Fate of injected glucagon taken up by rat liver *in vivo*

Degradation of internalized ligand in the endosomal compartment

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The uptake and processing of glucagon into liver endosomes were studied *in vivo* by subcellular fractionation. After injection of [¹²⁵I]iodo-Tyr¹⁰glucagon and [¹²⁵I]iodo-Tyr¹³glucagon to rats, the uptake of radioactivity into the liver was maximum at 2 min (6% of the dose/g of tissue). On differential centrifugation, the radioactivity in the homogenate was recovered mainly in the nuclear (N), microsomal (P) and supernatant (S) fractions, with maxima at 5, 10 and 40 min, respectively; recovery of radioactivity in the mitochondrial-lysosomal (ML) fraction did not exceed 6% and was maximal at 20 min. On density-gradient centrifugation, the radioactivity associated first (2–10 min) with plasma membranes and then (10–40 min) with Golgi-endosomal (GE) fractions, with 2–5-fold and 20–150-fold enrichments respectively. Subfractionation of the GE fractions showed that, unlike the Golgi marker galactosyltransferase, the radioactivity was density-shifted by diaminobenzidine cytochemistry. Subfractionation of the ML fraction isolated at 40 min showed that more than half of the radioactivity was recovered at lower densities than the lysosomal marker acid phosphatase. Throughout the time of study, the [¹²⁵I]iodoglucagon associated with the P, PM and GE fractions remained at least 80–90% trichloroacetic acid (TCA)-precipitable, whereas that associated with other fractions, especially the S fraction, became progressively TCA-soluble. On gel filtration and h.p.l.c., the small amount of degraded [¹²⁵I]iodoglucagon associated with GE fractions was found to consist of monoiodotyrosine. Chloroquine treatment of [¹²⁵I]iodoglucagon-injected rats caused a moderate but significant increase in the late recovery of radioactivity in the ML, P and GE fractions, but had little effect on the association of the ML radioactivity with acid-phosphatase-containing structures. Chloroquine treatment also led to a paradoxical decrease in the TCA-precipitability of the radioactivity associated with the P and GE fractions. Upon h.p.l.c. analysis of GE extracts of chloroquine-treated rats, at least four degradation products less hydrophobic than intact [¹²⁵I]iodoglucagon were identified. Radio-sequence analysis of four of these products revealed three cleavages, affecting bonds Ser²–Gln³, Thr⁵–Phe⁶ and Phe⁶–Thr⁷. When GE fractions containing internalized [¹²⁵I]iodoglucagon were incubated in iso-osmotic KCl at 30 °C, a rapid generation of TCA-soluble products was observed, with a maximum at pH 4. We conclude that endosomes are a major site at which internalized glucagon is degraded, endosomal acidification being required for optimum degradation.

INTRODUCTION

It is now well established that, upon interaction with the hepatocyte, its major target cell, glucagon undergoes receptor-mediated endocytosis and degradation [1–5]. However, the subcellular sites at which glucagon degradation occurs, and the role of endocytosis in glucagon degradation, are not yet firmly established. Studies on intact isolated hepatocytes have enabled the identification of two pathways of glucagon degradation: one chloroquine-sensitive pathway, shown to result in the cleavage of glucagon to low-molecular-mass peptides and suggested to occur in lysosomes [6]; and one chloroquine-insensitive pathway, shown to result in the removal of the three *N*-terminal residues of the hormone and suggested to occur in a superficial region of the hepatocyte [3]. Studies with isolated liver plasma membranes have shown that, after binding to its receptor, glucagon is processed to fragments that remain in part membrane-associated [7,8]; the Tyr¹³–Leu¹⁴ bond was identified as one major site of cleavage [9].

Previous studies using subcellular fractionation have shown that, upon uptake by the liver *in vivo*, [¹²⁵I]iodoglucagon undergoes progressive accumulation in low-density endocytic structures physically distinct from lysosomes [10,11]. It was also shown that the [¹²⁵I]iodoglucagon associated with these structures retained apparent integrity [11], making their involvement in glucagon degradation questionable. However, those studies used

analytical methods that would not have allowed the detection of subtle changes in the glucagon molecule.

In the present study, the uptake *in vivo* of [¹²⁵I]iodoglucagon into liver endocytic structures has been studied by using [¹²⁵I]iodo-Tyr¹⁰- and [¹²⁵I]iodo-Tyr¹³-glucagons as radio-labelled probes and h.p.l.c. as a method to assess [¹²⁵I]iodoglucagon integrity. In addition, because of the possibility that acidity may be required for optimum degradation of glucagon in endosomes, the effects of chloroquine treatment on [¹²⁵I]iodoglucagon processing *in vivo* and of a low pH on [¹²⁵I]iodoglucagon degradation in a cell-free system have been examined. The results of these studies clearly identify the endosomal compartment as one major site at which internalized glucagon is degraded. Furthermore, three cleavage sites affecting the *N*-terminal region of glucagon have been identified by radio-sequence analysis.

MATERIALS AND METHODS

Materials

Pig glucagon was from Novo Research Industries (Copenhagen, Denmark). Carrier-free Na¹²⁵I was from Amersham International (Amersham, Bucks., U.K.). Lactoperoxidase was from Calbiochem. BSA was from Miles. Trypsin, chymotrypsin, horseradish peroxidase (HRP; type VI), bacitracin, *N*-ethylmaleimide, diaminobenzidine and 1,10-

Abbreviations used: TCA, trichloroacetic acid; TFA, trifluoroacetic acid; HRP, horseradish peroxidase; GalBSA-HRP, galactosylated BSA conjugated to HRP.

phenanthroline were from Sigma. Acetonitrile and trifluoroacetic acid (TFA) of h.p.l.c. grade were from Baker Chemical Co. Chloroquine sulphate was from Specia (Paris, France). Other chemicals were of analytical grade.

