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Intracellular localization of ^{125}I-labeled insulin in hepatocytes from intact rat liver

(insulin receptor/autoradiography/ultrastructure)

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ABSTRACT **We have shown that ^{125}I-labeled insulin initially localizes to the plasma membrane of isolated rat hepatocytes. The ligand is subsequently internalized and preferentially localizes to lysosomal structures. Further, we have observed that labeled insulin localizes to regions of the cell rich in lysosomal and Golgi elements. In the present study in intact rat liver we found that, approximately 10 min after a pulse injection of ^{125}I-labeled insulin, 56% of the label was internalized by the cell. When all grains are considered there is a preferential localization of grains to the biliary pole of the cell and these grains are almost all internalized and preferentially associated with lysosomes. These data, therefore, demonstrate that the lysosome–Golgi-rich area of the isolated hepatocyte corresponds to the biliary pole of the cell and there is a movement of the labeled hormone from its initial binding site on the plasma face of the cell membrane toward the biliary pole of the cell.**

Direct binding studies of the interaction of polypeptide hormones with cells have, for the most part, been carried out "*in vitro*," using iodinated ligands and isolated cells or subcellular components (1). While it would be desirable to study these processes "*in vivo*," there are technical problems, particularly involving the specificity of the binding process. Once a system is well characterized *in vitro*, however, it may be possible to answer specific questions by using *in vivo* techniques. We have used quantitative electron microscope autoradiography to study the morphologic events involved in the binding of ^{125}I-labeled insulin (^{125}I-insulin) to isolated rat hepatocytes and have observed that the radioactivity initially associates with the plasma membrane of the cell and that a fraction of the radioactivity is subsequently internalized as a constant function of the binding process. The labeled material progressively associates with lysosomal structures as a function of incubation time and temperature (2–5). In addition, we have found that autoradiographic grains associated with lysosomes are found in regions of the cell where the volume density of such structures and Golgi elements are high.§ Because the polarity of the hepatocyte is no longer detectable in a preparation of isolated cells, it is impossible to know how labeled lysosomes and Golgi elements relate to the cell in the intact organ. To answer this question, we have injected ^{125}I-insulin intraportally, fixed the liver by perfusion, and quantitatively analyzed the distribution of ^{125}I-insulin in the intact liver by autoradiography.

MATERIALS AND METHODS

Perfusion Conditions and Reagents. Six to 8-week-old normal Wistar rats fed ad lib were anesthetized by an intraperitoneal injection of Nembutal; the portal vein was exposed and atraumatically canulated. ^{125}I-Insulin [80–100 μCi $\approx$ 100 $\times$ 10^6 cpm (1 Ci = 3.7 $\times$ 10^{10} becquerels) prepared at a specific activity of 250 μCi/μg by a modification of the chloramine-T method (6) and purified by filtration on a Sephadex G-50 column at 4°C] was pulse injected into the portal vein in a total volume of 300 μl (for 20 sec) and allowed to circulate in the whole animal for 5–9 min at 37°C. The inferior vena cava and suprahepatic veins were cut and the liver was washed with Krebs–Ringer bicarbonate buffer containing 3% bovine serum albumin and glucose at 3 g/liter for 1½–4 min at 20°C (flow rate: 12 ml/min). This was followed by perfusion of 4% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (7–12 min at 20°C). The mean whole liver weight was 7.5 g (6.2–8.8 g) and the average liver contained 20 $\times$ 10^6 cpm (as extrapolated from 90 mg of tissue). A similar liver perfused with a mixture of 100 μCi of ^{125}I-insulin and 15 μg of unlabeled insulin (to approach nonspecific binding) contained 6 $\times$ 10^6 cpm per total liver at the end of the perfusion.

