BINDING AND UPTAKE OF $^{125}$I-INSULIN INTO RAT LIVER HEPATOCYTES AND ENDOTHELIUM

An In Vivo Radioautographic Study

J. J. M. BERGERON, R. SIKSTROM, A. R. HAND, and B. I. POSNER

From the Departments of Anatomy and Medicine, McGill University, Montreal, Quebec, H3A 2B2, Canada. Dr. Hand's present address is the Laboratory of Biological Structure, National Institute of Dental Research, the National Institutes of Health, Bethesda, Maryland 20014.

ABSTRACT

Electron microscope radioautography has been used to study hormone-receptor interaction. At intervals of 3, 10, and 20 min after the injection of $^{125}$I-insulin, free hormone was separated from bound hormone by whole body perfusion with modified Ringer's solution. The localization of bound hormone, fixed in situ by perfusion with glutaraldehyde, was determined.

At 3 min, $^{125}$I-insulin has been shown to be exclusively localized to the hepatocyte plasmalemma (Bergeron et al., 1977, Proc. Natl. Acad. Sci. U. S. A., 74:5051-5055). In the present study, quantitation indicated that $10^6$ receptors were present per cell and distributed equally along the sinusoidal and lateral segments of the hepatocyte plasmalemma. At later times, label was found in the Golgi region. At 10 min, both secretory elements of the Golgi apparatus and lysosome-like vacuoles were labeled, and at 20 min the label was especially concentrated over the latter vacuoles. Acid phosphatase cytochemistry showed that the vacuoles did not react and therefore were presumed not to be lysosomal. These Golgi vacuoles may constitute a compartment involved in the initial degradation and/or site of action of the hormone. Control experiments were carried out at all time intervals and consisted of parallel injections of radiolabeled insulin with excess unlabeled hormone. At all times in controls, label was diminished over hepatocytes and was found primarily over endothelial cells and within the macropinocytotic vesicles and dense bodies of these cells. Kupffer cells and lipocytes were unlabeled after the injection of $^{125}$I-insulin with or without excess unlabeled insulin.

KEY WORDS insulin receptor, Golgi apparatus, lysosomes

The initial interaction of $^{125}$I-radiolabeled insulin with the hepatocyte plasmalemma was recently demonstrated in vivo by electron microscope radioautography (4). Little is known about the fate of labeled polypeptide hormones after binding to the surface of their target cells. In the present study, we have approached the problem by examining the distribution of label at different times after $^{125}$I-insulin administration. We have demon-
strated that label initially bound to the plasma-
lemma is subsequently found within the hepatocyte, and especially within the Golgi region.\(^1\)

**MATERIALS AND METHODS**

**Animals**

The studies were carried out with 14 young male Sherman rats (40-55 g body weight; fed ad libitum) bred from the McGill Department of Anatomy colony.

**Tracer**

\(^{131}\)I-insulin (porcine, 24.4 U/mg; Connaught Laboratories, Willowdale, Ontario) was freshly prepared immediately before each experiment. The iodination was carried out using Chloramine T as described previously (33). The reaction mixture was diluted with 2.5% bovine serum albumin in 25 mM Tris HCl, pH 7.4, and 0.1-ml portions were injected into the portal vein.\(^2\) The quality of each \(^{131}\)I-insulin preparation was tested by measuring its specific binding as described elsewhere (5, 32). The specific activity of the injected \(^{131}\)I-insulin varied from 134 to 160 \(\mu\)Ci/\(\mu\)g.

**Injection, Perfusion, and Fixation**

Under nembutal anesthesia, animals received 0.1 ml of radiolabeled hormone (from \(138.6 \times 10^6\) to \(177 \times 10^6\) dpm) by intraportal injection. At 3, 10, and 20 min after injection of label, the animals were whole body perfused by gravity (1.2 M height) through the left ventricle (21-gauge needle; Venoset 78 no. 1881 intravenous set; Abbott Laboratories, North Chicago, Ill.) with lactated Ringer’s solution (Abbott Laboratories) to remove unbound hormone. The perfusion with modified Ringer’s solution was carried out until the liver blanched (30 s maximum) and was followed by freshly prepared fixative (2.5% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer, 0.1% sucrose, pH 7.3) for 10 min flowing through the “Y” joint in the Venoset. Subsequent fixation and processing were carried out as described previously (5). Control animals received the same quantity of freshly prepared \(^{131}\)I-insulin along with 50 \(\mu\)g of unlabeled insulin.