Reverse-phase h.p.l.c. was performed on a Waters model 600 liquid chromatograph equipped with a model U6K sample injector fitted with a 1 ml loop, a guard column and an analytical micro Bondapak C18 column (0.4 cm × 30 cm; Waters), or an analytical Ultrasphere C18 ion-pairing column (5 µm particle size; 0.46 cm × 25 cm; Beckman). Eluates were monitored on-line for A_{280} with a LC spectrophotometer and for radioactivity with a Berthold LB 504 gamma detector connected to an Apple IIe computer.

Automated Edman degradation was carried out on an Applied Biosystems sequenator model 470 A equipped with on-line identification of amino acid phenylthiohydantoin. At each cycle, the radioactivity released from [^{125} I]iodoglucagon or its degradation products was monitored.

Preparation, purification and characterization of [^{125} I]iodoglucagon

[^{125} I]iodoglucagon was prepared by using lactoperoxidase as described by Sonne *et al.* [12], with a molar ratio of iodine to glucagon of 1:4. Routinely, incorporation of ^{125} I into peptide as determined by trichloroacetic acid (TCA) precipitation was in the range 60–80%, corresponding to a specific radioactivity of 60–80 mCi/mg (about 0.2 atom of iodine per molecule).

Purification of radioiodinated products was achieved by reverse-phase h.p.l.c. in two steps. The iodination mixture was first chromatographed on an analytical micro Bondapak C18 column, with as eluent a mixture of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), pumped at a rate of 2 ml/min. Elution was carried out for 30 min with a linear gradient of 30–40% solvent B. The two major radioactive products in the eluate were collected and, after addition of BSA (1%, w/v), freeze-dried. One of these products, which was eluted at 21 min (see the Results section), was further analysed by chromatography on an Ultrasphere C18 ion-pairing column exactly as described by Hagopian & Tager [2]. The two major products in the eluate were collected and freeze-dried.

The iodotyrosine composition of the radioiodinated products was assessed by Pronase hydrolysis, followed by chromatography on the micro Bondapak C18 column by isocratic elution with 15% solvent B. The distribution of ^{125} I between Tyr¹⁰ and Tyr¹³ of glucagon was assessed by two independent methods: sequential trypsin and chymotrypsin hydrolysis, and radio-sequence analysis. Trypsin hydrolysis of [^{125} I]iodoglucagons was carried out as described by Rojas *et al.* [13]. After isolation by h.p.l.c., the tryptic peptides were hydrolysed by chymotrypsin (0.1 mg/ml) in 25 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-CaCl₂ and 1 mg of BSA/ml for 16 h at 37 °C, and re-chromatographed; column and elution conditions were as described for Pronase hydrolysates. Radio-sequence analysis was carried out by automated Edman degradation as described above.

The binding of [^{125} I]iodoglucagon derivatives (0.01–1 nM) to liver plasma membranes was measured as described previously [14], with results analysed in accordance with Scatchard [15].

Animals and injections

Male Sprague-Dawley rats weighing 180–200 g were obtained from Charles River France and were fasted for 18 h before death. [^{125} I]iodoglucagon [(20–100) × 10⁶ c.p.m.] was diluted into 0.5 ml of 0.15 M-NaCl containing 0.1% BSA and injected over 15 s into the penis vein under light ether anaesthesia. Except when indicated, a mixture of isomers labelled at positions 10 and 13 (peak B of first chromatographic step; see the Results section)

was used. Rats were killed from 30 s to 40 min after injection. In studies with chloroquine, this drug was dissolved in water and given as two intraperitoneal injections (2.5 mg/100 g body wt. each) at 75 and 15 min before injection of [^{125} I]iodoglucagon.

Liver subcellular fractionation

Livers were homogenized and submitted to subcellular fractionation by using established procedures with slight modifications [16]. To minimize the degradation of [^{125} I]iodoglucagon which occurs in homogenates during fractionation, *N*-ethylmaleimide (2 mM), bacitracin (1 mg/ml) and 1,10-phenanthroline (5 mM) were routinely included in the homogenization medium. Nuclear (N), mitochondrial-lysosomal (ML), microsomal (P) and supernatant (S) fractions were isolated from homogenates in 0.25 M-sucrose by differential centrifugation [17]. Light (GEI), intermediate (GEi) and heavy (GEh) Golgi-endosomal fractions (respective densities 1.03–1.08, 1.08–1.11 and 1.11–1.18 g/cm³) were isolated from the microsomal fraction by discontinuous-density-gradient centrifugation [18]. Analytical subfractionation of Golgi-endosomal fractions was performed by the diaminobenzidine-induced density-shift method of Courtoy *et al.* [19]. Plasma membranes were isolated from homogenates in 1 mM-bicarbonate as described by Neville [20] up to step 11. In some experiments, the ML fraction was subfractionated by centrifugation in analytical sucrose density gradients as described previously [16]. After isolation, subcellular fractions were resuspended in 5 mM-Tris/HCl buffer, pH 7.4, and analysed for protein, marker enzymes and/or radioactivity. In preparative procedures, results of radioactivity determinations were expressed as percentage of injected dose per mg of protein, relative specific radioactivity [(c.p.m./mg of protein in fraction)/(c.p.m./mg of protein in homogenate)] and/or recovery (100 × total c.p.m. in fraction/total c.p.m. in homogenate). In analytical procedures, results of biochemical and radioactivity determinations were expressed as recoveries as a function of density.

Characterization of the radioactivity associated with subcellular fractions

This was done by three analytical methods: precipitation by 5% TCA; gel filtration on Sephadex G-25; and h.p.l.c. With the latter method, [^{125} I]iodoglucagon labelled at positions 10 or 13 (peaks I or II of second chromatographic step; see the Results section) were used as radioligands. For gel-filtration studies, Golgi-endosomal fractions were acidified with acetic acid (25%, v/v) and applied on a 60 cm × 1 cm column of Sephadex G-25 (superfine) equilibrated and eluted with 5% acetic acid containing 0.5 mg of BSA/ml. For h.p.l.c. studies, fractions were first acidified with acetic acid (23%) and freeze-dried. The dry residue was resuspended in 10% acetonitrile and centrifuged for 1 h at 20000 g. The supernatant, which contained 95% of the radioactivity, was chromatographed on the micro Bondapak C18 column, with as eluent a mixture of solvents A and B. Elution was carried out for 68 min with a linear gradient of 10 to 50% solvent B. Under these conditions, monoiodotyrosine and intact [^{125} I]iodoglucagon were eluted at 4 and 57 min respectively.

