Degradation of endocytosed insulin in rat liver is mediated by low-density vesicles

Richard J. PEASE, Geoffrey D. SMITH* and Timothy J. PETERS

Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

(Received 5 November 1984/21 January 1985; accepted 7 February 1985)

Uptake of ^{125}I -insulin by the liver of intact rats is followed by rapid translocation of label to low-density vesicles. Subcellular-fractionation studies indicate that, although the ^{125}I associated with these vesicles is predominantly trichloroacetic acid-precipitable, there is an acid-soluble component arising from processing of the hormone *in vivo*. H.p.l.c. analysis indicates that the acid-precipitable ^{125}I is not intact iodoinsulin, but may correspond to 'clipped insulin'. Isolated low-density vesicles degrade the acid-precipitable iodopeptide intravesicularly when incubated at 37°C in iso-osmotic medium at pH 7. The rate constant for intravesicular degradation is consistent with the rate of insulin clearance by the liver *in vivo*. Pretreatment of the rats with chloroquine resulted in a decrease in proteolysis of the iodopeptide within the isolated vesicles.

Studies of hormone processing by various cell and organ preparations have suggested that the ligand is delivered to its site of degradation by receptor-mediated endocytosis (Terris & Steiner, 1975, 1976; Asoli & Puett, 1978; Gliemann & Sonne, 1978; Draznin *et al.*, 1981; Duckworth *et al.*, 1981; Plas & Desbuquois, 1982; Neal *et al.*, 1983; Dennis & Aronson, 1984). Ligands binding to the plasma membrane and those remaining free in solution both gain access to the cell in similar vesicles. Early in endocytosis segregation occurs, so that the free contents are destined for lysosomes, whereas membrane markers recycle via the Golgi apparatus (Farquhar, 1983). Hormones bind to membrane receptors and are delivered to the Golgi/endoplasmic-reticulum/lysosome locus of the cell, where acidification (Tycko & Maxfield, 1982) and prelysosomal processing may dissociate the ligand from its receptor (Wall *et al.*, 1980; Posner *et al.*, 1981; Anderson *et al.*, 1982), facilitating the transfer of the ligand to lysosomes and the recycling of the receptor back to the plasma membrane (Geisow, 1982; Palade, 1982). In some tissues, this segregation may be incomplete. In rat liver, *in vivo*, ligands may be rapidly transferred to secretory compartments after endocytosis (Regoezci *et al.*, 1982; Khan *et al.*, 1982).

Iodoinsulin is translocated to low-density vesi-

cles, and autoradiography of the vesicles has suggested that they include very-low-density-lipo-protein-packed secretory vesicles and Golgi-associated saccules (Khan *et al.*, 1982). These workers have assumed that the secretory vesicles subsequently fuse with the lysosomes, where degradation of the ligand occurs (Posner *et al.*, 1982).

Studies from our laboratory have followed the endocytosis of iodoinsulin by perfused rat liver and have shown that translocation to low-density vesicles (Smith & Peters, 1982) is followed by the rapid appearance of ^{125}I -labelled degradation products (Smith *et al.*, 1984). Fractionation studies have indicated that ^{125}I -labelled degradation products are associated with the low-density vesicles (Smith *et al.*, 1984), suggesting that proteolysis may occur within the vesicles. In contrast, no evidence was obtained from these studies for the association of ^{125}I with lysosomes. In the present studies we have investigated the degradation of insulin by rat liver *in vivo*, and show that, after endocytosis, it is translocated to low-density vesicles, within which it is rapidly degraded.

Materials and methods

Portal-vein injections

[A14- ^{125}I]iodotyrosine]insulin was prepared essentially by the method of Welinder *et al.* (1983). Lactoperoxidase-iodinated insulin (Jorgensen &

* To whom reprint requests should be addressed.

Larsen, 1980) was chromatographed on a column of Hypersil-WP 300-butyl (Shandon, Runcorn, Cheshire, U.K.) and eluted with 23.5% (v/v) acetonitrile in 0.25M-triethanolamine/phosphate buffer, pH 3.0, at a flow rate of 1 ml/min. The ^{125}I -insulin produced was found to have a dissociation constant of 1.7 nmol/l in an isolated hepatocyte competitive-binding assay (S. Juul, personal communication).

