RECEPTOR-MEDIATED INTERNALISATION OF INSULIN IN INTACT RAT LIVER

A biochemical study

Bernard DESBUQUOIS, Jacques WILLEPUT and Agnès HUET de FROBERVILLE
Unité 30 INSERM, Hôpital des Enfants-Malades, 75015 Paris, France

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1. Introduction

There is wide acceptance that the initial step in the interaction of insulin with hepatocytes is binding to specific receptors at the cell surface. The fate of insulin following receptor binding has been studied with the use of a ^{125}I-labeled ligand in isolated hepatocytes [1–3], isolated perfused liver [4,5] and intact liver in vivo [6–8]. Ultrastructural studies have shown that insulin initially localized in the plasma membrane and was progressively translocated within the cell [2,3,7,8]; it preferentially associated with lysosomes in isolated hepatocytes [3] and with Golgi elements and lysosome-like structures of the Golgi region in liver in vivo [8]. Biochemical studies have demonstrated that tissue or cell-associated insulin was rapidly degraded to fragments that were in major part released extracellularly [1–6].

Based on these findings, it has been proposed that insulin was internalised by adsorptive pinocytosis [3]; this process would mediate the degradation of the hormone, and also explain the insulin-induced loss of receptors. To examine these issues, we have injected ^{125}I-labeled insulin to rats, analysed by subcellular fractionation the distribution of the labeled material taken up by the liver, and characterized the latter biochemically. We have also analysed the subcellular distribution of the insulin binding sites, following injection of native hormone.

2. Materials and methods

Porcine insulin (Eli Lilly and Co) was labeled with ^{125}I (the Radiochemical Centre, Amersham) at spec. act. 800–1100 Ci/mmol using a modification [9] of the chloramine T method.

Experiments were performed in fed, male Sprague Dawley rats (Charles River France) weighing 200 ± 20 g. ^{125}I-Labeled insulin (10–20 pmol) and/or unlabeled insulin, diluted into 0.5–1 ml physiological saline buffered to pH 7.4, were injected over <10 s in the penis vein under ether anesthesia. Shortly before sacrifice, the abdominal cavity was exposed through a median incision, and at the indicated times (measured from the end of injection), the liver was quickly removed and the fractionation procedures were immediately initiated (at 4°C). The total particulate fraction and the microsomal fraction were prepared from homogenates in 0.25 M sucrose as in [10]. The microsomal fraction was subfractionated by discontinuous density gradient centrifugation [11]. This procedure, carried out after ethanol treatment as described originally, resulted in the isolation of three subfractions of densities 1.031–1.077, 1.077–1.110 and 1.110–1.167 (referred to, respectively, as light, intermediate and heavy 'Golgi' fractions), and of a residual microsomal fraction (density 1.167). A fraction enriched in plasma membranes (density 1.188) was isolated from a low-speed sediment of homogenates in hypotonic medium as in [12], up to step 11. The subcellular fractions as isolated here were comparable to those obtained in earlier work with respect to enzymatic [13,14] and insulin-binding [15] activities.

Following isolation, subcellular fractions were analysed for protein [16] and, whenever indicated, radioactivity or in vitro insulin binding [17]. In addition, bound radioactivity was examined for sponta-

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neous release from the fractions in vitro, and was characterized biochemically. Experimental details are given in the appropriate table or figure legends.

3. Results

3.1. *Uptake of ^{125}I-labeled insulin by crude subcellular fractions*

Rapid uptake of radioactivity into liver subcellular fractions occurs following injection of ^{125}I-labeled insulin, confirming earlier reports [18,19]. Uptake is maximal by 1–2 min and subsequently declines with an apparent halflife of 6–10 min. At maximum, the recovery of radioactivity in the homogenate is ~0.02% of the dose injected/mg protein (3.6% of the dose/g liver). 80% of this material is associated with the particulate fraction, predominantly microsomal elements.

A marked inhibition of uptake of radioactivity occurs when native insulin is injected simultaneously with labeled insulin (table 1). At maximally effective doses, the former inhibits labelling of the particulate and microsomal fractions by ≥90%, and that of the homogenate, by 80%. In contrast, peptide hormones which are chemically unrelated to insulin do not inhibit, and even slightly enhance, labeling. Thus, insulin uptake by the liver in vivo demonstrates apparent specificity and saturability, and is presumably receptor-mediated.