Cell-free degradation studies

Golgi-endosomal fractions were prepared 20 min after injection of [^{125} I]iodoglucagon as described above, except for the absence of inhibitors of degradation in the homogenization medium. Immediately after isolation, fractions were resuspended (0.1–2 mg/ml of protein) in 150 mM-KCl buffered with 25 mM-citrate/phosphate, pH 2–8.5, and incubated at 30 °C with constant shaking. At various times, the integrity of [^{125} I]iodoglucagon

in the incubation medium was assessed by precipitation with 5% TCA.

Protein and enzyme assays

Protein concentration was measured as described by Lowry *et al.* [21], with BSA as a standard. Acid phosphatase was assayed as described by Trouet [22]. Alkaline phosphodiesterase and *N*-acetyl- β -D-glucosaminidase were assayed by the method of Touster *et al.* [23], with 3 mM-*p*-nitrophenyl 5'-thymidylate and 6 mM-*p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as substrates respectively. Galactosyltransferase activity was measured as described by Beaufay *et al.* [24], with ovalbumin as acceptor.

RESULTS

Purification and characterization of mono[^{125} I]iodoglucagon

Fractionation of the glucagon iodination mixture by reverse-phase h.p.l.c. on the C18 micro Bondapak column allowed the isolation of two major radioiodinated products, which were eluted at 14 and 21 min (Fig. 1, panel *a*). These products were well resolved from native glucagon, which was eluted at 17 min. When individually collected and rechromatographed, products

A and B were eluted at the same position as observed initially (results not shown). However, upon treatment with chloramine-T (1 mM) at 23 °C for 5 min, peak B was converted into a product with the same retention time as product A (results not shown). Thus product A is probably an oxidized form of product B.

The chemical identity of peak B was examined by Pronase hydrolysis and sequential trypsin and chymotrypsin hydrolysis (results not shown). After hydrolysis by Pronase, about 95% of the radioactivity was eluted on h.p.l.c. as monoiodotyrosine. With trypsin hydrolysates, the radioactivity recovered in the h.p.l.c. eluate was equally distributed between two major components, with retention times of 3 and 8 min respectively. On rechromatography after chymotrypsin hydrolysis, the former was converted into an unknown product which was eluted at 7.5 min, whereas the latter was converted into a product which co-eluted with monoiodotyrosine. These results suggest that the tryptic peptides eluted at 3 and 8 min consist respectively of fragments 1–12 and 13–17 of glucagon, and that peak B consists of two equimolar populations of mono[^{125} I]iodoglucagons labelled at positions 10 and 13.

Further purification of peak B was then achieved by h.p.l.c. on an Ultrasphere ion-pairing C18 column under the conditions described by Hagopian & Tager [2]. As expected, the radioactivity eluted from the column was equally distributed between two components (I and II), with retention times of 30 and 34 min (Fig. 1, panel *b*). Radio-sequence analysis showed that the radioactivity associated with these peptides was released during cycles 13 and 10 of Edman degradation respectively (Fig. 2). These results extend those obtained by enzymic hydrolysis and show that peaks I and II are [[^{125} I]iodo-Tyr¹³]glucagon and [[^{125} I]iodo-Tyr¹⁰]glucagon respectively.

Biochemical characterization of liver subcellular fractions

Recoveries of protein and marker enzymes in liver subcellular fractions of normal rats are shown in Table 1. The N and P fractions accounted together for 90% of alkaline phosphodiesterase, a plasma membrane marker; the ML and P fractions, for about 70% and 20%, respectively, of acid phosphatase and *N*-acetyl- β -D-glucosaminidase, two lysosomal markers; and the P fraction, for 90% of galactosyltransferase, a Golgi marker. Golgi-endosomal fractions accounted together for 10% of galactosyltransferase, but for only 1% of alkaline phosphodiesterase, 0.6% of acid phosphatase and 0.4% of *N*-acetyl- β -D-glucosaminidase. From the data shown in Table 1, the maximal enrichment in lysosomal markers in Golgi-endosomal fractions can be estimated as 3-fold, a value at least 20 times lower than that reported for purified lysosomes [25]. Plasma membranes accounted for about 10% of alkaline phosphodiesterase, but for only 0.1% of lysosomal markers. These data essentially agree with previously published studies [16,17,20,25,26].

As previously reported [27], chloroquine treatment led to a 2-fold increase in the recovery of acid phosphatase in Golgi-endosomal fractions (results not shown).

Time course of uptake of [^{125} I]iodoglucagon into liver subcellular fractions of untreated and chloroquine-treated rats

After injection of [^{125} I]iodoglucagon, the uptake of radioactivity into liver homogenates was maximal at 2 min (Fig. 3). At this time, about 0.03% of the dose injected was recovered per mg of protein (about 6% of the dose per g of liver). Homogenate radioactivity declined rapidly thereafter, with a half-life of about 10 min. At no time after injection did chloroquine treatment significantly affect the uptake of radioactivity.

The subcellular distribution of the radioactivity in liver homogenates was first examined by using differential centrifugation (Fig. 4 and Table 2). At early times, about half of

Fig. 1. H.p.l.c. elution profiles of the ^{125} I-labelled products generated upon radioiodination of glucagon

After iodination of glucagon with ^{125} I, the entire iodination mixture was chromatographed on a micro Bondapak C18 column with as eluent a gradient of acetonitrile in 0.1% TFA (panel *a*). The arrow indicates the position of elution of native glucagon. The peak B eluted at 21 min was then collected, freeze-dried and chromatographed on an Ultrasphere ion-pairing C18 column exactly as described by Hagopian & Tager [2] (panel *b*). The broken lines indicate the concentration of acetonitrile in the eluent. Details of the methods are described in the Materials and methods section.