Sprague-Dawley rats (approx. 300 g), fed *ad libitum*, were anaesthetized with intraperitoneal injection of phenobarbitone (50 mg/kg body wt.). ^{125}I -insulin (40 fmol; 1 μCi ; 1 ml) was injected into the portal vein over a period of 30 s, and subsequently (0–10 min) the liver was excised. The administration of ^{125}I -insulin increased circulating immunoassayable insulin by less than 10% (B. Chowdhary, unpublished work). In some experiments, rats were pretreated with two intraperitoneal doses of chloroquine (each 50 mg/kg body wt.) (Sigma Chemical Co.), 2 h and 1 h before the intraportal insulin injection.

Preparation of homogenates

Portions of liver (2 g) were minced and then homogenized in 10 ml of 0.25M-sucrose containing 1 mM- Na_2EDTA , pH 7.2, and 22 mM-ethanol (sucrose medium), with ten strokes each of a loose and a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.), and filtered through a 50 μm -mesh nylon filter. In some experiments, bacitracin (5 mg/ml; Sigma) was included in the homogenizing medium.

Analytical fractionation

Rat liver homogenates were fractionated by isopycnic sucrose-gradient centrifugation in a Beaufay zonal rotor, as described by Smith & Peters (1980), and the results were analysed as activity-volume distributions (Leighton *et al.*, 1968). In some experiments, bacitracin (2.5 mg/ml) was included in the sucrose solutions.

Preparative discontinuous-density-gradient centrifugation

Liver homogenate (2.5 ml) was layered on to a discontinuous gradient of 5 ml of sucrose ($\rho = 1.09 \text{ g}\cdot\text{cm}^{-3}$) and 10 ml of sucrose ($\rho = 1.14 \text{ g}\cdot\text{cm}^{-3}$), both solutions containing 22 mM-ethanol and 1 mM- Na_2EDTA , pH 7.2 (Pease *et al.*, 1984).

After centrifugation at 100 000g for 1 h at 4°C in the 3 $\times$ 25 rotor of an MSE SS65 centrifuge, the upper (soluble) layer and lower (low-density membrane) interfaces were aspirated in a volume of 2.5 ml, and the pellet was resuspended in 2 ml of sucrose medium.

Proteolysis of endogenous ^{125}I -insulin

Low-density membranes prepared from rats injected *in vivo* with ^{125}I -insulin were slowly diluted with an equal volume of 0.25M-sucrose containing 20 mM-imidazole/HCl buffer, pH 7, and incubated at 37°C for up to 1 h. In some experiments the buffer of the incubation mixture was varied. In other experiments, bacitracin (5 mg/ml), insulin (0.5 mg/ml) and/or 0.1% (w/v) Brij 35 (Sigma) was included in the incubation medium.

H.p.l.c. of the ^{125}I -labelled material from low-density vesicles

Low-density membranes were diluted in 0.25M-sucrose/20 mM-imidazole, pH 7, layered over 1 ml of Maxidens (Nyegaard, Oslo, Norway), and centrifuged for 15 min at 100 000g in an MSE 10 $\times$ 10 angle rotor. The membranes were resuspended in 0.5 ml of 0.1% Brij 35, 10 ml of 40 mM-HCl was added, and they were dispersed with ten strokes of the tight-fitting pestle in a Dounce homogenizer. After incubation for 1 h at 0°C, the membranes were pelleted by centrifugation (100 000g for 15 min) and the supernatant was collected, freeze-dried and redissolved in 0.5 ml of 25% acetonitrile in 0.25M-triethylammonium/phosphate buffer, pH 3.0 (elution buffer). Samples (200 μl) were loaded on a column (10 cm $\times$ 5 mm internal diam.) of Hypersil-WP 300-butyl, and the column was run at 1 ml/min on a Varian model 5000 chromatograph. Native insulin was used as a calibration standard.

Gel-exclusion chromatography of the trichloroacetic acid-soluble iodinated products

Liver homogenate was prepared in the presence of bacitracin (5 mg/ml) from a rat injected 2.5 min previously with ^{125}I -insulin. A portion (5.0 ml) of this homogenate was subjected to isopycnic centrifugation on a sucrose density gradient in a Beaufay zonal rotor as described above. The sucrose solutions contained 2.5 mg of bacitracin/ml. The fraction of density $1.12 \text{ g}\cdot\text{cm}^{-3}$, containing the peak of radiolabelled material, was treated with an equal volume of 20% trichloroacetic acid, centrifuged, and the supernatant chromatographed on Sephadex G-10 as described below. An additional portion (7.5 ml) of the homogenate was used to prepare low-density membranes as described above. These were incubated in iso-osmotic medium for 1 h, treated with an equal volume of trichloroacetic acid, followed by 100 μg of albumin/ml; the mixture was centrifuged (2500g for 10 min), and a sample of the supernatant analysed by gel filtration on Sephadex G-10.