Table 1
Uptake of radioactivity by the total particular fraction of liver following injection of ^{125}I-labeled insulin alone or in association with unlabeled peptide hormones

Unlabeled hormones (nature and dose[a])	Radioactivity bound (%)
Pork insulin, 2.6	25 ± 3
Pork insulin, 8.6	13 ± 4
Pork insulin, 86	8 ± 2
Beef–pork glucagon, 14.3	114 ± 12
Human growth hormone, 6.2	103 ± 6
1–24 Corticotropin, 21.3	115 ± 3

[a] nmol/100 g body wt

Rats were sacrificed 90 s after injection. Experiments routinely included 2 rats which received ^{125}I-labeled insulin alone (referred to as controls), and 2–4 rats which received labeled insulin at the same dose and unlabeled hormone. Within each experiment, uptake of radioactivity in the latter was expressed as the percentage of that observed in the controls. The results are the mean ± SEM of such percentages (3 determinations). Results of similar experiments with the microsomal fraction (not shown) were identical. Uptake of radioactivity in control rats was, respectively, 0.027 ± 0.001 (total particulate fraction) and 0.090 ± 0.004 (microsomal fraction) percent of injected dose per mg protein (mean ± SEM, 10 determinations)

3.2. *Kinetics of association of radioactivity with plasma membranes and microsomal subfractions*

Initial studies showed that, early after injection, the radioactivity associated with liver particles was recovered in the microsomal, and, to a lesser extent, nuclear fractions (80% and 20% of the total, respectively), whereas later on, microsomes became progressively more labeled. We therefore kinetically characterised the labeling of microsomal subfractions and of plasma membranes isolated from a nuclear pellet.

Specific labeling of plasma membranes and of the microsomal subfraction of highest density occurs immediately after injection, is maximal at 90 s, and becomes greatly reduced at 8 min (fig.1). At early times, labeling of the plasma membranes exceeds by 5-fold that of the microsomal elements. In contrast, labeling of the lighter, Golgi fractions, does not occur until 90 s, is maximal at 4 (heavy fraction) and 8 (light and intermediate fractions) min, and clears up by 24 min. The maximal labeling of the heavy and intermediate fractions exceeds by 5-fold that of the light fraction, and by 20-fold that of the residual microsomal fraction.

Recoveries of radioactivity in the fractions (expressed as the percentage of the dose injected recovered per g liver) were calculated using the protein yields indicated in legend to fig.1. Of note are the following findings:

(i) At 15 and 90 s, the radioactivity associated with the microsomal subfraction of high density (0.9–1.1%) exceeds by 6-fold that associated with the plasma membranes (0.15–0.17%);

(ii) At 4 and 8 min, the Golgi-associated radioactivity is recovered predominantly in the heavy fraction (1.1–1.2%) and, to a lesser extent, in the intermediate fraction (0.3%);

(iii) The recoveries of radioactivity in the three 'Golgi' fractions at 90 s and 4 min (1.2 and 1.5%) closely correspond to the summed recoveries of radio-