Fig. 2. Automated radio-sequence analysis of radioiodinated glucagons I (panel *a*) and II (panel *b*)

Radioiodinated glucagons I and II isolated by two-step h.p.l.c. (Fig. 1) were submitted to radio-sequence analysis as described in the Materials and methods section. For each labelled peptide, the radioactivity liberated as the corresponding amino acid phenylthiohydantoin is plotted against the cycle of Edman degradation.

Table 1. Distribution of protein and marker enzymes in subcellular fractions obtained from liver homogenates

Nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), supernatant (S), light (GE_L), intermediate (GE_i) and heavy (GE_h) Golgi-endosomal fractions and plasma membranes (PM) were prepared and examined for enzyme activities as described in the Materials and methods section. Recoveries of enzymes were calculated from relative specific activities and average protein yields, with results expressed as the percentage of activity in the homogenate.

Constituent	Subcellular fractions							
	N	ML	P	S	GE _L	GE _i	GE _h	PM
Protein	14.9 ± 3.2	15.7 ± 3.6	26.3 ± 1.3	41.5 ± 2.1	0.023 ± 0.01	0.050 ± 0.01	0.13 ± 0.03	0.92 ± 0.08
Alkaline phosphodiesterase	32.5 ± 4.8	7.1 ± 1.0	57.6 ± 4.7	3.6 ± 0.6	0.058 ± 0.01	0.37 ± 0.07	0.96 ± 0.15	11.9 ± 1.50
Acid phosphatase	2.5 ± 0.3	66.1 ± 7.8	20.9 ± 2.5	10.4 ± 0.9	0.034 ± 0.01	0.15 ± 0.02	0.40 ± 0.12	0.11 ± 0.03
<i>N</i> -Acetyl-β-D-glucosaminidase	4.5 ± 0.9	75.8 ± 9.9	17.2 ± 1.4	2.4 ± 0.3	0.034 ± 0.01	0.083 ± 0.01	0.24 ± 0.04	0.19 ± 0.04
Galactosyltransferase	—	—	91 ± 6	12 ± 2	0.98 ± 0.17	2.34 ± 0.32	7.27 ± 1.22	—

Fig. 3. Time course of uptake of [¹²⁵I]iodoglucagon into liver homogenates from control (□) and chloroquine-treated (■) rats

Animals were killed at the indicated times after injection of an equimolar mixture of [¹²⁵I]iodo-Tyr¹⁰- and [¹²⁵I]iodo-Tyr¹³-glucagons (about 7×10^6 c.p.m.). The results shown are means ± S.E.M. of 6 determinations.

the radioactivity was associated with the N and P fractions, the remainder being associated with the S fraction. Up to 20 min, there was a progressive decrease in the amount of the radioactivity associated with the N fraction, along with an increase in that present in the P fractions; the amount of radioactivity associated with the ML fraction increased only slightly, and that associated with the S fraction remained relatively unchanged. After 20 min, however, the amount of radioactivity associated with the ML and P fractions decreased, whereas that associated with the S fraction increased proportionately. Chloroquine treatment did not affect the distribution pattern of the radioactivity in the first 20 min, but caused an increase in the proportion of radioactivity associated with the ML and P fractions at 40 min, along with a correlative decrease of that associated with the S fraction.

The uptake of radioactivity into plasma membranes and Golgi-endosomal fractions derived from the P fraction was then studied (Fig. 5 and Table 3). Uptake of radioactivity into plasma membranes was maximal at 5 min, with a 5-fold enrichment and a 5% recovery. Uptake of radioactivity into Golgi-endosomal fractions occurred more slowly and reached a maximum by 20 min, with enrichments of about 25- (light fraction), 150- (intermediate fraction) and 75-fold (heavy fraction), and corresponding recoveries of 0.5, 5.5 and 5.1%. Chloroquine treatment did not affect the uptake of the radioactivity into plasma membranes, but caused a decrease in the enrichment of the radioactivity in Golgi-endosomal fractions at 5–20 min. However, because of higher protein recoveries, recoveries of radioactivity in Golgi-endosomal fractions of chloroquine-treated rats

Fig. 4. Distribution of the radioactivity in subcellular fractions obtained from liver homogenates by differential centrifugation in control (a) and chloroquine-treated (b) rats

Liver homogenates obtained at various times after injection of an equimolar mixture of [^{125} I]iodo-Tyr ^{10}] and [^{125} I]iodo-Tyr ^{13}] glucagons were fractionated by differential centrifugation as described in the Materials and methods section. Subcellular fractions are plotted from left to right in the order of their coefficient of sedimentation: nuclear fraction (N), mitochondrial-lysosomal fraction (ML), microsomal fraction (P) and supernatant (S). On the abscissa, each fraction is represented cumulatively by its protein content, expressed as the percentage of total homogenate protein. On the ordinate, each fraction is represented separately by the enrichment (relative specific activity) of the radioactivity, expressed by reference to the radioactivity in the homogenate. The results shown are means $\pm$ S.E.M. of 6 determinations.

Table 2. Recoveries of radioactivity in the nuclear (N), mitochondrial-lysosomal (ML), microsomal (P) and supernatant (S) fractions in control and chloroquine-treated rats

Recoveries were calculated from relative activities (shown in Fig. 4) and protein yields with results expressed as the percentage of the radioactivity in the homogenate. Asterisks indicate a significant difference between control and chloroquine-treated animals (** $P < 0.01$; *** $P < 0.001$).