Portions (1.0 ml) of the supernatant after trichloroacetic acid precipitation of samples were

chromatographed on a column (1.1 cm $\times$ 30 cm) of Sephadex G-10. The column was equilibrated and eluted with 4M-guanidinium chloride (Aldrich Chemical Co., Gillingham, Dorset, U.K.) at a flow rate of 15 ml/h.

Assays

Marker enzymes were assayed as described by Smith & Peters (1980) and Shah *et al.* (1984). Insulin proteinase was measured from the release of acid-soluble peptides from ^{125}I -insulin by the method of Brush & Jehring (1979). Protein was measured by a modification of the Lowry method (Schacterle & Pollack, 1973) with bovine serum albumin as standard. For determination of trichloroacetic acid-soluble and -precipitable ^{125}I , membranes were treated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid, 100 μg of albumin was added, and the mixture then centrifuged at 2500g for 10 min, and the supernatants and precipitates were counted for radioactivity with a Beckman 7000 gamma counter. Treatment of the membranes with Triton X-100 (0.1%, w/v) before trichloroacetic acid precipitation gave no alteration in the distribution of radioactivity. Addition of further amounts of albumin (300 μg) to the supernatant from the trichloroacetic acid-precipitation step, followed by re-centrifugation at 2500g for 10 min, gave no significant amounts of radioactivity in the pellet. Similarly, extraction of the pellet from the first acid precipitation with 0.1% Triton X-100, followed by re-precipitation with trichloroacetic acid, resulted in complete re-precipitation of the radioactivity.

Results

Uptake of ^{125}I -insulin by rat liver

^{125}I -insulin was injected into the portal vein of anaesthetized littermate rats; the liver was removed and 1–2 g of perilobular tissue was counted for ^{125}I radioactivity. The results in Fig. 1 show the ^{125}I in the liver at various intervals after injection. Association is maximal at 60 s, followed by rapid clearance from the liver. Similar activities of ^{125}I per g wet wt. were found for perilobular tissue from each liver lobe (results not shown).

Analytical fractionation

Fractionation on continuous sucrose gradients of liver homogenates prepared 2 min after injection (Fig. 2) shows trichloroacetic acid-precipitable ^{125}I associated primarily with low-density membranes, but also with a second component coincident with the plasma-membrane marker 5'-nucleotidase. Acid-soluble ^{125}I is associated with the cytosolic fraction and with low-density membranes. The results show no association of ^{125}I

Fig. 1. Association of ^{125}I -insulin with rat liver. ^{125}I -insulin was injected into the portal vein of male littermate rats over 30 s. Subsequently, perilobular tissue was removed and counted for ^{125}I . The results from two separate experiments are shown.

with the lysosomal marker, *N*-acetyl- β -glucosaminidase.

To determine whether the acid-soluble ^{125}I associated with the low-density membranes represents insulin that has been processed *in vivo*, homogenization and fractionation were carried out in sucrose solutions containing bacitracin (5 mg/ml and 2.5 mg/ml respectively) sufficient to inhibit the degradation of exogenously added iodoinsulin by over 90% (results not shown). The results (Fig. 3) demonstrate that ^{125}I that has been processed *in vivo* is associated with these vesicles.

Discontinuous-sucrose-density-gradient centrifugation

Fractions of equilibrium density $< 1.09 \text{ g} \cdot \text{cm}^{-3}$, $1.09\text{--}1.14 \text{ g} \cdot \text{cm}^{-3}$ and $> 1.14 \text{ g} \cdot \text{cm}^{-3}$ were prepared as described in the Materials and methods section. The distribution of trichloroacetic acid-soluble and -precipitable ^{125}I and of protein is shown in Table 1. Acid-precipitable ^{125}I is associated primarily with low-density membranes, but also with the pellet. Acid-soluble ^{125}I is associated with the soluble fraction, with a small component in the low-density membranes. Inclusion of bacitracin in the homogenate does not change the distribution of total ^{125}I , but a greater proportion of the ^{125}I in the soluble fraction is recovered in the acid-precipitable form. The results show that, under the harsher conditions of discontinuous