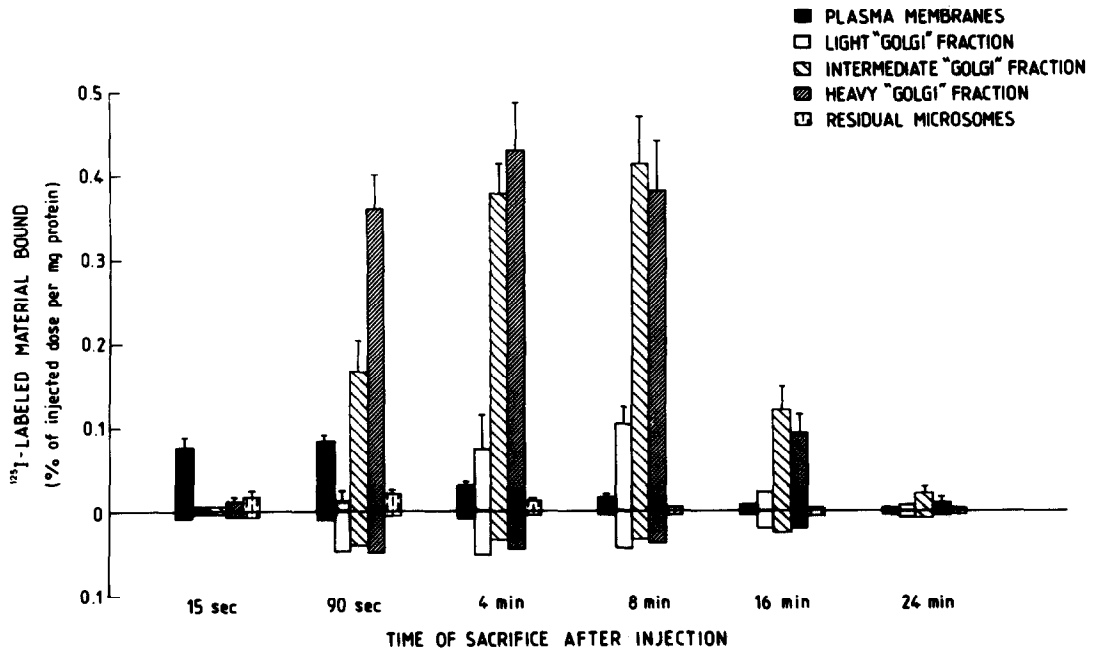

Fig.1. Time course of association of radioactivity with plasma membranes and microsomal subfractions after intravenous injection of ^{125}I-labeled insulin. Experiments were performed on groups of 3 rats. Two rats, which received labeled insulin alone, served to determine total binding; one animal, which received labeled insulin at the same dose and unlabeled insulin (80 nmol) served to determine nonspecific binding. Specific binding (bars above the abscissa) was calculated by subtracting nonspecific (bars below the abscissa) from total binding. Results are the mean ± SEM of 3 (15 s), 4 (90 s, 4 min and 8 min) and 2 (16 and 24 min) such experiments. Recoveries of radioactivity (not shown) can be calculated using the following protein yields, expressed as mg/g liver (wet wt): plasma membranes, 2; light, intermediate and heavy 'Golgi' fractions, 0.14, 0.6 and 3, respectively; residual microsomal fraction, 52. These values are derived from our own experiments, except that of the heavy 'Golgi' fraction, which has been recalculated from [14].

activity in the plasma membranes and the residual microsomal fraction at 15 and 90 s (1.1 and 1.3%). The latter finding suggests that most of the labeled material initially associated with the membranes of high density is subsequently translocated to those of lower density*.

3.3. *In vitro release of the radioactivity associated with the subcellular fractions*

Although compatible with an endocytic process, the results just described do not exclude a redistribution of insulin–receptor complexes at the cell surface. We therefore examined the ability of the labeled material associated with the fractions to spontaneously dissociate in vitro (fig.2). No more than 15–20% of the radioactivity associated with the 'Golgi' fractions is released into the medium in 1 h at 23°C. In contrast, the material associated with the membranes of highest density rapidly dissociates, with an apparent halflife of 20 min; this process is kinetically comparable to the dissociation of insulin–membrane complexes formed in vitro [17,20]. These results suggest that a reversal of the membrane configuration occurs upon insulin translocation.

3.4. *Nature of the radioactivity associated with the subcellular fractions*

To determine whether internalised insulin underwent degradation, we biochemically characterised the labeled material associated with the fractions (fig.3).

* This conclusion is valid only if one assumes that insulin–membrane complexes formed in vivo do not dissociate upon fractionation and that the determination of recoveries is correct