Subcellular fraction	Treatment	Time of killing after injection (min)				
		2	5	10	20	40
N	Control	16.3 $\pm$ 3.69	15.1 $\pm$ 2.29	10.4 $\pm$ 0.86	7.95 $\pm$ 0.79	5.68 $\pm$ 1.06
	Chloroquine	24.9 $\pm$ 4.48	17.6 $\pm$ 1.86	13.7 $\pm$ 1.53	11.1 $\pm$ 2.27	8.21 $\pm$ 0.73
ML	Control	2.06 $\pm$ 0.12	1.61 $\pm$ 0.15	2.37 $\pm$ 0.07	6.22 $\pm$ 0.44	3.45 $\pm$ 0.24
	Chloroquine	1.54 $\pm$ 0.14	1.84 $\pm$ 0.13	2.06 $\pm$ 0.33	7.72 $\pm$ 0.57	8.86 $\pm$ 1.45**
P	Control	27.9 $\pm$ 1.60	34.8 $\pm$ 4.04	47.1 $\pm$ 2.00	43.3 $\pm$ 0.73	22.0 $\pm$ 0.90
	Chloroquine	21.1 $\pm$ 3.68	34.7 $\pm$ 0.81	40.6 $\pm$ 2.84	46.3 $\pm$ 2.14	30.9 $\pm$ 0.51***
S	Control	39.1 $\pm$ 1.33	30.7 $\pm$ 2.15	28.2 $\pm$ 2.12	29.8 $\pm$ 1.09	56.0 $\pm$ 5.27
	Chloroquine	37.5 $\pm$ 2.61	32.9 $\pm$ 2.17	27.7 $\pm$ 0.88	26.1 $\pm$ 3.61	43.7 $\pm$ 5.71

at 5 and 10 min were decreased to a lesser degree, and recoveries at 20 and 40 min were, in fact, generally increased.

Subfractionation of Golgi-endosomal and ML fractions on analytical sucrose density gradients

To assess further the localization of the radioactivity associated with Golgi-endosomal fractions, the latter were subfractionated by the diaminobenzidine-induced density-shift protocol [19], after which the distribution of the radioactivity was compared with that of galactosyltransferase. When Golgi-endosomal fractions loaded by the GalBSA-HRP conjugate were incubated

with diaminobenzidine in the absence of H_2O_2 , the radioactivity migrated as a peak of density of about $1.11 \text{ g} \cdot \text{cm}^{-3}$, which coincided with the peak for galactosyltransferase (Fig. 6). In contrast, when the same incubation was carried out in the presence of H_2O_2 , the peak of radioactivity was shifted to a density of about $1.15 \text{ g} \cdot \text{cm}^{-3}$, the distribution of galactosyltransferase remaining unaffected. These results identify endosomes as the structures containing glucagon in Golgi-endosomal fractions.

To determine whether the radioactivity present in the ML fraction at late times was associated with lysosomes, its dis-

Fig. 5. Distribution of the radioactivity in plasma membranes and Golgi-endosomal fractions in control and chloroquine-treated rats

Plasma membranes (PM) and light (GEI), intermediate (GEi) and heavy (GEh) Golgi-endosomal fractions were prepared from liver homogenates obtained at various times after injection of an equimolar mixture of [^{125} I]iodo-Tyr ^{10} - and [^{125} I]iodo-Tyr ^{13} -glucagons; experimental details are described in the Materials and methods section. The results shown are means $\pm$ S.E.M. of 4–6 determinations. Key: ▨, control; ■, chloroquine-treated.

tribution on sucrose gradients was studied and compared with that of acid phosphatase. As shown in Fig. 7(a), the radioactivity was broadly distributed throughout the gradient with a peak at densities 1.11–1.13 $\text{g} \cdot \text{cm}^{-3}$, and a median density of 1.165 $\text{g} \cdot \text{cm}^{-3}$. In contrast, acid phosphatase was recovered mainly at densities below 1.15 $\text{g} \cdot \text{cm}^{-3}$, with a median density of 1.203 $\text{g} \cdot \text{cm}^{-3}$. In chloroquine-treated rats (Fig. 7b), both the radioactivity and acid phosphatase were shifted towards lower densities (median densities of 1.128 and 1.169 $\text{g} \cdot \text{cm}^{-3}$ respectively), but the relative amount of radioactivity overlapping with acid phosphatase was little affected. Comparison of the density-distribution histograms indicates that, regardless of chloroquine treatment, at least 55–60% of the radioactivity associated with the ML fraction is intrinsic to structures which do not contain acid phosphatase, presumably endosomes.

Fig. 6. Effects of diaminobenzidine and H_2O_2 treatment on the density distribution of [^{125} I]iodoglucagon (a) and galactosyltransferase (b) associated with Golgi-endosomal fractions

Golgi-endosomal fractions were isolated from rats killed 10 min after co-injection of [^{125} I]iodoglucagon (equimolar mixture of [^{125} I]iodo-Tyr ^{10} and [^{125} I]iodo-Tyr ^{13} isomers) and GalBSA-HRP conjugate. These fractions were then treated with diaminobenzidine in the absence or presence of H_2O_2 and subfractionated by centrifugation in continuous sucrose density gradients. In each subfraction, radioactivity (a) and galactosyltransferase activity (b) were determined, with results expressed as the percentage of the total recovered per normalized density interval of 0.01 $\text{g} \cdot \text{cm}^{-3}$. Arrows on the abscissa indicate the median densities of constituents in the absence (thick arrow) or presence (thin arrow) of H_2O_2 . Key: □, control; ▨, diaminobenzidine-treated.

Characterization of the radioactivity associated with liver subcellular fractions

The integrity of the [^{125} I]iodoglucagon associated with liver subcellular fractions was first characterized by TCA precipitation (Fig. 8). At 2 min, about 90% of the radioactivity associated with the homogenate and the crude sedimentable fractions was TCA-precipitable, but only 70% of that associated with the S fraction was TCA-precipitable. Later, there was a progressive

Table 3. Recoveries of radioactivity in plasma membranes (PM) and in light (GEI), intermediate (GEi) and heavy (GEh) Golgi-endosomal fractions in control and chloroquine-treated rats

Recoveries were calculated from relative specific activities (shown in Fig. 5) and protein yields, with results expressed as the percentage of the radioactivity in the homogenate. Asterisks indicate a significant difference between control and chloroquine-treated animals (* $P < 0.05$; ** $P < 0.01$).