Preparation for Electron Microscopy and Autoradiography. Livers sat overnight at room temperature in 4% glutaraldehyde and were then transferred into 0.1 M phosphate buffer, pH 7.4, where they remained for 3–4 days until processed. Pieces of tissue were excised from the three main lobes of the liver as well as from the intermediate lobe and caudate process (7). After three successive washes in phosphate buffer, the pieces of tissue were postfixed in 0.1 M osmium tetroxide, pH 7.4, for 2 hr at room temperature, dehydrated in graded ethanol, and embedded in Epon. Semithin sections ($\approx$1 μm thick) were prepared and coated with an Ilford L4 emulsion by the method of Caro *et al.* (8) for optical autoradiography. After 1 week the sections were developed in Kodak D 19. For electron microscope autoradiography, thin sections were made always in the perilobular region. The sections deposited on copper grids were coated with Ilford L4 emulsion by the loop method of Caro *et al.* (8). After 3–5 weeks the grids were developed in Microdol X. Samples were examined in a Philips EM 300 electron microscope. Photographs were taken at a magnification (×9000) calibrated with a reference grid (2160 lines per mm).

Sampling and Analysis of Data. For each lobe analyzed six different Epon blocks were cut, and for each block two grids were prepared. A total of 60 grids was prepared for each liver analyzed. In the two livers perfused with ^{125}I-insulin, grains were photographed on cells judged to be well preserved: this gave totals of 675 and 405 grains, respectively. The quantitative determinations were carried out on positive prints at a final magnification of ×27,000.

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§ Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A. & Orci, L., *Experientia*, in press.

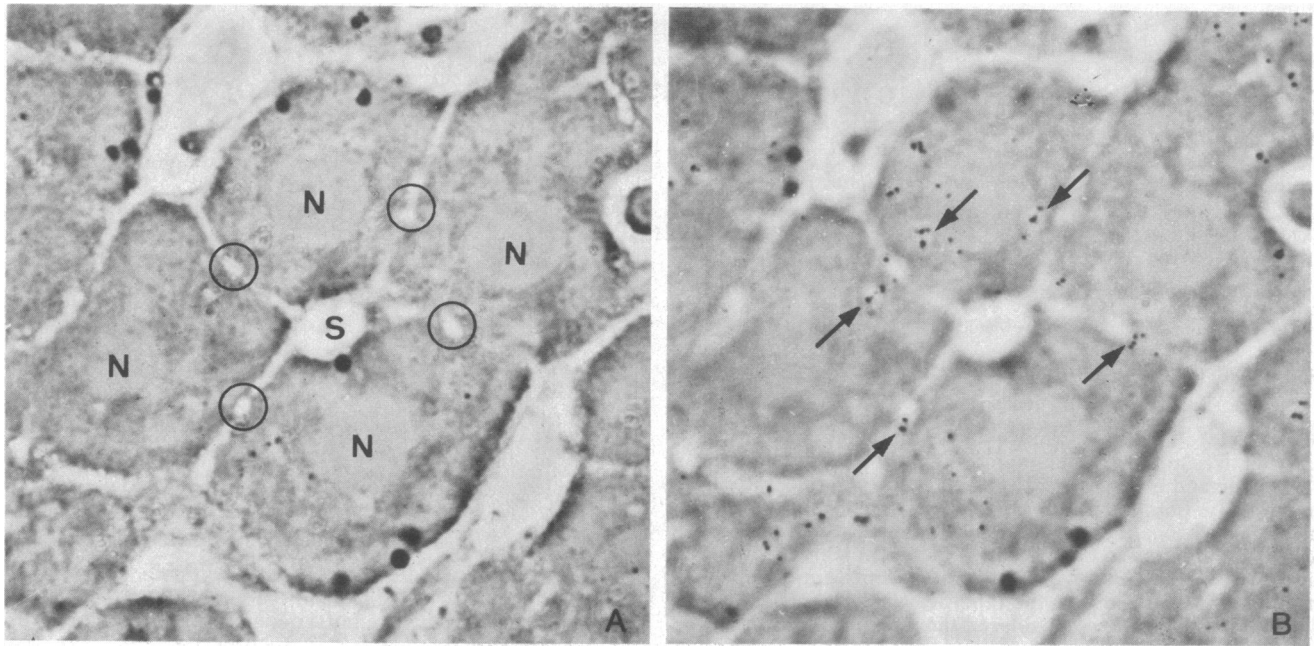

FIG. 1. Light microscope autoradiogram of a semithin section of liver fixed after pulse injection and recirculation of ^{125}I-insulin for 5 min. Epithelial cells [with round nuclei (N), sinusoids (S), and bile canaliculi (within circles)] are easily detectable. In *A* the photograph was focused on the section while in *B* the focus was on the autoradiographic grains, which are often found in clusters close to bile canaliculi (arrows). (×1600.)