Fig. 2. Density-gradient fractionation of rat liver homogenates

^{125}I -insulin was injected into male rats, and after 2 min the liver was removed and fractionated as described in the Materials and methods section. The results show the density-gradient increments, the volume-frequency distributions of the marker enzymes malate dehydrogenase (mitochondria and cytosol), *N*-acetyl- β -glucosaminidase (lysosomes), 5'-nucleotidase (plasma membrane), neutral α -glucosidase (endoplasmic reticulum), lactate dehydrogenase (cytosol) and galactosyltransferase (Golgi apparatus) and the relative frequency distributions of trichloroacetic acid-soluble (—) and -precipitable (—) ^{125}I . The results are means of four experiments.

Table 1. Association of ^{125}I with low-density membrane fraction

Rats were injected with ^{125}I -insulin, their livers were homogenized after 2 min and membranes were prepared as described in the Materials and methods section. The results show the percentages of recovered radioactivity and protein from the homogenate and the trichloroacetic acid (TCA)-precipitability of ^{125}I . The results show means $\pm$ s.d. for three to four experiments (n.d., not determined).

Fraction	Control homogenate			Homogenate + bacitracin (5 mg/ml)	
	^{125}I recovery (%)	^{125}I TCA ppt. (%)	Protein recovery (%)	^{125}I recovery (%)	^{125}I TCA ppt. (%)
Soluble ($<1.09\text{ g}\cdot\text{cm}^{-3}$)	17.2 ± 2.6	29.5 ± 4.7	35 ± 0.5	28.4 ± 4.9	70 ± 10.5
Low-density membranes ($1.09\text{--}1.14\text{ g}\cdot\text{cm}^{-3}$)	34.4 ± 4.2	88.3 ± 1.6	5.2 ± 0.5	41.2 ± 8.1	85.7 ± 3.1
Pellet ($>1.14\text{ g}\cdot\text{cm}^{-3}$)	18.3 ± 2.9	89.9 ± 2.5	50.5 ± 1.7	11.7 ± 1.5	n.d.

Fig. 3. Sucrose-density-gradient fractionation in the presence of bacitracin

^{125}I -insulin was injected into male rats, and after 2 min liver was homogenized in sucrose medium containing 5 mg of bacitracin/ml, and fractionated on a sucrose density gradient containing 2.5 mg of bacitracin/ml. The results, means of two experiments, show the volume–relative-frequency plots of trichloroacetic acid-soluble (----) and -precipitable (—) ^{125}I .

gradient centrifugation, acid-soluble ^{125}I and some acid-precipitable ^{125}I is lost from the low-density vesicles, with acid-precipitable ^{125}I being subsequently degraded, presumably by the soluble proteinases of the homogenate. Similar distributions of ^{125}I between the fractions were found from homogenates prepared 30 s to 4.5 min after portal-vein injection (results not shown).

Incubation of low-density vesicles

Low-density vesicles isolated from rats that had been injected 2 min before removal of the liver were incubated as described in the Materials and methods section. A progressive degradation of trichloroacetic acid-precipitable ^{125}I was observed, with maximal initial rates between pH 6 and 7 (Fig. 4). A similar time course of degradation was observed in vesicles prepared 30 s to 4 min after portal injection (results not shown). Fig. 5 shows the effects of disruption of the vesicles with Brij, and inclusion of bacitracin or insulin in the incubation mixture. Bacitracin markedly inhibits the degradation of iodo-peptide by disrupted vesicles, but not by intact vesicles, suggesting that proteolysis is intravesicular. By contrast, insulin at a concentration of 0.9 mM did not decrease the rate of proteolysis of iodo-peptide either by disrupted or by intact vesicles. The insulin proteinase activity of low-density membranes was assayed by the

Fig. 4. pH optimum of vesicular proteolysis

^{125}I -insulin was injected intraportally into rats and, after 2 min, liver was fractionated by discontinuous-density-gradient centrifugation. Low-density vesicles were incubated at 37°C in 10 mM-imidazole (a) or -Mes (b) buffer at pH 5 (■), 6 (○), 7 (●) or 8 (□), and samples removed and mixed with trichloroacetic acid. The results show the percentage of acid-precipitable ^{125}I associated with the vesicle at zero time that is degraded to acid-soluble products during incubation.