**Quantitative Radioautography**

Light and electron microscope radioautography of embedded tissues were performed as described previously (4). Fine grain development was carried out by the Agfa-Gevaert “solution physical technique” as described before for Golgi fractions (5). Ifford L-4 emulsion (Ifford Photo, Don Mills, Ontario) was used for most of the experiments reported in this study. In some experiments, the Sakura NR-H2 emulsion (obtained from Dr. K. Imamoto, Shiga University, Shiga, Japan) was used,\(^3\) in which case the exposure was carried out under helium (23), and development was carried out with Kodak D19B. This resulted in filamentous grains (22).

Radioautographs were analyzed by scoring the structure directly underlying the grain (24). The diameter of filamentous grains resulting from the development of the Ifford L-4 emulsions in Kodak D19B was \(\approx 260\) nm. The diameter of the developed silver fine grain by the solution physical technique was \(\approx 40\) nm. Fine grains are formed either singly or in small clusters of two to three silver deposits (22), providing potential difficulty in scoring. Thus, quantification of the distribution of fine grains was carried out by scoring the structure underlying each silver deposit as well as under each silver crystal as described by Kopriwa (22) as follows: A transparency of a circle with a diameter equivalent to the average size of the silver bromide crystal (0.14 \(\mu\)m) in the emulsion was superimposed over clusters of silver deposits to group them into crystals. The structure underlying such a crystal was then scored.

The scoring was divided into grains exclusively over defined cellular compartments and those over two, over three, etc. compartments. These shared grains were then assigned to structures on the basis of the original proportion of exclusive grains over the same structures. Shared grains were a minor proportion of the total in most circumstances.

To quantify the number of receptors over the hepatocyte plasmalemma at the 3-min time point, the procedure outlined by Fertuck and Salpeter (15) was followed. Briefly, the analysis was carried out on two sets of micrographs, all taken at an initial magnification of 10,000 and photographically enlarged to 30,000. The first set (method A) of electron micrographs was taken at random (and thus included several micrographs with no grains and no plasmalemma). The second set (method B) was taken only wherever there were grains. A two-dimensional grid made by photocopying \(1 \times 1\) cm graph paper onto a transparency (equivalent to 0.335 \(\mu\)m spacing when corrected for magnification and photocopying error) was placed over each of the electron micro-

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\(^{1}\) These observations were presented in part at the 17th annual meeting of the American Society for Cell Biology and published in abstract form (3).

\(^{2}\) The proportion of radioactivity in free \(^{131}\)I was between 18.8 and 27.7% of the total injected radioactivity for the various experiments. When equivalent doses of \(^{131}\)I alone or \(^{131}\)I with unlabeled insulin (50 \(\mu\)g) were injected, little (2% of that seen with \(^{131}\)I-insulin) binding or uptake by the liver was observed when assessed biochemically and no radioautographic reaction at the light microscope level was observed.

\(^{3}\) For a considerable period of time, successive batches of Ifford L-4 emulsion were unsatisfactory for radioautography.
graphs, and the number of intersections with the plasmalemma was noted (only cross sections of plasmalemma were scored). For both methods A and B, the length of plasmalemma was then determined from equation 1 of Fertuck and Salpeter (15). From this value, the concentration of bound insulin was determined by dividing the total number of grains by the length of plasmalemma. The grain density (grains/square micrometer of plasmalemma) was then calculated by dividing by the thickness of the section (50 nm, determined on profile sections). Using the value of 60% as the efficiency of detection of 125I sources, with monolayer Ilford L-4 emulsion (14), and taking into account specific radioactivity of 125I-insulin at the beginning of the exposure period, degree of iodination of insulin (theoretical [125I]monoiiodoinsulin, 368 μCi/μg) duration of exposure, half-life of 125I and Avogadro number, the number of insulin binding sites per unit plasmalemma and thence per hepatocyte was determined.

The receptor distribution along the hepatocyte plasmalemma was also determined for the 3-min time point of the experimental sections. Thus, the plasmalemma was demarcated on the micrographs into the sinusoidal surface, lateral surface, and the membrane of the bile canalculus. As for the calculation of receptor number, all grains were used in the analysis; the grain nearest the defined portion of the plasmalemma was attributed to a source from that plasmalemma compartment (4). The analysis was carried out on micrographs taken at random (method A) as well as micrographs taken only where grains were found (method B).

Electron Microscope Cytochemistry

Cytochemical reactions for acid phosphatase using cytidine monophosphate as substrate or trimetaphosphate as substrate (30 min of incubation at 37°C) were carried out on "chopper" sections of liver as described by Hand and Oliver (17) and Doty et al. (11). Radioautography was carried out on the cytochemically reacted tissue using Sakura NR-H2 emulsion as described above.