Subcellular fraction	Treatment	Time of killing after injection (min)				
		2	5	10	20	40
PM	Control	3.68 $\pm$ 0.65	5.18 $\pm$ 0.57	4.40 $\pm$ 0.49	1.74 $\pm$ 0.25	1.24 $\pm$ 0.09
	Chloroquine	3.09 $\pm$ 0.44	3.81 $\pm$ 0.39	3.92 $\pm$ 0.64	1.75 $\pm$ 0.06	1.12 $\pm$ 0.10
GEI	Control	0.03 $\pm$ 0.003	0.13 $\pm$ 0.03	0.35 $\pm$ 0.05	0.39 $\pm$ 0.04	0.46 $\pm$ 0.05
	Chloroquine	0.04 $\pm$ 0.004	0.11 $\pm$ 0.02	0.20 $\pm$ 0.03	0.72 $\pm$ 0.10*	0.68 $\pm$ 0.12
GEi	Control	0.09 $\pm$ 0.01	0.89 $\pm$ 0.17	3.68 $\pm$ 0.35	5.47 $\pm$ 0.51	3.35 $\pm$ 0.26
	Chloroquine	0.11 $\pm$ 0.02	0.50 $\pm$ 0.07	2.81 $\pm$ 0.54	7.33 $\pm$ 1.12	5.03 $\pm$ 0.18**
GEh	Control	0.46 $\pm$ 0.01	0.89 $\pm$ 0.20	3.05 $\pm$ 0.26	5.10 $\pm$ 0.44	2.06 $\pm$ 0.33
	Chloroquine	0.34 $\pm$ 0.07	0.97 $\pm$ 0.18	1.83 $\pm$ 0.26	3.55 $\pm$ 0.35	2.78 $\pm$ 0.15

Fig. 7. Density distribution of the radioactivity (□) and of acid phosphatase (▨) associated with the mitochondrial-lysosomal fractions from control (a) and chloroquine-treated (b) rats

A mitochondrial-lysosomal fraction was isolated from control and chloroquine-treated rats 40 min after injection of [^{125} I]iodoglucagon. It was subfractionated by centrifugation on linear sucrose density gradients as described in the Materials and methods section. In each subfraction, radioactivity and acid phosphatase activities were determined, with results expressed as the percentage of total recovered per normalized density interval of $0.01 \text{ g} \cdot \text{cm}^{-3}$. Arrows on the abscissa indicate the median densities of the radioactivity (thin arrow) and acid phosphatase (thick arrow). The results shown illustrate one representative experiment; similar results have been obtained in another identical experiment.

loss of [^{125} I]iodoglucagon integrity in most subcellular fractions, which was especially marked in the S fraction. However, the decrease in TCA-precipitability of the radioactivity in the P fraction occurred only after 20 min, and was preceded by a slight increase in TCA-precipitability. The radioactivity associated with Golgi-endosomal fractions was about 70–80 % TCA-precipitable at 5 min, and 80–95 % TCA-precipitable at later times, precipitability being higher in the heavy and intermediate fractions than in the light fraction. The radioactivity associated with plasma membranes remained at least 95 % TCA-precipitable regardless of time.

Chloroquine treatment little affected the TCA-precipitability of the radioactivity in homogenates and in the N, ML and S fractions, except for a significant increase in TCA-precipitability in homogenates at 40 min. Unexpectedly, at most times after injection, chloroquine treatment caused a slight but significant decrease in the TCA-precipitability of the radioactivity associated with the P fraction and the Golgi-endosomal fractions.

The integrity of the [^{125} I]iodoglucagon associated with Golgi-endosomal fractions of control and chloroquine-treated rats was further characterized by gel filtration and h.p.l.c., by using [^{125} I]iodoglucagon labelled at either the 10 or the 13 position (Fig. 9). In control rats, about 95 % of the radioactivity was recovered as intact [^{125} I]iodoglucagon; most of the remainder was eluted at the same position as monoiodotyrosine. In chloroquine-treated rats, there was a slight decrease in the

Fig. 8. TCA-precipitability of the radioactivity associated with liver subcellular fractions in control (□) and chloroquine-treated (■) rats

Samples of homogenate (Hm) and subcellular fractions prepared at various times after injection of [^{125} I]iodoglucagon (Figs. 3–5) were precipitated with 5 % TCA as described in the Materials and methods section. The results shown are means $\pm$ S.E.M. of 6 determinations. At least 98 % of the radioactivity associated with plasma membranes (not shown on the Figure) was TCA-precipitable.

radioactivity recovered as intact [^{125} I]iodoglucagon, along with the appearance of degradation products distinct from monoiodotyrosine. On gel filtration, these products were eluted as one single broad component, half-way between intact [^{125} I]iodoglucagon and monoiodotyrosine. On h.p.l.c., at least four degradation products less hydrophobic than [^{125} I]iodoglucagon, numbered sequentially 1–4, were identified; their retention times were 13, 28, 40 and 43 min respectively. The elution profile of these products was the same whether [^{125} I]iodo-Tyr ^{10}]glucagon or [^{125} I]iodo-Tyr ^{13}]glucagon was used as ligand, indicating similar processing of the two ligands.