Fig. 5. Effects of bacitracin and of insulin on vesicular proteolysis

Vesicles were incubated, as in Fig. 4, at pH 7 in the presence or absence of 0.1% Brij and of bacitracin (5 mg/ml) or insulin (0.5 mg/ml). Results show control (○) and experimental (●) incubations for: (a) incubation in absence of Brij with and without bacitracin; (b) incubation in presence of Brij with and without bacitracin; (c) incubation in absence of Brij with and without insulin; (d) incubation in presence of Brij with and without insulin. Results are averaged from two experiments.

method of Brush & Jehring (1979). Incubations in the presence and absence of 0.1% Brij showed the activity to be 80% latent and to have an apparent K_m for insulin of 3–4 μ M at pH 7.2.

Association of undegraded 125 I with low-density vesicles

Samples of low-density vesicles, incubated for up to 60 min, were re-centrifuged on a second discontinuous gradient to separate membrane-associated 125 I from free 125 I. The results (Table 2) shows that trichloroacetic acid-precipitable 125 I remains associated with the membrane fraction throughout incubation. Acid-soluble material is released into the supernatant, although a constant proportion of acid-soluble material is associated with the vesicles throughout incubation. Acid-soluble label was present in both the vesicles and

the supernatant, suggesting intravesicular proteolysis followed by release of degraded label.

H.p.l.c. of iodopeptides of the low-density vesicles

Low-density membranes were re-centrifuged over Maxidens, to yield pellets containing 90% acid-precipitable 125 I. The 125 I was released by acid treatment (89% of membrane-associated label) and was analysed by h.p.l.c. The results (Fig. 6) show the label to be associated with a peptide less hydrophobic than 125 I-insulin. The elution volumes of insulin and iodoinsulin were unchanged by acid treatment and freeze-drying.

Gel-exclusion chromatography

In order to investigate further the nature of the trichloroacetic acid-soluble radiolabel, samples obtained both from the analytical gradients and

after incubation of low-density vesicles were precipitated with trichloroacetic acid and chromatographed on Sephadex G-10. Fig. 7 shows the elution profile of the acid-soluble radiolabelled material immediately after isolation by isopycnic centrifugation, when intravesicular processing is at a minimum (30% acid-soluble products), and after incubation of low-density membranes for 1 h at 37°C, when intravesicular processing will have reached a plateau (63% acid-soluble products). The labelled material in both cases is within the included volume of the column, indicating $M_r < 500$. The elution profile obtained after 30 min

incubation of low-density membranes (not shown) was very similar to that seen after 60 min.

Effect of chloroquine pretreatment on low-density vesicle degradation

Rats were pretreated with chloroquine at doses that do not affect uptake of insulin but which block insulin processing *in vivo* (Posner *et al.*, 1982), and low-density vesicles were isolated and incubated *in vitro*. The results (Fig. 8) show that chloroquine both decreases the acid-soluble ^{125}I associated with isolated vesicles and delays the subsequent

Fig. 6. *H.p.l.c. analysis of iodopeptides associated with low-density vesicles*

Iodopeptides were obtained by acid extraction of low-density vesicles and analysed by h.p.l.c. The results show the elution profiles of ^{125}I -insulin (.....), of acid-treated freeze-dried iodoinsulin (—) and of extracted iodopeptides (----). The void volume of the column (V_0), the elution position of native insulin (Insulin), and the elution (Wash) with buffer containing 60% acetonitrile are shown.

Table 2. *Association of undegraded iodopeptide with incubated vesicles*

Low-density vesicles from rats injected with ^{125}I -insulin were incubated for 0–60 min at 37°C, pH 7. Portions (4 ml) of low-density membranes were layered on 10 ml of sucrose medium ($\rho = 1.17 \text{ g}\cdot\text{cm}^{-3}$) and centrifuged at 100000g for 20 min in a 3×25 rotor. The distribution (%) of ^{125}I , trichloroacetic acid-soluble (sol) and -precipitable (ppt), between the supernatant and the membranes collected at the interface ($\rho < 1.17 \text{ g}\cdot\text{cm}^{-3}$) is shown. The results are means of two experiments.