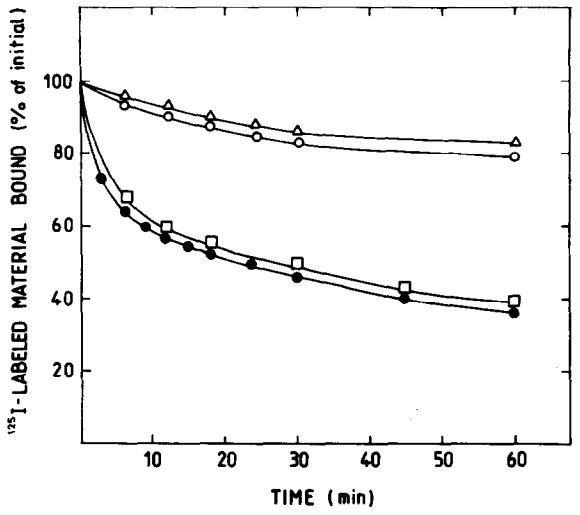

Fig.2. Time course of in vitro release of radioactivity associated with plasma membranes (•), 'Golgi' intermediate (△) and heavy (○) fractions and residual microsomes (□) after labeling in vivo. Plasma membranes and residual microsomes were isolated in rats sacrificed 30 s after injection of labeled insulin; 'Golgi' fractions were obtained 4 min after injection. Particulate material was suspended (10–20 μg/ml protein) into 50 mM Tris–HCl buffer (pH 7.4) containing 1 mg/ml albumin, and incubated at 23°C. At the indicated times aliquots of these suspensions (110–130 μg plasma membrane and microsomal protein; 25–30 μg Golgi protein) were filtered on Millipore membranes [17] to determine radioactivity bound. Results (mean of 5 separate experiments) are expressed as the percentage of the radioactivity bound at zero time; they were not corrected for nonspecific binding.

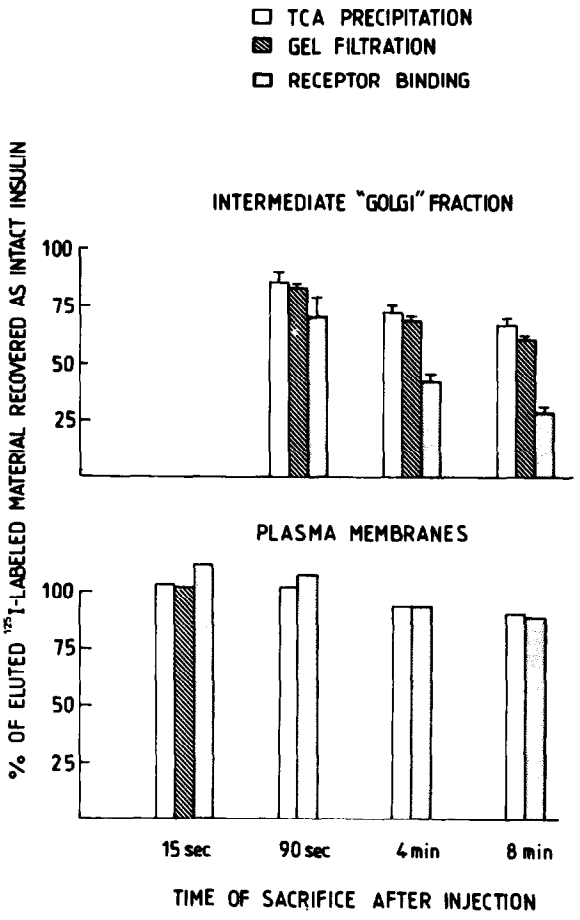

Fig.3. Characterisation of the labeled material associated with subcellular fractions after labeling in vivo. Plasma membranes and the Golgi intermediate fraction (4–8 and 1.5–3 mg protein, respectively) obtained in rats which had received labeled insulin were suspended into 1–2 ml 30% acetic acid, or 2–3 ml 0.2 N HCl containing 2% albumin and 0.2% bacitracin. These suspensions were incubated for 20 min at 30°C, and subsequently centrifuged for 20 min at 40 000 × g; ~95% of the radioactivity bound initially was recovered in the supernatant. The material extracted by acetic acid was analysed by gel filtration as shown in fig.4. That extracted by HCl was tested for precipitation by 5% trichloroacetic acid and ability to bind specifically to liver membranes, following neutralisation by 1 M Tris. To measure binding, the extracted material (5–25 pM) was incubated with microsomal protein (0.5 mg/ml) for 24 h at 4°C. Results (expressed by reference to appropriate controls) are the mean ± SEM of 3–5 determinations. The material extracted from the residual microsomal fraction at 15 and 90 s retained full integrity, and that extracted from the heavy 'Golgi' fraction was degraded to the same extent as with the intermediate fraction (not shown).

The material recovered from the plasma membranes is clearly indistinguishable from insulin by all criteria, just as that recovered from insulin–membrane complexes formed in vitro [17]. In contrast, a large fraction of the material recovered from the 'Golgi' fractions is degraded hormone. This degraded fraction, when estimated by receptor-binding, is ~30% at 90 s, 58% at 4 min and 70% at 8 min; it is less when assessed by physical techniques. On gel filtration (fig.4), the degraded material is eluted in the positions of monoiodotyrosine and, to a lesser extent, of the A chain of insulin; a minor component is also present in the void volume. These results indicate that internalised insulin is cleaved into several, as yet unidentified, low molecular weight peptides; cleavage may occur, at least in part, by proteolysis.