Radioactivity Determinations

These were carried out with a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) operating at an efficiency of 41.5%.

RESULTS

Loss of Label

The retention of the bound radiolabeled insulin in tissue during fixation, dehydration, and embedding was assessed by quantifying the losses in radioactivity after the perfusion with glutaraldehyde. In one experiment (Table I), all fixative and alcohol solutions were combined and counted for radioactivity. The majority of the radioactivity remained in the Epon-embedded blocks. In a second experiment (data not shown), each of the fixatives, block stains, dehydration solvents, and Epon was assessed for radioactivity. Losses were found to occur primarily in the glutaraldehyde immersion step of the procedure.

An assessment of radioactivity in liver and thyroid was determined for the time intervals under study (Table II). About half the label was lost from the liver by 20 min (as determined both biochemically and radioautographically; Table II). However, with time, label was concentrated in the thyroid presumably in the form of thyroglobulin (26, 27). We therefore conclude that any free 125I

| Time after injection | 3 min | 10 min | 20 min |
|---------------------|-------|--------|--------|
| Label originally present in diced liver sample* | 9.6 × 10⁸ † | 7.1 × 10⁸ | 3.3 × 10⁸ |
| Label lost to solvents | 0.3 × 10⁶ | 0.3 × 10⁶ | 0.2 × 10⁶ |
| Label remaining in Epon-embedded blocks of tissue | 8.2 × 10⁵ | 4.2 × 10⁵ | ND† |

Experimental rats received intraperitoneally 0.1 ml of 125I-insulin (177 × 10⁶ dpm; sp act, 135 μCi/μg). At various times after injection, the animals were perfused sequentially with lactated Ringer's (30 s) and glutaraldehyde as described in Materials and Methods. Small pieces of the left median lobe of liver were removed, blotted, and weighed in a vial of glutaraldehyde preweighed on an analytical balance. A portion of the weighed liver sample was removed and processed for electron microscopy. The remaining portion was blotted, weighed as before, and radioactive content was determined with the gamma counter.

* Determined from adjacent portion of liver sample and corrected for differences in weight.
† Radioactivity units, disintegrations per minute.
§ Not determined.
TABLE II
Biochemical and Radioautographic Determination of Radioactivity Present in Perfused Liver and Thyroid Tissues after the Injection of \(^{125}\)I-insulin

| Time after injection | 3 min | 20 min |
|----------------------|-------|--------|
| Weight of rat (g)    | 45    | 45     |
| \(^{125}\)I-insulin injected (dpm)* | \(177 \times 10^6\) | \(177 \times 10^6\) |
| Weight of fixed liver (g) | 2.24 | 2.28 |
| Total radioactivity in liver (dpm) | \(30 \times 10^6\) | \(14.9 \times 10^6\) |
| Concentration of radioactivity in fixed liver (dpm/g wt) | \(13.4 \times 10^6\) | \(6.5 \times 10^6\) |
| Concentration of radioactivity in fixed thyroid (dpm/g wt) | \(4.96 \times 10^6\) | \(18.3 \times 10^6\) |
| Grains per 11.4 x 11.4 /~m frame over liver sections after 14-d exposure‡ | \(8.9 \pm 0.7\) | \(3.1 \pm 0.4\) |

* As described in the legend to Table I.
‡ 21-d Interval between injection of isotope and coating with emulsion.
¶ ± SEM.

in the reaction mixture is rapidly removed from the circulation by the thyroid. Similarly, any labeled \(^{125}\)I released from the liver as a consequence of hormone degradation would also rapidly find its way to the thyroid.

Light Microscope Radioautography

The experimental samples and their controls (in which 50 /~g of unlabeled insulin was coinjected with the same amount of labeled insulin) are depicted in Figs. 1-6.

The specificity of labeling at an early time interval (3 min) is illustrated by Fig. 1 with grains observed over the hepatic parenchyma but not to any appreciable extent over the cellular and noncellular components of the portal area. With respect to the parenchyma at the early interval, a peripheral localization of grains was observed over hepatocytes. At later time intervals (Figs. 3 and 5), fewer grains were observed over the cell periphery and clusters of grains were found over hepatocyte perinuclear areas.

The control samples (Figs. 2, 4, and 6) demonstrated a grain distribution considerably different from that of the experimentals (Figs. 1, 3, and 5). At all time intervals, grains were observed over cells lining the sinusoidal lumina and were reduced or absent over the hepatocytes.