Quantitative Evaluation. For each liver analyzed, the association of the labeled material to the plasma membrane of hepatocytes was assessed quantitatively by the method of Salpeter *et al.* (9, 10). According to this method, the normalized number of grains was plotted as a function of the distance between the center of all grains photographed and the closest plasma membrane. Grains were then divided in two categories on the basis of their proximity to the plasma membrane delimiting a bile canaliculus (biliary region) or to the plasma membrane limiting all other regions of the cell (so-called "nonbiliary" regions). In each of these two regions, the percentage of grains related to the plasma membrane—i.e., situated within ±250 nm of the membrane, a distance assumed to include about 80% of the grains related to a line source (10)—as well as the percentage of grains internalized—i.e., percentage of grains situated at a distance greater than 250 nm on the inside of the plasma membrane—were calculated. Grains internalized in biliary regions were frequently over lysosomal structures, and this possible association was assessed quantitatively by analyzing the grain distribution around the lysosomal membrane by the Salpeter method (10, 11). In nonbiliary regions, the preferential association of grains to intracellular organelles was less obvious, and the probability circle method (12, 13) was applied as described (5).

In order to evaluate the size of lysosomal structures and small vesicles (for definition see *Results*), the shape of these intracytoplasmic structures was approximated by circles and the diameters of the circles were determined by comparison with calibrated circles. Values measured were corrected to account for the sectioning artifact according to Weibel (14).

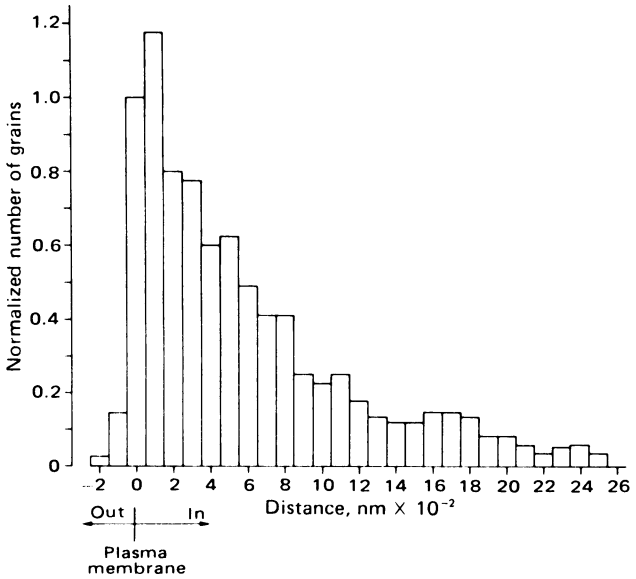

FIG. 2. Distribution of autoradiographic grains around the plasma membrane of hepatocytes after pulse intraportal injection and recirculation of ^{125}I-insulin for 5 min in an intact liver. The normalized number of grains is plotted as a function of the distance between the grain center and the plasma membrane. This histogram is representative of one experiment, but identical results were obtained with the second liver perfused. Note the difference in the histograms representing plasma membrane localization in isolated hepatocytes and cultured human lymphocytes as shown in ref. 3. Number of grains analyzed: 675.

Table 1. Relationship of autoradiographic grains to the biliary poles of the hepatocytes

Exp.	Grains analyzed	Grains related to biliary poles, %*	Cell surface involved in biliary poles, %†	Ratio
1	675	37	13	2.5
2	405	48	13	3.7

* A grain was considered related to a biliary pole if its center was closer to the plasma membrane delimiting a biliary region (included within two opposed junctional complexes in a cross section) than to the plasma membrane limiting another region of the cell.