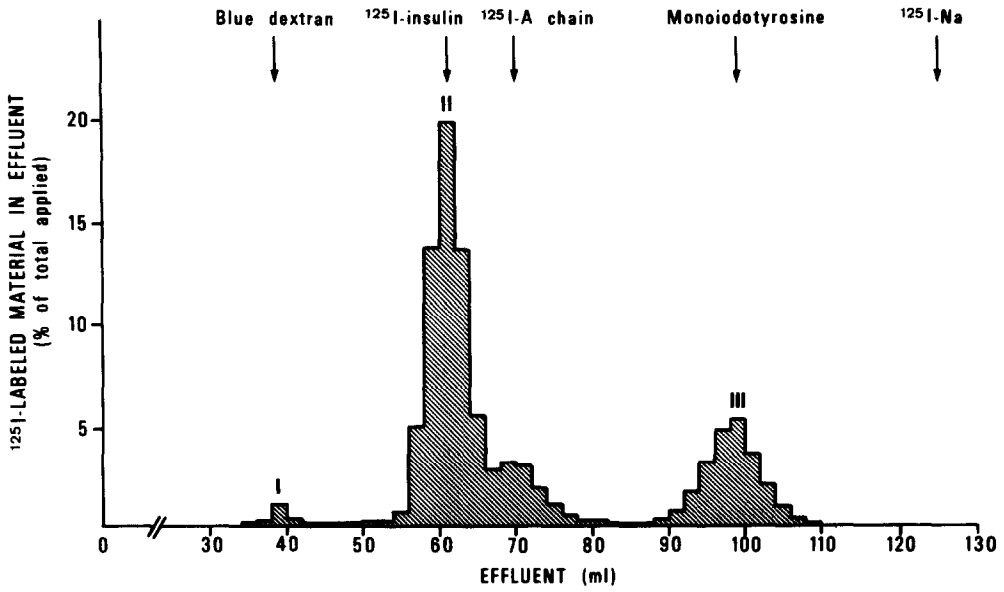

Fig.4. Sephadex G-75 gel filtration elution profile of the radioactivity associated with the intermediate 'Golgi' fraction after labeling in vivo. The intermediate 'Golgi' fraction (2 mg protein) was isolated 4 min after injection of 15 pmol ^{125}I-labeled insulin. The labeled material bound (0.11 pmol) was extracted by 30% acetic acid and applied to a column (1.6 × 72 cm) of Sephadex G-75 equilibrated with the same solvent. Fractions (2 ml) were collected and analysed for radioactivity. Arrows designate the positions at which different markers are eluted.

3.5. *Insulin-binding activity in subcellular fractions obtained in rats injected by excess native insulin*

Results described so far provide no information on the fate of the receptors. We therefore examined the subcellular distribution of the in vitro insulin binding activity, following in vivo injection of native insulin at a maximal dose (table 2). The latter treatment clearly increases the ability of the 'Golgi' fractions to bind insulin, whereas, concomittantly, binding activity decreases in the membranes of higher density. Within the time interval tested, these changes are maximal by 4–8 min. At maximum, binding activity is enhanced by 1.5-fold in the heavy and intermediate 'Golgi' fractions, and 2.5-fold in the light fraction; it is reduced by ~2-fold in the plasma membrane and the residual microsomal fraction**. These results provide direct evidence that internalisation of the receptors accompanies, and presumably mediates, that of insulin.

** It is unlikely that receptor occupancy by injected insulin explained the reduced binding activity in the high density membranes, since this change was clearly delayed relative to label uptake. However, partial receptor occupancy, as well as reduced accessibility of the binding sites to extracellular hormone, may have lead to an underestimation of the binding activity in the heavy and intermediate Golgi fractions

4. Discussion

Earlier studies have demonstrated the feasibility of labeling receptors in intact tissues and cells by their ligands; complexes formed were shown to remain stable upon subsequent subcellular fractionation [10]. Here we have used this approach to assess the fate of insulin–receptor complexes formed in rat liver in vivo with the following results:

1. ^{125}I-labeled insulin injected as a pulse specifically associates with the particulate fraction of liver;
2. Particle-associated radioactivity is progressively translocated from membranes of density 1.17–1.19, recovered in nuclear and microsomal fractions, to microsomal elements of density 1.03–1.17;
3. The material which is translocated becomes less dissociable in vitro and looses in part the properties of insulin;

Table 2
Insulin binding activity in subcellular fractions of rat liver following intravenous injection of native insulin at a maximal dose

Subcellular fraction	^{125}I-labeled insulin bound specifically				
	Control rats (n = 10)	Insulin-injected rats			
	(cpm/μg protein)	15 s (n = 3)	90 s (n = 3)	4 min (n = 7)	8 min (n = 4)
		(% of radioactivity bound in controls)			
'Golgi' fractions					
Light	50 ± 5		189 ± 31	186 ± 11	218 ± 30
Intermediate	126 ± 10	95 ± 6	112 ± 6	158 ± 22	145 ± 13
Heavy	128 ± 7	99 ± 18	134 ± 26	156 ± 8	152 ± 17
Residual microsomal fraction	95 ± 5	81 ± 7	81 ± 12	51 ± 3	46 ± 3
Plasma membranes	475 ± 25	105 ± 7	78 ± 7	55 ± 5	47 ± 4

Subcellular fractions obtained from rats injected by physiological saline or native insulin (25 nmol/100 g body wt) were incubated over a range of 2–3 protein concentrations (25–100 μg/ml) with ^{125}I-labeled insulin (0.2 nM) in 0.3 ml 50 mM Tris–HCl buffer (pH 7.4) containing 0.5% albumin and 0.05% bacitracin. After 24 h at 4°C, binding was determined by filtration procedures [17]. Routinely, 3 sets of fractions were tested per experiment: two sets obtained from insulin-injected rats, and one set obtained from control rats. Within each experiment, binding in the insulin-injected animals has been expressed as the percentage of that in the control rat. Results are the mean ± SEM of such percentages. The changes observed at 4 min are statistically significant when analysed by the paired t-test ($p < 0.05$ for the Golgi intermediate fraction; $p < 0.001$ for other fractions)

4. Following injection of excess native insulin, hormone binding activity decreases in the membranes of high density, and, concomitantly, increases in those of low density. These results provide biochemical evidence that, upon interaction with liver cells in vivo, insulin is internalised and simultaneously degraded. Furthermore, they suggest that it is the insulin–receptor complex which is internalised.

The membranes of high density which are labeled initially are presumably derived from the hepatocyte surface. In support of this is the finding that label distribution somewhat resembles that reported for plasma membrane marker enzymes and in vitro insulin binding activity [15,21]. The higher recovery of radioactivity in the microsomal fraction, as compared to that in the membranes prepared from the nuclear fraction, suggests that the former contains most membranes derived from the blood sinusoidal face of the hepatocytes.

The nature of the low density elements to which insulin is translocated is not obvious. In support of their assignment to the Golgi apparatus is the recent demonstration [22] that, when similar subcellular fractions labeled by ^{125}I-prolactin in vivo were examined by electron microscopic autoradiography, a large proportion of the label was found over authentic Golgi structures. On the other hand, morphological and biochemical evidence has been presented [23,24] that such fractions may also contain sinusoidal plasma membranes. In addition, recent ultrastructural studies analyzing the localisation of ^{125}I-labeled insulin in hepatocytes in situ [8] have shown that less radioactivity was associated with Golgi structures than found in the 'Golgi' fractions in this work. 3 min after insulin injection, only plasma membranes were labeled, and at 10 min, ⩽25% of the cell-associated radioactivity was found over Golgi structures; the remaining was recovered over lysosome-like vacuoles that did not stain for acid phosphatase [8]. Thus, it would appear

that a large fraction of the internalised radioactivity is present in structures unrelated to the Golgi apparatus, presumably pinocytic vesicles. Lysosomes are also a likely candidate; however, we recovered little or no radioactivity in the mitochondrial–lysosomal fraction, a finding consistent with the absence of label over identifiable lysosomes in ultrastructural studies [8].

The results of the dissociation experiments suggest that, while the insulin associated with the plasma membranes is bound to receptors on the external surface of the membrane, that associated with the 'Golgi' fractions is sequestered inside inverted membrane vesicles, either still receptor-bound, or free in the lumen of the vesicles. It is of relevance that inversion of plasma membrane vesicles by in vitro procedures affects the dissociation of previously bound ligands in a similar manner [25].