The four major chloroquine-sensitive products were characterized by radio-sequence analysis (Fig. 10). When [^{125} I]iodo-Tyr ^{10}]glucagon was used as a radioligand, the radioactivity associated with products 1, 2, 3 and 4 was released mainly during cycles 4, 5, 5 and 8 of Edman degradation, respectively. With [^{125} I]iodo-Tyr ^{13}]glucagon, the radioactivity associated with products 3 and 4 was released during cycles 8 and 11, respectively. In addition, minor amounts of radioactivity associated with product 4 were released during cycles 6 and 7 with [^{125} I]iodo-Tyr ^{10}]glucagon and during cycles 9 and 10 with [^{125} I]iodo-Tyr ^{13}]glucagon. These results identify residues Thr ^{7} , Phe ^{6} , Phe ^{6} and Gln ^{3} as the main N-terminal residues of products

Fig. 9. Gel-filtration (*a,b*) and h.p.l.c. (*c,d*) elution profiles of the radioactivity associated with Golgi-endosomal fractions from control (*a* and *c*) and chloroquine-treated (*b* and *d*) rats

A total Golgi-endosomal fraction was isolated from rats killed 10 min after injection of about $(30-50) \times 10^6$ c.p.m. of [^{125} I]iodoglucagon. For gel-filtration studies, an equimolar mixture of [^{125} I]iodo-Tyr¹⁰ and [^{125} I]iodo-Tyr¹³ isomers was injected, whereas for h.p.l.c. studies the [^{125} I]iodo-Tyr¹⁰ isomer was used. The radioactivity was extracted and submitted to gel-filtration on Sephadex G-25 or to h.p.l.c. on a micro Bondapak C18 column as described in the Materials and methods section. Monoiodotyrosine and intact [^{125} I]iodoglucagon were eluted at 4 and 57 min respectively. The major degradation products identified in h.p.l.c. eluates of chloroquine-treated rats, numbered sequentially 1-4, were eluted at 13, 28, 40 and 43 min respectively. An identical elution profile was obtained when [^{125} I]iodo-Tyr¹³]glucagon was used as a ligand (not shown on the Figure).

1, 2, 3 and 4 respectively. They also suggest that products 2 and 3, which are eluted differently although having the same *N*-terminal residue, extend to different lengths beyond Tyr¹⁰.

Cell-free degradation of [^{125} I]iodoglucagon associated with endosomal fractions

The inability to detect large amounts of [^{125} I]iodoglucagon degradation products in Golgi-endosomal fractions may result from their rapid diffusion out of the endosomes, either *in vivo* or *in vitro* during subcellular fractionation. Accordingly, Golgi-endosomal fractions containing internalized [^{125} I]iodoglucagon were resuspended in iso-osmotic KCl buffered to pH 2-8.5 and examined for their ability to degrade this ligand in a subsequent incubation *in vitro* (Fig. 11). A rapid degradation of [^{125} I]iodoglucagon, as judged from the release of TCA-soluble products, occurred in the pH interval 3-5, with a maximum at pH 4. The rate of degradation (about 10 % of the total/min) was a linear function of time up to 30 % of the total degraded. It was, however, unaffected by the concentration of cell fraction protein in the incubation medium (results not shown), suggesting that degradation occurred intraluminally.

DISCUSSION

Previous studies using subcellular fractionation have shown that, when injected peripherally [10] or infused intraportally [11],

Fig. 10. Automated radio-sequence analysis of ^{125} I-labelled peptides isolated from Golgi-endosomal fractions of chloroquine-injected rats

^{125} I-labelled degradation products isolated by h.p.l.c. from Golgi-endosomal fractions of chloroquine-treated rats (Fig. 9) were submitted to automated radio-sequence analysis as described in the Materials and methods section. Panels (*a*), (*b*), (*c*) and (*d*) show radio-sequence analysis of peptides 1, 2, 3 and 4 generated from [^{125} I]iodo-Tyr¹⁰]glucagon respectively (Fig. 9*d*). Panels (*e*) and (*f*) show results obtained with peptides 3 and 4 generated from [^{125} I]iodo-Tyr¹³]glucagon respectively.

[^{125} I]iodoglucagon is taken up in part into the liver and accumulates under a relatively intact form in non-lysosomal low-density endocytic structures. In the present studies, the involvement of these structures in the processing of glucagon has been further assessed *in vivo* and in a cell-free system using [^{125} I]iodoglucagon labelled at positions 10 or 13 as probes and reverse-phase h.p.l.c. as a method to assess [^{125} I]iodoglucagon integrity. It has been found that, although virtually absent in freshly extracted endosomal fractions of control rats, degradation products are present in endosomal fractions from chloroquine-treated rats and are rapidly generated in isolated endosomes maintained at a low pH. Furthermore, three cleavage sites on the *N*-terminal side of the Tyr¹⁰ residue of glucagon have been identified. These data suggest that the endosomal compartment is a major site of glucagon degradation in the hepatocyte.

The use of the lactoperoxidase method of radioiodination and of a two-step h.p.l.c. purification procedure allowed the isolation of mono[^{125} I]iodoglucagons labelled at positions 10 and 13 that were not oxidized and were free of contamination by native glucagon. For convenience, most experiments described in this work were carried out with an equimolar mixture of isomers labelled at positions 10 and 13, and only when radio-sequence analysis was performed were the separated isomers used. This

Fig. 11. Cell-free degradation of $[^{125}\text{I}]$ iodoglucagon associated with Golgi-endosomal fractions as a function of time (a) and pH (b)

A total Golgi-endosomal fraction was isolated from livers of rats killed 20 min after injection of about 30×10^6 c.p.m. of an equimolar mixture of $[^{125}\text{I}]$ iodo-Tyr ^{10} - and $[^{125}\text{I}]$ iodo-Tyr ^{13} -glucagon. After resuspension, this fraction was incubated in buffered 0.15 M-KCl at 30 °C under various time and pH conditions, after which the release of TCA-soluble radioactivity was measured. (a) Time course of $[^{125}\text{I}]$ iodoglucagon degradation at pH 4 (■), 5 (□) and 6.5 (▲); (b) $[^{125}\text{I}]$ iodoglucagon degradation as a function of pH of the medium.

was of little importance, since, as previously shown in studies with isolated hepatocytes [2], the two isomers were processed identically by liver subcellular fractions.