† Ref. 17.

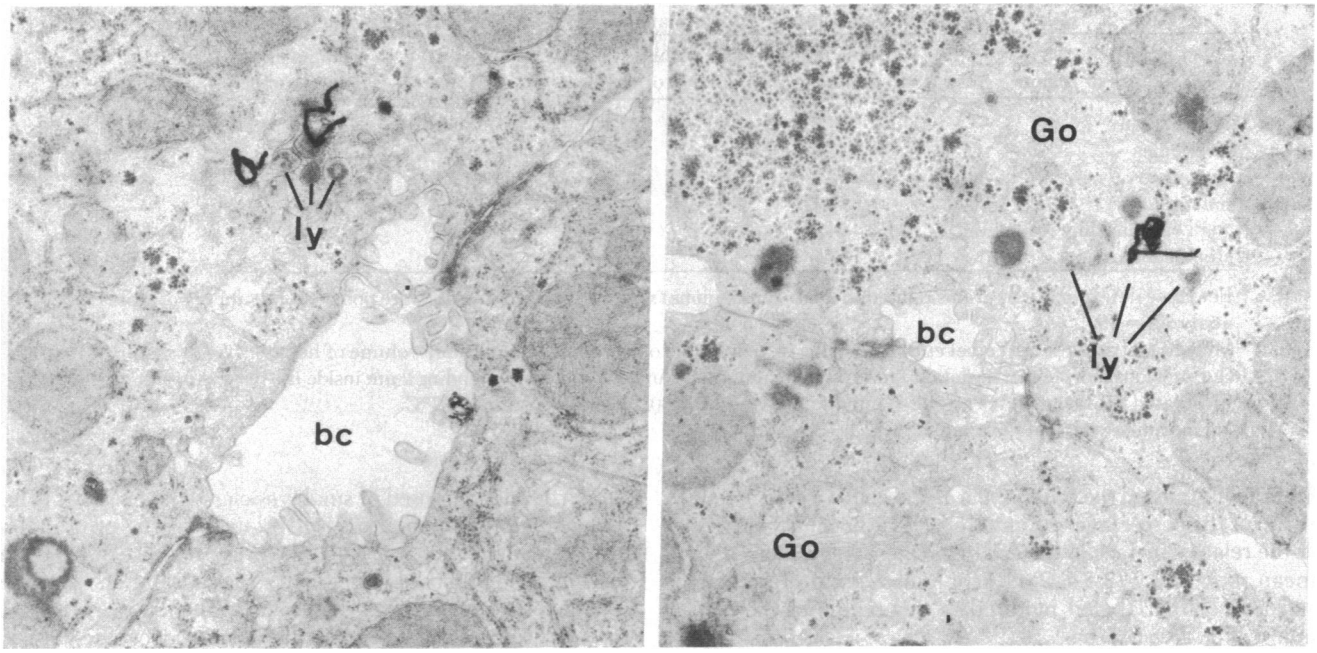

FIG. 3. Thin sections of hepatocytes from liver perfused with ^{125}I-insulin. These selected sections show autoradiographic grains overlying lysosomal structures (ly) at the biliary pole of the hepatocyte. bc, Bile canaliculus; Go, Golgi apparatus. (×26,000.) Lysosome structures are considered as membrane-bounded intracytoplasmic organelles—i.e., dense bodies—multivesicular bodies, autophagosomes, glycogenosomes, and heterophagosomes.

RESULTS

General Characteristics of the Perfused Liver. As described by others (15, 16) the different cell types and their respective relationships are well preserved at the end of the perfusion (Fig. 1) and the various subcellular components are easily detected by the electron microscope (Fig. 3).