Electron Microscope Radioautography

Experimental Sections—Early Time Intervals: Evidence has been previously obtained (4) that at 3 min after the injection of \(^{125}\)I-insulin, label is located exclusively on the hepatocyte plasmalemma. We have now attempted to quantify the number of binding sites marked by this procedure. This was carried out by placing a two-dimensional grid over the electron micrographs (Fig. 7) and determining the density and number of binding sites per cell as outlined in Table III and Materials and Methods. In addition, the distribution of binding sites along the various domains of the hepatocyte plasmalemma was determined as described in Table IV. These data were compared (Table IV) to recent estimates (7) of surface area of the three physiologic poles of the hepatocyte plasmalemma.

Experimental Sections—Later Time Intervals: Direct grain counting indicated significant intracellular localization of the grains at both 10 and 20 min postinjection (Tables V and VI). Of note at the 10-min intervals was the proportion of grains over the Golgi region (Figs. 8 and 9, and Table V). The large filamentous Ilford grains rendered it difficult to identify the structures beneath the grains. However, with fine-grain development it was possible to be more precise in scoring grains. It was found (Table V, right side) that grains overlay elements scored as Golgi (containing low density lipoprotein-like particles [1, 12] and thus included GERL [29]) but also overlaid structures scored as lysosome-like vacuoles (Fig. 9, Table V). Also of note was the finding that with the fine grains, small, uncoated, irregular cytoplasmic vesicles accounted for some of the labeling previously ascribed to the free cytoplasm when counting the larger filamentous grains. One problem in counting the fine grains was whether to count individual silver deposits or groups of silver deposits the size of an Ilford L-4 crystal. Thus, fine grains were counted using both conventions, with little difference noted in the distribution (Table V).

At the 20-min interval, the highest proportion of grains was observed over the lysosome-like...
Figure 1 Experimental section of liver perfused 3 min after the injection of $^{125}$I-insulin ($138.6 \times 10^6$ dpm, 160 $\mu$Ci/$\mu$g). Markedly fewer grains are found over the portal space (right side) than the hepatic parenchyma (left side). The portal vein (PV), bile duct (BD), hepatic arteriole (HA), lymphatics (Lym), and surrounding connective tissue elements have few grains. Grains are observed mainly over boundaries of hepatocytes (H). The grains over the sinusoidal lumina (S) can be attributed to radiation scatter of light microscope autoradiographs of $^{125}$I sources (4). The clear areas within hepatocytes are glycogen deposits (Gly). There was a 15-d interval before entry of the sections into light microscope radioautography, and the duration of exposure was 18 d. $\times$ 1,000.

Figure 2 Control for experiment of Fig. 1. All operations were identical to that described for Fig. 1 except that the injection contained in addition 50 $\mu$g of unlabeled insulin. The grain distribution is considerably different from that in Fig. 1. Grains are reduced over hepatocytes (H) and are primarily found in clusters (arrows) over cells lining the sinusoidal lumina (S). Fat droplets (F) are well preserved by the fixative. $\times$ 1,000.
**Figure 3** Experimental section; perfused 10 min after the injection of $^{14}$I-insulin ($159.8 \times 10^6$ dpm, 144 $\mu$Ci/µg). There was a 21-d interval before the sections were dipped in emulsion. Exposure, 22 d. Grains are generally scattered over hepatocytes (H) with little preferential location. $\times 1,000$.

**Figure 4** Control for experiment of Fig. 3. All operations were identical to that described for Fig. 3 except that the injection contained 50 $\mu$g of unlabeled insulin. As seen in Fig. 2, the grains (arrows) are localized in clusters and preferentially over cells lining the sinusoidal lumina (S). The sinusoids are clearly reduced in volume. As in all the figures, the clear areas within hepatocytes (H) reflect accumulations of glycogen (Gly). $\times 1,000$. 

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Figure 5 Experimental section 20 min after the injection of $^{131}$I-insulin (141.4 × 10^6 dpm, 134 $\mu$Ci/ $\mu$g). There was a 14-d interval before dipping into emulsion, and the exposure period was 14 d. Grains are found in clusters (arrows) within hepatocytes (H), particularly in juxtanuclear positions of hepatocytes. There is, as well, a scattering of grains elsewhere. The sinusoidal lumina (S) appear reduced in volume. × 1,000.

Figure 6 Control for experiment of Fig. 5 carried out identically except the injection contained an additional 50 $\mu$g of unlabeled insulin. Grains (arrows) as for all the other control experiments are found in clusters overlying lining cells of the sinusoidal lumina (S). These lumina are again reduced in volume. Few grains overlie hepatocytes (H). × 1,000.
Quantitation of $^{125}$I-Insulin-Binding Sites on Hepatocyte Plasmalemma

| Sampling procedure* | No. of grains | No. of line hits | Receptor density | Receptors per cell |
|---------------------|---------------|------------------|------------------|-------------------|
| Method A            | 368           | 10,805           | 26$^f$           | $1.3 \times 10^5$ |
| Method B            | 340           | 11,008           | 20               | $1.0 \times 10^5$ |

$^{125}$I-insulin was injected and, after 3 min, the animal was perfused and liver tissue was processed as described in the legend to Fig. 1.