The time-dependent changes in the subcellular distribution of $[^{125}\text{I}]$ iodoglucagon in control rats indicate that this ligand undergoes endocytosis. At early times, the sedimentable radioactivity in the homogenate was for the most part associated with the N and P fractions, a distribution typical for plasma-membrane markers, and was concentrated 6-fold in partially purified plasma membranes. Later, it accumulated progressively in the P and ML fractions, with a 20–150-fold concentration in low-density P subfractions. On analytical sucrose-density gradients, the distribution of radioactivity associated with the low-density P subfractions was density-shifted by diamino-benzidine cytochemistry, whereas the distribution of galactosyltransferase was unaffected. On similar gradients, at least 60 % of the radioactivity associated with the ML fraction at a late time was found to be associated with low-density structures that did not contain acid phosphatase. These findings confirm and extend previous results [10,11] and identify low-density endosomes as one major subcellular site at which $[^{125}\text{I}]$ iodoglucagon is concentrated upon endocytosis.

The time-dependent decrease in the TCA-precipitability of the radioactivity associated with liver homogenates and crude subcellular fractions indicates that $[^{125}\text{I}]$ iodoglucagon taken up by the liver is progressively degraded. In addition, the rapid generation of TCA-soluble products in the S fraction suggests that this process occurs intracellularly, at least in part. However, throughout the time of study the radioactivity associated with the P, PM and Golgi-endosomal fractions remained at least 80–90 % TCA-precipitable; a paradoxical increase in TCA-precipitability was even observed at early post-injection times, probably reflecting a rapid movement of monoiodotyrosine and/or short peptides out of the liver. Furthermore, on gel filtration and h.p.l.c., most of the $[^{125}\text{I}]$ iodoglucagon associated with Golgi-endosomal fractions retained integrity, monoiodotyrosine being the only degradation product detectable.

The inability to document extensive entry of internalized glucagon into lysosomes, also observed with insulin [26], and the

fact that $[^{125}\text{I}]$ iodoglucagon associated with endosomes retains apparent integrity, could result from a rapid diffusion of degradation products out of these organelles. One theoretical way to overcome this drawback would be to link $[^{125}\text{I}]$ iodoglucagon to a labelled molecule that does not diffuse through the membrane of endocytic structures, such as $[^{14}\text{C}]$ sucrose [28] or $[^{125}\text{I}]$ iodotyramine-cellobiose [29]. Although this approach has been successfully applied to high-molecular-mass proteins, such as asialoglycoproteins [30] and formaldehyde-treated albumin [31], it is unlikely to be applicable to a low-molecular-mass peptide such as glucagon. So far, most derivatives of glucagon have shown a drastic loss in the affinity of this peptide for its receptor [32].

In a number of ligand-receptor systems, the weak base chloroquine has been shown to increase the accumulation of internalized ligand in acidic cell compartments, presumably by inhibiting the dissociation of ligand-receptor complexes and/or the subsequent degradation of dissociated ligand [33]. At variance with previous observations with insulin [26,27,34] and prolactin [26,34,35], chloroquine treatment caused only a minimal and late increase in glucagon accumulation in the liver endocytic structures and a paradoxical decrease in TCA-precipitability of the ligand associated with these fractions. In gel-filtration and h.p.l.c. studies, chloroquine treatment led to the appearance of multiple low-molecular-mass degradation products less hydrophobic than $[^{125}\text{I}]$ iodoglucagon; radio-sequence analysis of four of these products identified cleavages at bonds Ser ^{2} -Gln ^{3} , Thr ^{5} -Phe ^{6} and Phe ^{6} -Thr ^{7} . These findings indicate that a low pH may be required for the complete degradation of intermediate products to monoiodotyrosine. Alternatively, certain endosomal proteases may retain some activity at neutral pH.

Several ligands undergoing endosomal degradation in intact cells or tissues, such as mannoseylated serum albumin in macrophages [36,37] and insulin in liver [38,39], have been shown to undergo further degradation in cell-free endosomes maintained at pH below neutrality. In agreement with these observations, when Golgi-endosomal fractions containing $[^{125}\text{I}]$ iodoglucagon were incubated under iso-osmotic conditions at 30 °C, $[^{125}\text{I}]$ iodoglucagon was rapidly converted into TCA-soluble products, with a maximum at pH 4. This observation reinforces the view that, at the low pH which prevails in endosomes, the TCA-soluble products generated from $[^{125}\text{I}]$ iodoglucagon *in vivo* rapidly diffuse out of these organelles.

Although insulin internalized in liver cells is taken up and degraded in the same endocytic structures as glucagon [33,40], the fate of these peptides differs in several respects. First, in time studies, insulin associates more rapidly with endosomes than does glucagon, and is also more rapidly cleared from these structures [26,34,41,42]. Secondly, presumably because of their larger size, insulin degradation products, unlike glucagon products, remain in part associated with endosomes [41–43]. Thirdly, the endosomal retention of undegraded ligand caused by chloroquine treatment is higher for insulin than for glucagon [26,27]. Finally, in cell-free endosomes, the pH for maximal insulin degradation is about 5–6 [38,39], a value higher than the pH for maximal glucagon degradation; this appears to reflect different sensitivities of ligand-receptor complexes to dissociation by a low pH (F. Authier & B. Desbuquois, unpublished work).

Because the integrity of the glucagon sequence beyond tyrosine-13 was not examined in the present work, it is difficult to compare the specificity of the endosomal degrading activity with that of other glucagon-degrading activities reported previously. However, none of the cleavage sites identified in this study correspond to the cleavage site described in studies with isolated dog and rat hepatocytes, which was shown to affect the Gln ^{3} -Gly ^{4} bond [2,3]. Likewise, the results described here do not

support the involvement of the glucagon-receptor-linked protease recently identified in dog plasma membranes, which was shown to cleave the Tyr¹³-Leu¹⁴ bond [8,9]. Insulin protease, which cleaves the bond Thr⁵-Phe⁶ [44], might be a better candidate, although it does not account for the cleavages at the 2-3 and 6-7 positions. Additional work is required to identify the cleavage sites in the glucagon sequence beyond tyrosine-13 and to characterize the glucagon-degrading activity associated with liver endosomes.

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Received 14 May 1990/6 August 1990; accepted 20 August 1990