Autoradiography: Quantitative Evaluation. When the liver is fixed approximately 10 min after the pulse injection of ^{125}I-insulin and prepared for autoradiography, over 90% of developed grains are found associated with parenchymal cells and only a few are related to endothelial cells. In addition, grains frequently appear associated with the biliary pole of the hepatocytes (Fig. 1). When a large number of grains are photographed and the data are expressed in the form of a grain distribution histogram (Fig. 2), about 56% of the total number of grains are found within the cell (i.e., beyond 250 nm of the plasma membrane). Most of these grains are localized in a limited region of the cell periphery extending from 300 to 1800 nm from the plasma membrane. Although the percent of grains internalized is greater than in our *in vitro* studies (3), this is consistent with the pulse injection carried out here versus continuous incubation in the *in vitro* study (3). These data are, therefore, in good agreement with our previous results in isolated hepatocytes (3, 5).

All developed grains photographed were then divided in two categories on the basis of their proximity to the plasma membrane delimiting a bile duct—i.e., a membrane portion included within two opposed junctional complexes in cross section (see Table 1)—or to other regions of the cell membrane. Of the total number of grains (Table 1) a mean of 43% (37–48%) was found closer to a bile duct membrane than to other regions of the cell membrane. To determine if there is a preferential lo-

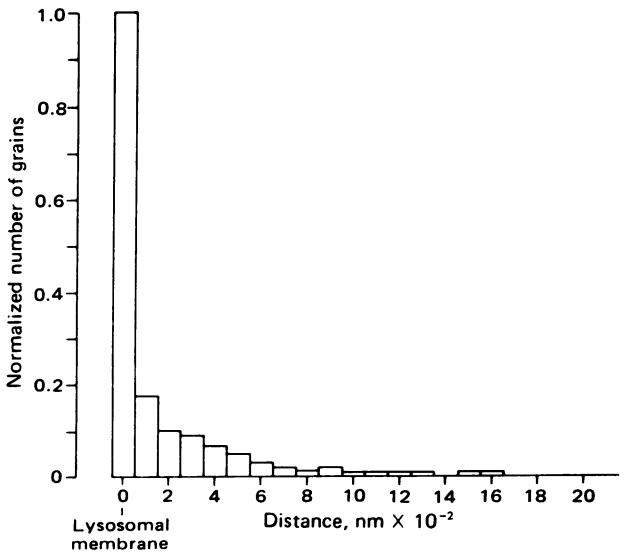

FIG. 4. Distribution of autoradiographic grains around lysosomal membranes. Lysosomes were selected because they lay closer to a biliary pole of the cell (see Table 1). The normalized number of grains is plotted as a function of the distance between the grain center and the lysosomal membrane. Number of grains analyzed: 360. For further details of this form of analysis see ref. 18.

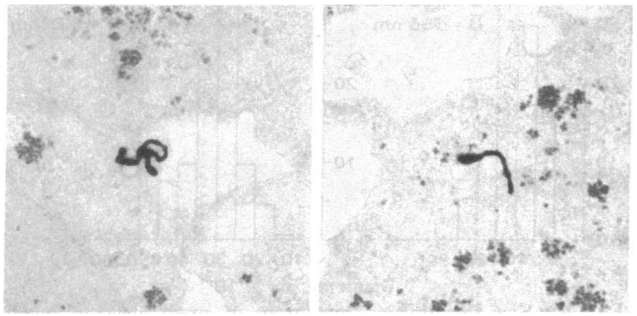

FIG. 5. Electron microscope autoradiographs of thin sections of hepatocytes from a liver fixed after perfusion with ^{125}I-insulin. These selected pictures show autoradiographic grains associated with the nonbiliary pole of the hepatocytes and overlying small electron-lucent vesicles. (×14,400.)

Table 2. Relationship of autoradiographic grains related to nonbiliary regions to hepatocyte organelles*

Structure or region	Grains related to organelles, %	Volume density of organelles†	Ratio	Volume density of organelles‡	Ratio
Mitochondria	18.2	23.7	0.8	23.3	0.8
Microbodies	1.1	1.4	0.8	1.4	0.8
Lysosomes	12.7	0.8	15.9	2.1	6.1
Small vesicles§	6.1	—	—	—	—
Rough endoplasmic reticulum	4.3	9.4	0.5	3.8	1.1
Cytoplasm matrix	57.6	64.7	0.9	69.4	0.8

* For these determinations grains related to nonbiliary regions from the two experiments were pooled: total number of grains analyzed = 400.