Earlier studies have shown that degradation of insulin by intact liver cells [1,5], unlike by isolated membranes [26], is receptor-mediated. Although showing that receptor-bound insulin is degraded intracellularly, our results do not exclude the possibility that degradation also occurs at the cell surface, since in this case the degradation products would not be expected to remain associated with the membranes. Whether the degradation which occurs intracellularly is catalysed by cell surface enzymes internalised concomitantly with the insulin–receptor complex, or by intracellular enzymes, possibly lysosomal, is as yet unknown.

We have not been able to biochemically identify internalised insulin–receptor complexes. However, the redistribution of insulin-binding activity which occurs after injection of excess native hormone clearly indicates that cell surface receptors are internalised upon occupation. Thus, it is unlikely that, as suggested [22], extracellular hormone would directly bind to pre-existing, intracellular receptors. Finally, our results suggest that internalisation of the receptors may account, at least in part, for the widely occurring 'down regulation' phenomenon. The fate of internalised receptors is as yet unknown; they may be degraded, or recycled back to the cell surface.

References

[1] Terris, S. and Steiner, D. F. (1975) J. Biol. Chem. 250, 8389–8398.
[2] Gorden, P., Carpentier, J. L., Freychet, P., Le Cam, A. and Orci, L. (1978) Science 200, 782–785.
[3] Carpentier, J. L., Gorden, P., Freychet, P., Le Cam, A. and Orci, L. (1979) J. Clin. Invest. 63, 1249–1261.
[4] Mortimore, G. E. and Tietze, F. (1959) Ann. NY Acad. Sci. 82, 329–337.
[5] Terris, S. and Steiner, D. F. (1976) J. Clin. Invest. 57, 885–896.
[6] Izzo, J. L., Bartlett, J. W., Roncone, A., Izzo, M. J. and Bale, W. F. (1967) J. Biol. Chem. 242, 2343–2355.
[7] Bergeron, J. J. M., Levine, G., Sikstrom, R., O'Shaughnessy, D., Kopriwa, B., Nadler, N. J. and Posner, B. I. (1978) Proc. Natl. Acad. Sci. USA 74, 5051–5055.
[8] Bergeron, J. J. M., Sikstrom, R., Hand, A. R. and Posner, B. I. (1979) J. Cell Biol. 80, 427–443.
[9] Cuatrecasas, P. (1971) Proc. Natl. Acad. Sci. USA 68, 1264–1268.
[10] Chang, K. J., Bennett, V. and Cuatrecasas, P. (1975) J. Biol. Chem. 250, 488–500.
[11] Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. 59, 45–72.
[12] Neville, D. M. (1968) Biochim. Biophys. Acta 154, 540–552.
[13] Pohl, S. L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849–1856.
[14] Bergeron, J. J. M., Ehrenreich, J. H., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. 59, 73–88.
[15] Bergeron, J. J. M., Posner, B. I., Josefsberg, Z. and Sikstrom, R. (1978) J. Biol. Chem. 253, 4058–4066.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Cuatrecasas, P., Desbuquois, B. and Krug, F. (1971) Biochem. Biophys. Res. Comm. 44, 333–339.
[18] Lee, N. D. and Williams, R. H. (1954) Endocrinology 54, 5–19.
[19] Lee, N. D. and Wiseman, R. (1959) Endocrinology 65, 442–450.
[20] Kahn, C. R., Freychet, P., Roth, J. and Neville, D. M. (1974) J. Biol. Chem. 249, 2249–2257.
[21] Horvat, A., Li, E. and Katsoyannis, P. G. (1975) Biochim. Biophys. Acta 382, 609–620.
[22] Josefsberg, Z., Posner, B. I., Patel, B. and Bergeron, J. J. M. (1979) J. Biol. Chem. 254, 209–214.
[23] Touster, O., Aronson, N. N., Dulaney, J. T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–618.
[24] Wisher, M. H. and Evans, W. H. (1975) Biochem. J. 146, 375–388.
[25] Bennett, V. and Cuatrecasas, P. (1973) Biochim. Biophys. Acta 311, 362–380.
[26] Freychet, P., Kahn, R., Roth, J. and Neville, D. M. (1972) J. Biol. Chem. 247, 3953–3961.