* The micrographs were taken at random (method A) or only where there were grains (method B). The exposure time for the radioautographs analyzed in method A was 78 d, and 45 d for those of method B.

$^f$ The value of 5.128 $\mu m^2$ was chosen as the surface area of the hepatocyte (uncorrected value under the electron microscope) calculated from references 8 and 41.

$^f$ Units are receptors per $\mu m^2$ of hepatocyte plasmalemma. As the bile canaliculus is unlabeled (see Table IV), then the receptor density over the sinusoidal and lateral surfaces is ~10% higher.

Vacuoles (Fig. 10 and Table VI) although grains were still present over trans-Golgi vesicles. Of note was the finding that grains were not found over easily identifiable lysosomes, i.e. classical dense or residual bodies at the 10- or 20-min intervals. The small Sakura filamentous grains provided less difficulty in scoring structures than the larger Ilford filamentous grains. Thus, a clearer delineation of grains over the Golgi region was possible.

**Cytochemistry**

Acid phosphatase cytochemistry was carried out on liver sections 20 min after the injection of $^{125}$I-insulin. The reaction clearly marked the lysosomes but surprisingly, grains were not associated with these lysosomes but rather, with unreactive vacuoles or unreactive components of the Golgi apparatus (Figs. 11-13). A quantitative analysis was carried out. This analysis was restricted to the
TABLE IV

| Sampling Procedure | A  | B  | Area* |
|--------------------|----|----|-------|
| No. of grains      | 371| 718|       |
| Sinusoidal surface | 86 | 84.6| 72    |
| Lateral surface, % | 14 | 15.2| 14.5  |
| Bile surface, %    | nil| 0.2 | 13    |

Micrographs of experimental sections were taken either at random (A) or only where there were grains (B). Grains were allocated to the closest portion of hepatocyte plasmalemma. All grains, regardless of location in the micrographs, were used in the analyses.

* From Blouin (7) uncorrected for systematic errors due to section thickness effect (40).

Golgi region of the hepatocyte and only to those cells which showed a strong cytochemical reaction. Both the original negatives and printed positive images of the micrographs were carefully analyzed. This was carried out to prevent the underestimation of grains over reactive lysosomes by obliteration of the grain image from the cytochemical reaction deposit. Nevertheless, of 146 grains analyzed, 87.6% were over structures negative for cytochemical acid phosphatase activity, 8.2% were over strongly reactive structures, and 4.1% over weakly reactive vesicles (usually containing very low density lipoprotein-like particles and probably Golgi secretory droplets and/or components of GERL [29]).

"Excess" Unlabeled Insulin as Control

The nonspecific control consisted of the injection of excess unlabeled insulin to saturate the high affinity receptors and thus competitively reduce the labeling of these receptors by $^{125}$I-insulin. Such a control was previously shown (4) to demonstrate grains primarily over endothelial cells, the hepatocytes being largely unlabeled.

In the present study, quantitative analysis was carried out on sections of control animals fixed 10 min after injection. Grains were found over both macropinocytotic vesicles and dense bodies of endothelial cells (Fig. 14). Quantitative analysis (Table VII) was carried out and the scoring of Ilford filamentous grains and fine grains was compared. Although little difference in scoring the proportion of grains over hepatocytes vs. endothelial cells was noted, there were considerable differences between the two techniques when scoring grains over structures present within endothelial cells. Thus, of the filamentous grains over endothelial cells, 49.7% were attributed to macropinocytotic vesicles and dense bodies; while of the fine grains either 83.2% (silver crystals, convention I) or 87.7% (silver deposits, convention II) of the endothelial grains were attributed to these same structures. Clearly, when considering a small attenuated cell such as the endothelial cell, fine grain development leads to a more precise scoring of radioactive sources.

DISCUSSION

Free hormone was separated from bound hormone by the whole-body perfusion technique. This was concluded from analysis of radioautographs which showed few grains over sinusoidal lumina, blood vessels, and lymphatics. The bound hormone was adequately fixed as judged by the low level of solvent extraction of radioactivity in processing for electron microscopy. That this bound hormone was associated with saturable sites was concluded from the low level of labeling over hepatocytes when excess unlabeled insulin was coinjected with $^{125}$I-insulin. As injected $^{125}$I-insulin is rapidly cleared from the circulation (19), it therefore follows from the above considerations that radioautography can provide valuable information on the number, distribution, and fate of receptor-bound insulin molecules in a target tissue such as liver (see also reference 20).