Incubation time (min)	^{125}I (%)			
	Supernatant		Membranes	
	sol	ppt	sol	ppt
0	13	8	22	57
15	23	11	26	40
30	30	8	27	35
60	45	5	23	27

Fig. 7. *Chromatography of trichloroacetic acid-soluble iodinated degradation products on Sephadex G-10*
The elution profiles of acid-soluble iodinated material obtained immediately after analytical fractionation (—) and from a low-density membrane fraction after 60 min incubation (----) are compared with those of ^{125}I -insulin (.....) and ^{125}I (—). The void volume (V_0) of the column was determined with Blue Dextran.

Fig. 8. Effect of chloroquine pretreatment on vesicular proteolysis

Rats were pretreated for 2h with 100mg of chloroquine/kg body wt., then injected with ^{125}I -insulin, and liver was removed after 2min. Low-density vesicles were isolated and incubated in sucrose medium containing 10mM-imidazole, pH7. Results show the trichloroacetic acid-soluble ^{125}I as a percentage of the total ^{125}I , and are means $\pm$ S.D. for six control animals (○) and for four chloroquine-treated animals (●).

degradation of acid-precipitable label within low-density membranes.

The delay seems consistent with diffusion of chloroquine from the lumen of the vesicles into the incubation medium. Conversely, when chloroquine (0.5–2.0mM) was included in the incubation of vesicles from untreated animals, a normal rate of processing was observed for 5min, followed by marked inhibition of degradation (results not shown). This confirms that chloroquine can diffuse across the membranes of incubated vesicles *in vitro*.

Discussion

These studies have followed the hepatic processing of [A14- ^{125}I]iodotyrosine]insulin after injection into the portal vein of male rats. The liver was confirmed to be a major site of iodoinsulin processing, and the time course of association of ^{125}I with the liver was similar to that reported by Pilch *et al.* (1983) and by Posner *et al.* (1981).

The subcellular localization of ^{125}I was investigated by continuous sucrose-density-gradient centrifugation. About 70% of the ^{125}I was trichloroacetic acid-precipitable and was associated with low-density membranes and, to a lesser extent, with membranes coincident with plasma membranes. Acid-soluble ^{125}I was associated with cytosolic fractions, and with the low-density membranes, and represented ^{125}I that had been processed *in vivo*, and was not due to artifactual degradation during processing.

Low-density vesicles were prepared by discontinuous-density-gradient centrifugation. Enzymic and morphological characterization of these vesicles has been reported previously (Pease *et al.*, 1984). These vesicles were enriched in ^{125}I , although some acid-precipitable and most of the acid-soluble ^{125}I was lost from the vesicles under the harsher conditions of discontinuous-density-gradient centrifugation, as compared with continuous-gradient centrifugation in the Beaufay rotor. To determine whether these vesicles could participate in insulin degradation *in vivo*, they were incubated in buffered medium at 37°C and the degradation of endogenous ^{125}I -peptide to trichloroacetic acid-soluble products was monitored. Degradation was maximal at pH6–7. Two results show that proteolysis is intravesicular. Firstly, degradation of the iodo-peptide by Brij-disrupted, but not by intact, vesicles was inhibited by bacitracin. Similar results were obtained when 1mM- AgNO_3 (Burghen *et al.*, 1972) was substituted for bacitracin (results not shown). Secondly, trichloroacetic acid-precipitable iodo-peptide remained associated with the membranes throughout incubation. In contrast, exogenous insulin at concentrations 250-fold above the K_m for insulin of the insulinase activity of these membranes did not compete with the proteolysis of the iodo-peptide either by intact or by Brij-disrupted vesicles. The lack of effect of insulin on the proteolysis of Brij-disrupted vesicles implies that the iodo-peptide within the vesicles is not iodoinsulin. Figs. 5(a) and 5(b) also show that the rate of degradation is increased in the presence of detergent and not decreased as might be expected from substrate dilution on disruption of the vesicles. This is probably due to contact between insulin and extra-vesicular proteinases.

