 1980; McCaleb & Donner, 1981; Corin & Donner, 1982) and adipose (Olefsky *et al.*, 1979; Huber *et al.*, 1980) tissue may exist in either of two states of affinity. These receptors are in equilibrium with one another, and occupancy favours the accumulation of the higher-affinity binding state. The occupancy-mediated affinity increase of the insulin receptor (Olefsky *et al.*, 1979; Huber *et al.*, 1980) may be a precursor step for subsequent hormone processing. Half-maximal inhibition of degradation occurs in the presence of 10 nM-insulin (Fig. 1), a hormone concentration at which the insulin receptor would be saturated (Gammeltoft *et al.*, 1978; Donner, 1980). This suggests that at high concentrations non-receptor uptake accounts for some degradation regardless of the affinity state of the receptor.

The different effects of chloroquine on the processing of hormone bound to sites of low or high affinity suggests different pathways of degradation for receptor- and non-receptor-bound insulin. Krupp & Lane (1982) found that not all of the degradation of insulin by chick liver cells was inhibited by chloroquine. In order to relate insulin degradation to receptor recycling, it was necessary to correct for insulin degradation that was not suppressed by chloroquine (Krupp & Lane, 1982). The data in this paper also suggest that, without correction for non-insulin-receptor-mediated uptake, hormone degradation cannot be used as a measure for receptor recycling.

The concentration-dependence of degradation and the effect of glucagon on insulin uptake offer insight into the nature of the non-receptor system. The observation that the proportion of degraded hormone increases as insulin interacts with low-affinity sites suggests that such uptake is related to processing. Molecular species which degrade insulin and are distinct from the insulin receptor have been characterized in liver cell membranes (Freychet *et al.*, 1972; Krupp & Livingston, 1980). Glutathione–insulin transhydrogenase (Varandani, 1973) and insulin proteinase (Duckworth, 1979; Yokono *et al.*, 1982) are in the hepatocyte plasma membrane, although the predominant sites at which they are localized are intracellular (Brush & Jering, 1979; Ansorge *et al.*, 1973). Degradation by glutathione–insulin transhydrogenase will be favoured at high hormone concentrations, since the K_m for the interaction of insulin with this enzyme is in the micromolar range (Ansorge *et al.*, 1973). Since degradation at the plasma membrane is limited, internalization of insulin during non-receptor-mediated processing may be a requisite step in the actions of glutathione–insulin transhydrogenase. Insulin and glucagon competitively interact with glutathione–insulin transhydrogenase (Varandani

et al., 1975). The inability of glucagon to compete for insulin binding to high-affinity sites, while inhibiting low-affinity uptake, both implicates glutathione–insulin transhydrogenase in the processing of insulin and precludes the possibility that insulin–receptor binding is an obligatory step preceding degradation at high hormone concentration. Alternatively, high concentrations of insulin may bind to receptors for insulin-like growth factors (Kahn *et al.*, 1981). This would be consistent with our observations if the glucagon used in the present study was contaminated with insulin-like growth factors and if binding to insulin-like growth-factor receptors leads to proportionately greater degradation than did binding to the insulin receptor. We cannot exclude the latter explanation, but favour the former, owing to the well-recognized role of insulin proteinases in the degradation of insulin. We have, however, described two independent systems leading to insulin degradation; one is insulin-receptor-mediated and the other is not.

The effects of degradation on the assay of insulin uptake and release are substantial (Donner, 1980; McCaleb & Donner, 1982). Association curves suggest more intact ^{125}I-labelled insulin than is present. Fragments of insulin are released more rapidly than insulin during dissociation, and this may affect kinetic determinations of receptor affinity. I (Donner, 1980) and others (Caro & Amatruda, 1980) previously reported that non-receptor-mediated insulin uptake and degradation may present the appearance of heterogeneous classes of receptor sites in equilibrium assays. The results presented here permit speculation that uptake related to glutathione–insulin transhydrogenase or uptake by binding to receptors that are entirely distinct from that for insulin gave rise to these observations. In either case extensive hormone degradation by whole cells renders the use of such equilibrium analysis inappropriate. However, minimal degradation is observed in membrane preparations, and curvilinear Scatchard plots from such systems may reflect the presence of two states of receptor affinity (Corin & Donner, 1982).

A small amount of insulin degradation was observed at 4°C in extracts from hepatocytes. The amount of degradation at low temperature was not sufficient to affect the integrity of the unbound pool of insulin (Fig. 2). This degradation may have resulted from uptake of insulin into a small number of non-viable cells which were abnormally permeable to insulin. These observations are not unique to liver cells, however, since similar results have been obtained with GH_3 pituitary tumour cells in culture (R. E. Corin, F. C. Bancroft, M. Sonenberg & D. B. Donner, unpublished work), nor are they specific for insulin, as CHO cells internalize receptor-bound low-density lipoprotein at 4°C (Anderson *et al.*, 1981). In many cells (Silverstein *et al.*, 1977), including the hepatocyte (Ose *et al.*, 1980), endocytosis may continue at low temperatures. Whether the degradation of insulin resulted from uptake by viable or non-viable cells, the integrity of bound radioactive label should be verified, even at low temperature.

The operation of receptor- and non-receptor-mediated pathways of insulin degradation may be of great importance. Chronic occupancy leads to down-regulation of receptor number (Kahn, 1976) and diminishes the efficacy of the receptor-mediated component of degradation (Philippe *et al.*, 1981). In contrast, glutathione–insulin transhydrogenase activity is induced by endogenous insulin (Varandani, 1974; Uete *et al.*, 1976; Hern & Varandani, 1980). Cells subjected to chronic exposure to high concentrations of hormone contain high enzyme levels (Varandani, 1974). The converse is true in tissues obtained from insulin-deficient animals (Uete *et al.*, 1976; Hern & Varandani, 1980). Therefore the two pathways of degradation are regulated in opposite ways by insulin. If both components of the degradative system were linked to the insulin receptor, compensation for a deficiency in one part of the processing mechanism by the other would not be possible.

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