Plasmalemma Receptors: Quantitation and Distribution

Based on published values of plasma volume (18), it can be calculated that an initial circulating concentration of $\sim 5 \times 10^{-8}$ M $^{125}$I-insulin was (half-distance, point-source) is expected to be greater than these values (35). A comparison of the simple scoring technique used here and more rigorous methods (i.e. references 6, 28, 36, 42) has shown no significant difference in determining the source of radiation (24).
The injected insulin and tissue processing was as described in the legend of Fig. 3. The enclosures indicate where differences were found between the scoring of Ilford filamentous grains and that of fine grains. The development procedures were as outlined in Materials and Methods (e.g., Fig. 8 for filamentous grain and Fig. 9 for fine grain). * The total numbers of grains analyzed were 382 filamentous grains and 196 fine grains (convention I) or 296 fine grains (convention II). 

No grains were observed over classical lysosomes, i.e., dense or residual bodies.

In addition, 6.4% of the grains were found over nonhepatocytes, i.e., over endothelial cells and lumina of sinusoids.

The experiment is described in the legend to Fig. 5. * The quantitation was carried out on sections coated with Sakura emulsion (see Materials and Methods) and represented the analysis of 223 grains.

† No grains were observed over classical lysosomes, i.e., dense or residual bodies. § In addition, 6.4% of the grains were found over nonhepatocytes, i.e., over endothelial cells and lumina of sinusoids.

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**Table V**

Electron Microscope Radioautographic Analysis of the 10-min Experimental Sections

| Hepatocyte Structure | Filamentous Grain | Fine Grain |
|----------------------|------------------|-----------|
|                      | % Total Grains   | % Total Grains |
| Plasmalemma          | 9.8              | 7.6        | 6.4        |
| Sub-plasmalemmal vesicles | 2.8        | 2.0        | 2.4        |
| Cytoplasm (near plasmalemma) | 16.8       | 6.6        | 7.1        |
| Small cytoplasmic vesicles | 9.7         | 11.5       |
| Cytoplasm (general) | 12.7            | 13.3       | 10.8       |
| Golgi apparatus     | 34.2            | 15.3       | 16.9       |
| Lysosome-like vacuoles§ | 17.3         | 19.9       |
| Rough endoplasmic reticulum | 0.7        | 5.6        | 4.0        |
| Smooth endoplasmic reticulum | 2.9        | 3.6        | 2.4        |
| Mitochondria        | 6.6             | 6.6        | 6.4        |
| Nucleus             | 1.0             | 1.5        | 1.0        |
| Glycogen            | 2.8             | 0.5        | 0.3        |
| All structures†     | 90.3%           | 89.6%      | 89.1%      |

The experiment is described in the legend to Fig. 5. * The quantitation was carried out on sections coated with Sakura emulsion (see Materials and Methods) and represented the analysis of 223 grains.

† No grains were observed over classical lysosomes, i.e., dense or residual bodies. § In addition, 6.4% of the grains were found over nonhepatocytes, i.e., over endothelial cells and lumina of sinusoids.

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**Table VI**

Electron Microscope Radioautographic Analysis of Experimental Sections from Livers Perfused 20 min after the Injection of 125I-Insulin

| Hepatocyte Structure | % Total Grains |
|----------------------|---------------|
| Plasmalemma          | 17.0          |
| Sub-plasmalemmal vesicles | 9.9          |
| Cytoplasm near plasmalemma | 12.6       |
| Small cytoplasmic vesicles | 1.8          |
| Cytoplasm (general)  | 4.9           |
| Golgi apparatus      | 5.8           |
| Lysosome-like vacuoles§ | 27.0         |
| Rough endoplasmic reticulum | 5.8        |
| Smooth endoplasmic reticulum | 2.2        |
| Mitochondria         | 3.1           |
| Nucleus              | 1.3           |
| Glycogen             | 1.8           |
| All Structures§      | 93.2          |
Figure 8: Experimental section. The animal was perfused 10 min after the injection of [125I]insulin (conditions described in the legend to Fig. 3). Filamentous grains are observed over the hepatocyte Golgi apparatus (G). 99 d exposure. × 30,000.

Figure 9: As for Fig. 8 but developed for fine grain radioautography. Two adjacent hepatocytes are shown in the region of the bile canaliculus (bc). Fine grains are shown over a lysosome-like vacuole (v) as well as over Golgi vesicles with very low density lipoprotein-like content (Gv). In the young rat, centrioles (c) are frequently prominent in the hepatocyte Golgi region (G). 163 d exposure. × 17,600.