H.p.l.c. confirmed that the membrane-associated iodo-peptide was not iodoinsulin. Poole *et al.* (1982) and Misbin *et al.* (1983) have both described a rapidly formed high- M_r intermediate of insulin processing by hepatocytes that is less hydrophobic than [A14- ^{125}I]iodotyrosine]insulin, and that has a structure consistent with initial cleavage by insulin proteinase. It seems probable that this iodo-peptide may be similar, and could arise by rapid cleavage of endocytosed insulin by a plasma-membrane-associated component of insulin proteinase (Yokono *et al.*, 1982). The nature of the proteolytic activity of the insulin-containing vesicles is unclear, and, since there is no reason to suppose that the principal degradative activity of detergent-dispersed membranes is the same activity that acts in the lumen of the vesicles, we have no means of identifying the physiological proteinase at present. However, lysosomal proteinases have been demonstrated in endocytic (Campbell &

Rome, 1983) and secretory (Quinn & Judah, 1978) compartments of rat liver.

Examination of the trichloroacetic acid-soluble products of intravesicular processing of iodinated insulin by Sephadex G-10 chromatography showed that this material had $M_r < 500$ and was therefore not merely the product of reductive cleavage of the A and B chains. Comparison with the elution volume of free iodide suggested that the reaction products were not the result of dehalogenase activity. These conclusions are valid at both early and late time points over the course of the intravesicular degradation.

Khan *et al.* (1982) have investigated the nature of the low-density vesicles by autoradiography of isolated Golgi fractions and have shown high- M_r iodopeptide in very-low-density-lipoprotein-containing Golgi secretory vesicles. Further, they have shown that pretreatment of rats with chloroquine at doses that inhibit insulin processing *in vivo* causes accumulation of iodopeptides in these vesicles, and they suggest that chloroquine blocks the transport of secretory vesicles to the lysosomes (Posner *et al.*, 1982). We have found that pretreatment of rats with chloroquine caused a marked delay in iodopeptide degradation by isolated vesicles. This suggests that chloroquine may directly inhibit degradation within the vesicles, inhibition being relieved as the chloroquine diffuses out, thus offering an alternative explanation of their results.

These experiments have shown that, after endocytosis of iodoinsulin, low-density vesicles degrade insulin-derived fragments *in situ*. From the descending limb of the clearance curve (Fig. 1) 1–4 min after injection, approx. 20% of the liver-associated ^{125}I is cleared/min. Analytical fractionation studies show that 70% of the trichloroacetic acid-precipitable ^{125}I is associated with low-density vesicles, and incubation of these vesicles shows that at 37°C and pH 7 the initial rate of degradation by these vesicles is approx. 6% of the included ^{125}I -peptide/min. Thus the rate of degradation by isolated vesicles can account for at least 20% of the total clearance from the liver observed *in vivo*.

Various workers have shown that the degradation of cell-bound insulin can be described as a first-order reaction. Thus Terris & Steiner (1975) and Juul & Jones (1982) obtained a rate constant of 0.03 min^{-1} for degradation of cell-bound insulin by intact hepatocytes. Jones *et al.* (1984) have calculated a value of 0.049 min^{-1} for degradation of receptor-bound insulin in humans.

Curve-fitting analysis of the present data (Simplex method; Nelder & Mead, 1965) indicated that the trichloroacetic acid-precipitable iodopeptide could be considered as two populations. One

($38 \pm 8\%$) did not appear to undergo degradation after isolation of the vesicles, the remaining iodopeptide undergoing degradation to acid-soluble products with an apparent first-order rate constant of $0.089 \pm 0.012\text{ min}^{-1}$ ($n = 12$).

The observed rate constant is consistent with the values reported from other systems discussed above. It would be expected that the constant for vesicular proteolysis would be greater than that for degradation of the total cell-bound pool, since not all of the insulin is in the degradative compartment. However, it would not be expected that the vesicular rate constant would be much greater than the rate constant for cell-bound insulin, or the undegraded label would not be seen to accumulate in low-density vesicles.

Thus we suggest that proteolysis in low-density vesicles of the endosome–Golgi axis may account for the physiological degradation of insulin in the liver.

We thank Dr. C. K. Lim for expert assistance with h.p.l.c., Ms. Taru Shah for technical support, and Ms. S. E. Ember for help in preparation of the manuscript. R. J. P. is in receipt of an M.R.C research studentship. This work was supported in part by the British Diabetic Association.