† Morphometric data of Weibel *et al.* (17) refer to an analysis of the entire cytoplasmic volume of hepatocytes *in situ*.

‡ Our previous morphometric data (5) refer to an analysis of a band of cytoplasm extending 2 μm inside the plasma membrane of isolated rat hepatocytes after 2–5 min of incubation with 0.1 nM ^{125}I-insulin at 37°C.

§ No morphometric data available.

calization of grains to the biliary pole of the cell, we calculated the ratio of the percentage of grains related to the biliary region over the relative surface of the cell involved in this region (17). A mean ratio of 2.7 (2.5–3.2) clearly indicates a preferential association of the labeled material to this region of the cell (Table 1, Fig. 3).

Ninety percent of autoradiographic grains associated with the biliary poles are found within the cells. Qualitatively, most of these grains are seen close to lysosomal structures (Fig. 3). When the relationship to lysosomal structures is analyzed quantitatively, a grain distribution histogram around the lysosomal membrane shows that over 70% of grains could be related to these structures (Fig. 4).

When grains were analyzed in the "nonbiliary" region of the cell, 44% (41%–47%) of these grains were found associated with the plasma membrane (i.e., ±250 nm from the plasma membrane). The relationship of internalized grains (i.e., >250 nm from the plasma membrane) to cytoplasmic organelles in the nonbiliary regions of the cell was assessed by the probability circle method (Table 2). A comparison of these results with previous morphometric data (5, 17) clearly shows that grains preferentially associate with lysosomal structures, whereas there is no preferential association with the other compartments that could be clearly defined at the magnification chosen in the rim of cytoplasm considered—i.e., lysosomes, mitochondria, rough endoplasmic reticulum, microbodies, and remaining cytoplasm matrix. Six percent of grains internalized in this region of the hepatocytes can be related to an additional intracytoplasmic compartment composed of small vesicles. These vesicles (Fig. 5) are membrane-bounded electron-lucent structures with a mean diameter of 156 nm. Their mean size and size distribution are compared to those of lysosomal structures in Fig. 6. On the basis of their size and their ultrastructural characteristics, the small vesicles were tentatively identified as endocytotic vesicles. Lysosomal structures with associated radioactivity showed a mean size and size distribution similar to those observed previously in isolated hepatocytes (5) and corresponded to a class of lysosomes of relatively small size (Fig. 6).

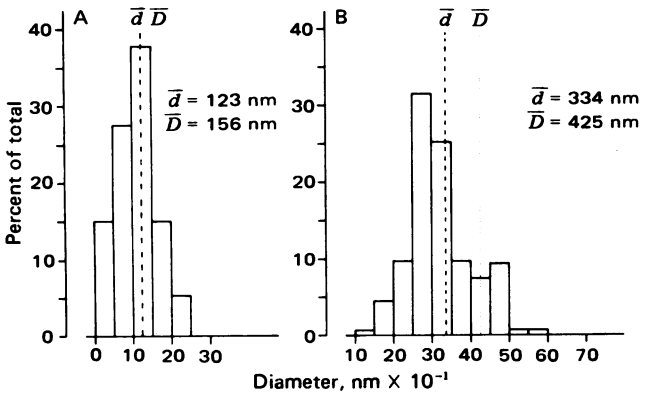

FIG. 6. Size distribution histogram of small electron-lucent vesicles (*A*) and lysosomal structures (*B*) related to autoradiographic grains at nonbiliary poles of the hepatocytes. The percents of lysosomal structures and small vesicles are plotted as functions of the diameters of their respective structures. $\bar{d}$, Calculated mean diameter; $\bar{D}$, mean diameter corrected according to Weibel (14).