Internalization of Label

Both the light and electron microscope radioautographs indicated an internalization of label. By 10 min, quantitative analysis of electron microscope autoradiographs showed considerable concentration of grains over Golgi elements. Such a

5 Similar experiments but using the technique of subcellular fractionation have demonstrated that injected 125I-
Figure 10  Section from experimental tissue fixed 20 min after ^Hl-insulin injection; conditions as described in the legend to Fig. 5. Filamentous grains (Sakura) are found over the inner periphery of a lysosome-like vacuole (v) adjacent to an autophagic vacuole (av). Exposure, 141 d. x 25,000.

The finding can be explained by the hypothesis of retrieval and reutilization of plasmalemma components during the process of protein secretion (13, 30). Our experimental data, however, showed in addition a progressive accumulation of grains over Golgi vacuoles of uncertain origin and function. Thus, by 20 min, the highest proportion of grains was over these vacuoles. As approximately half the radioactivity (Table II) was lost from the liver by this time, it was therefore reasoned that the vacuoles represented part of the degradation pathway of the hormone. Acid phosphatase cytochemistry failed to confirm the identification of the vacuoles as lysosomes, although local negative chemography cannot be formally ruled out. The enzymes considered to be part of the insulin degradation pathway (insulin glutathione transhydrogenase [39] and insulinase [25]) have been shown to have maximum activity at neutral pH and (at least for insulinase) to have a clear-cut "optimum" of pH 7.5 (10). Although insulinase has been ascribed a cytosolic location by subcellular fractionation (10, 25), this could be an artifact of homogenization of a potentially fragile organelle. Thus, the vacuoles described in the present study could represent a compartment containing the enzymes involved in the initial degradation of insulin and/or participate in the intracellular transmission of hormone action.

The absence of grains over clearly defined residual bodies may mean that 20 min is too early for hormone to appear in this compartment. As well, however, the acid pH of the lysosomal content would be expected to rapidly dissociate hormone from receptor (33). Thus, the hormone might not be fixed and therefore undetected by our techniques. Liver lysosome fractions do indeed have the capacity to degrade insulin (9) but whether they actually do so in vivo is not clear.

Other groups have suggested that insulin is internalized. Thus, using isolated hepatocytes, Terris and Steiner (38) have provided biochemical evidence that both insulin and receptor are internalized. Gordon et al. (16), also with hepatocyte cultures, have suggested a limited internalization of label as assessed by electron microscope radioautography. It should, however, be pointed out that this latter study was carried out under conditions of continuous labeling and would thus be expected to underestimate internalized label. Finally, at the level of the light microscope, a recent report by Schlessinger et al. (37) with fluorescent derivatives of insulin has documented internalization of hormone into endocytotic vesicles of fibroblasts.

Anderson et al. (2) have demonstrated the uptake of very low density lipoprotein bound to receptors on cultured fibroblasts. The internalization occurs via coated pits on the fibrocyte plasmalemma which pinch off to form coated vesicles which lose their coat and donate the very low density lipoprotein into the lysosome system of the fibrocyte. Such pits and coated vesicles are also found on the hepatocyte plasmalemma, not
Figure 11 Experimental sections perfused 20 min after injection of \( ^{131} \text{I}-\text{insulin} \) (see legend to Fig. 5), and incubated for acid phosphatase with trimetaphosphate as substrate (similar results were found with cytidine monophosphate). The micrograph is taken in the Golgi region (G) of a hepatocyte near the bile canaliculus (bc). Filamentous Sakura grains (indicated by arrows) are found over Golgi elements unreactive for acid phosphatase. In contrast, the strongly reactive vesicles are not overlaid by silver grains. Exposure, 141 d. \( \times 30,000 \).

Figure 12 Micrograph in the Golgi region (G) showing Sakura filamentous grains over the periphery of a vacuole (v) unreactive for acid-phosphatase activity. The labeled vacuole is immediately adjacent to a strongly reactive body (l). No grains are apparent over this lysosome. Exposure, 141 d. \( \times 30,000 \).