References

- Anderson, R. G. W., Brown, M. S., Biesiegel, U. & Goldstein, J. L. (1982) *J. Cell Biol.* **93**, 523–531
- Asoli, M. & Puett, D. (1978) *J. Biol. Chem.* **253**, 4892–4899
- Brush, J. S. & Jehring, A. (1979) *Endocrinology (Baltimore)* **104**, 1639–1643
- Burghen, G. A., Kitabchi, A. E. & Brush, J. S. (1972) *Endocrinology (Baltimore)* **91**, 633–642
- Campbell, C. H. & Rome, L. H. (1983) *J. Biol. Chem.* **258**, 13347–13352
- Dennis, P. A. & Aronson, N. N. (1984) *Metab. Clin. Exp.* **30**, 825–835
- Draznin, B., Solomons, C. C., Toothaker, D. R. & Sussman, K. E. (1981) *Endocrinology (Baltimore)* **108**, 8–17
- Duckworth, W. C., Runyan, K. B. & Wright, R. K. (1981) *Endocrinology (Baltimore)* **108**, 1142–1147
- Farquhar, M. G. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2407–2413
- Geisow, M. J. (1982) *Nature (London)* **295**, 649–650
- Gliemann, J. & Sonne, O. (1978) *J. Biol. Chem.* **253**, 7857–7863
- Jones, R. H., Sonksen, P. H., Boroujerdi, M. A. & Carson, E. R. (1984) *Diabetologia* **27**, 207–211
- Jorgensen, K. H. & Larsen, U. D. (1980) *Diabetologia* **19**, 546–554
- Juul, S. M. & Jones, R. H. (1982) *Biochem. J.* **206**, 295–299
- Khan, M. N., Posner, B. I., Khan, R. J. & Bergeron, J. J. M. (1982) *J. Biol. Chem.* **257**, 5969–5976

- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–512
- Misbin, R. I., Almira, E. C. & Buynitsky, S. J. (1983) *J. Biol. Chem.* **258**, 2157–2162
- Neal, G. W., Solomon, S. S., Shankar, T. P. & Duckworth, W. C. (1983) *Diabetologia* **25**, 404–410
- Nelder, J. A. & Mead, R. (1965) *Comput. J.* **7**, 308–313
- Palade, G. E. (1982) *Ciba Found. Symp.* **92**, 1–14
- Pease, R. J., Sharp, G. A., Smith, D. G. & Peters, T. J. (1984) *Biochim. Biophys. Acta* **774**, 56–66
- Pilch, P. F., Shia, M. A., Benson, R. J. J. & Fine, R. E. (1983) *J. Cell Biol.* **96**, 133–138
- Plas, C. & Desbuquois, B. (1982) *Biochem. J.* **202**, 333–341
- Poole, G. P., O'Connor, K. J., Lazarus, N. R. & Pogson, C. I. (1982) *Diabetologia* **23**, 49–53
- Posner, B. I., Bergeron, J. J. M., Josefsberg, Z., Khan, M. N., Khan, R. J., Patel, B. A., Sikstrom, A. & Verma, K. A. (1981) *Recent Prog. Horm. Res.* **37**, 539–579
- Posner, B. I., Patel, B. A., Khan, M. N. & Bergeron, J. J. M. (1982) *J. Biol. Chem.* **257**, 5789–5799
- Quinn, P. S. & Judah, J. D. (1978) *Biochem. J.* **172**, 301–309
- Regoezci, E., Chindemi, P. A., Debanne, M. T. & Charlwood, P. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2226–2230
- Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654–655
- Shah, T., Heywood-Waddington, D., Smith, G. D. & Peters, T. J. (1984) *Clin. Chim. Acta* **138**, 125–132
- Smith, G. D. & Peters, T. J. (1980) *Eur. J. Biochem.* **104**, 305–311
- Smith, G. D. & Peters, T. J. (1982) *Biochim. Biophys. Acta* **716**, 24–30
- Smith, G. D., Christensen, J. & Peters, T. J. (1984) *Biochem. Soc. Trans.* **12**, 1082–1083
- Terris, S. & Steiner, D. F. (1975) *J. Biol. Chem.* **250**, 8389–8398
- Terris, S. & Steiner, D. F. (1976) *J. Clin. Invest.* **57**, 885–896
- Tycko, B. & Maxfield, F. R. (1982) *Cell* **28**, 643–651
- Wall, D. A., Wilson, G. & Hubbard, A. L. (1980) *Cell* **21**, 79–93
- Welinder, B. S., Linde, S. & Brush, J. S. (1983) *J. Chromatogr.* **257**, 162–165
- Yokono, K., Roth, R. A. & Baba, S. (1982) *Endocrinology (Baltimore)* **111**, 1102–1107