DISCUSSION

Previous studies analyzing the fate of ^{125}I-insulin after its binding to isolated hepatocytes demonstrated a progressive translocation of the labeled material within the cell (2, 3). Moreover, we observed that internalized autoradiographic grains preferentially associated with lysosomal structures (4, 5) in regions of the cell cytoplasm quantitatively rich in lysosomal and Golgi elements.§ In the present study we have extended our observations to the intact liver with the following results: (*i*) Most of the ^{125}I-insulin pulse injected in the portal vein and recirculated for 5–9 min was detected as autoradiographic grains associated with parenchymal cells of the liver. (*ii*) Labeled material preferentially associates with the biliary pole of the hepatocyte. (*iii*) Most autoradiographic grains present at the biliary pole are related to lysosomal structures. (*iv*) Autoradiographic grains close to a nonbiliary region of the hepatocyte are preferentially associated with both the plasma membrane and lysosomal structures.

The results of our present study are, therefore, in agreement with our previous findings in isolated hepatocytes but allow a better understanding of the relationship of the labeled ligand to the hepatocyte within the intact liver. Thus, the lysosome–Golgi-rich areas in which autoradiographic grains are found in the isolated hepatocyte correspond to the peribiliary region of the hepatocyte in the intact liver. Taken together with our *in vitro* studies in isolated hepatocytes, these data suggest that labeled insulin initially localizes to the plasma face of the cell surface membrane and that the label "flows" towards the cytoplasm of the biliary pole of the cell. This flow might occur in the plane of the membrane or, after internalization, by way of small transfer vesicles or lysosomes. Obviously any combination of these events could occur; our present data do not allow for a quantitative distinction.

In most tissues, lysosomes are associated with a degradative activity of endocytosed material (19, 20). In hepatocytes, lysosomes have been shown to have an important role in protein catabolism (19, 20), and it has been suggested that lysosomes may play a role in insulin degradation (21, 22). Terris and

Steiner (23) showed that 60–90% of the radioactivity recovered in bile after perfusion of liver with ^{125}I-insulin was degraded, and Boynes *et al.* (24) have reported that less than 40% of the radioactive material appearing in the bile of rabbits injected *in vivo* with ^{125}I-insulin was precipitable by trichloroacetic acid and less than 10% was immunoreactive. Our data showing the association of the labeled hormone with lysosomes and its movement to the peribiliary region could represent the morphological correlate of the steps leading to its degradation or to the degradation of the insulin receptor complex. Bergeron *et al.* (25, 26) and Posner *et al.* (27) have demonstrated biochemically and morphologically that insulin binds to Golgi elements isolated by subcellular fractionation techniques. While these data demonstrate clearly that Golgi vesicles have binding sites for insulin, they do not directly relate to the route of internalized insulin in intact hepatocytes or liver. Using techniques similar to ours, Bergeron *et al.* (28) presented data in abstract form and have stated that labeled insulin localizes to lysosomes, Golgi apparatus, and other organelles in addition to the plasmalemma. Our studies show that internalized labeled insulin localizes to lysosome-rich areas that are close to Golgi elements, but an association of radioactivity to specific Golgi elements was not found.

Our findings differ from those of Goldfine *et al.* (29) for unexplained reasons; we have not found a preferential association of labeled insulin with either nuclei or endoplasmic reticulum in either cultured human lymphocytes or isolated hepatocytes.

Finally, it must be emphasized that our data relate only to the extracellular route of labeled insulin in intact cells and organs from initial binding to the plasma membrane through its internalization; our data neither support nor contradict possible mechanisms involved in the intracellular regulatory role of insulin.

Note Added in Proof. The studies of Bergeron *et al.* (28) have now been published in detail (30). In their study and in ours the major sites of preferential localization of ^{125}I-insulin are membrane-bounded structures that they refer to as "lysosome-like" and we refer to as "lysosomal structures." Our preliminary cytochemical studies (unpublished observations) yield a higher percentage of grains over structures containing acid phosphatase-positive material than reported by Bergeron *et al.* (30). Thus, we feel that these membrane-bounded structures most closely simulate lysosomes on morphologic, functional, and cytochemical grounds (5).

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