Figure 13 Three \( \text{trans} \) Golgi secretory droplets (or alternatively components of GERL) with very low density lipoprotein-like content are weakly reactive for acid-phosphatase (Gv). In addition, Sakura filamentous grains (arrows) overlie the periphery of the vesicles. The micrograph was taken in the Golgi region (G) next to the bile canaliculus (bc). Exposure, 141 d. \( \times 30,000 \).
FIGURE 14 Electron microscope radioautograph of control. The animal was perfused 10 min after the injection of \(^{125}\text{I}\)-insulin and an "excess" (50 \(\mu\text{g}\)) of unlabeled insulin (see legend to Fig. 4). Ilford fine grains are clustered over one pole of a macropinocytic vesicle (mv) of an endothelial cell (E) as well as over a dense body (db) of the same cell. Only few grains (arrows) overlie hepatocytes (H). The lipocyte (L) faces the space of Disse (D) between the two hepatocytes and is unlabeled. The lumen of the sinusoid is indicated (S). Exposure, 163 d. \(\times\) 15,000.

Excess Unlabeled Insulin as Control

The radioautographic technique based on the rationale of the biochemical specific binding assay (4) has localized insulin-specific binding sites. Thus, the sites should be labeled by \(^{125}\text{I}\)-insulin and competitively blocked by added unlabeled...
TABLE VII
Electron Microscope Radioautographic Analysis of Control Section Fixed 10 min after the Injection of ¹²⁵I-Insulin and Excess Unlabeled Insulin*

| Filamentous grain | Fine grain |
|-------------------|-----------|
|                   | Endothelial cells | Hepatocytes | Endothelial cells | Hepatocytes |
|                    | % Total grains | % Total grains | I | II | I | II |
| Plasmalemma        | 12.6 | 4.9 | 1.2 | 1.4 | 4.8 | 3.2 |
| Small vesicles     | 2.3  | 0.8 | 4.2 | 3.2 | 2.3 | 2.5 |
| Cytoplasm          | 21.1 | 10.1| 7.1 | 5.0 | 3.6 | 4.0 |
| Macropinocytotic vesicles | 15.2 | 44.0 | 46.0 | 47.3 | 2.4 | 2.2 |
| Dense bodies       | 23.1 | nil | 23.8 | 27.3 | nil | nil |
| Golgi apparatus    | nil  | 2.1 | nil | nil | 2.4 | 2.2 |
| Mitochondria       | 0.6  | 2.9 | nil | nil | 2.4 | 1.4 |
| Nucleus            | 2.2  | nil | 1.2 | 0.7 | 1.8 | 1.4 |
| All structures     | 77.1 | 20.8| 81.5 | 83.6 | 17.3 | 14.7 |

* All other conditions were identical to that described in the legend of Fig. 4.
† The distribution of 194 filamentous grains and 168 fine grains (Convention I) or 278 grains (Convention II) was analyzed.
‡ Conventions I and II as for Table V.
¶ Only found in endothelial cells.
§ The remaining grains were observed over lumina of sinusoids but not over Kupffer cells or lipocytes.

insulin at a concentration ("excess") greater than that required to saturate the receptors (reviewed in reference 31). As $-5 \times 10^{-6}$ M unlabeled insulin was injected in the case of controls, this should saturate and competitively inhibit ¹²⁵I-insulin binding to its receptors.

Quantitative radioautography indicated that much of the label over hepatocytes was inhibited by the coinjection of 50 µg of unlabeled insulin (control) at all the time intervals studied. However, such was not the case for the endothelial cells. In experimental animals, only low labeling over endothelial cells was observed. In the controls, about four-fifths of the labeling (the total grain density over control sections was always about one-half that of the experimentals—data not shown) was over endothelial cells and over the postulated lyosome system of this cell. The pinocytotic process was apparently activated by high concentrations of hormone, and grains visually appeared close to one pole of the membrane of the macropinocytotic vesicles (see reference 4 and Fig. 14 of this paper). This leads us to suggest that, far from representing nonspecific labeling, the findings suggest a low affinity receptor on the luminal surface of the endothelial cell although other explanations are possible. The biological

FIGURE 15 Section from experimental liver perfused min after the injection of ¹²⁵I-insulin (conditions described in legend to Fig. 1). The micrograph indicates a region close to the sinusoidal surface adjacent to the space of Disse (D) and an endothelial cell (E). Ilford filamentous grains overlie the periphery of the hepatocyte. However, the arrows indicate coated vesicles and coated invaginations of the cell surface which are a noteworthy feature of the hepatocyte periphery. It is speculated that these might be the carriers through which ¹²⁵I-insulin is internalized within the hepatocyte. Exposure, 78 d. × 20,000.
significance of the postulated receptor is as yet unknown.

Conclusions

Insulin initially binds to receptors dispersed over the sinusoidal and lateral surfaces of hepatocyte plasmalemma. The hormone is internalized into the Golgi region of the hepatocyte and especially into vacuoles whose functions are unclear but may represent a site of action and/or degradation of insulin.

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