

Binding, Internalization, and Lysosomal Association of ^{125}I -Human Growth Hormone in Cultured Human Lymphocytes: A Quantitative Morphological and Biochemical Study

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ABSTRACT ^{125}I -human growth hormone (^{125}I -hGH) binds specifically to receptors on cultured human lymphocytes (IM-9). When this process is studied by use of quantitative EM radioautography, under conditions of incubation at 15°C for 5 min, the ligand is localized to the plasma membrane of the cell. At 30° and 37°C , however, ^{125}I -hGH is progressively internalized by the cell as a function of time. The internalized ligand is found predominantly in the Golgi region of the cells, with a five-fold preferential localization to membrane-bounded structures with the morphological and cytochemical characteristics of lysosomes. Up to 59% of these lysosome-like structures are positive for the acid phosphatase reaction under the conditions of incubation at 37°C for 120 min. When the cell-associated radioactivity after 15–120 min of incubation at 37°C is extracted in 1 M acetic acid and filtered on a Sephadex G-100 column, 58–73% of the material elutes as intact hGH.

When cells are incubated with ^{125}I -hGH at 37°C for 15–120 min, separated from the incubation medium, and washed and diluted 100-fold, the percent ^{125}I -hGH dissociable decreases as a function of increasing time of incubation. When cells are incubated with ^{125}I -hGH for 15 min at 37°C and the radioactivity that dissociates from the cells during 15–90 min is studied, the labeled material appearing in the incubation medium is progressively degraded as a function of time of incubation. When the dissociation process is studied radioautographically, grains are found both in plasma membrane and intracellular compartments after 30 min of association, but after 30 and 120 min of dissociation a higher proportion of grains are in the intracellular compartment. After 120 min of association, there is less dissociation from either compartment and a preferential increase of grains in the intracellular compartment.

These data suggest that receptor-linked internalization of a polypeptide hormone provides a mechanism that couples degradation of the ligand with loss of the cell surface receptor.

When a polypeptide hormone or growth factor binds to specific receptors on cell surfaces, several diverse events occur. In target cells a biological response is initiated, in both target and nontarget cells the ligand is inactivated or degraded (38), and the hormone-receptor interaction regulates the ambient receptor concentration (16, 26).

By use of morphological probes, it has been shown directly that a variety of polypeptide hormones and growth factors bind

to specific sites on the cell surface and are subsequently internalized by the cell (1, 6, 10, 13, 17,–19, 22, 23, 31, 39). The function of this sequence of events is incompletely understood. For certain ligands, internalization is a necessary step in their biological action (2); it remains to be determined whether this process is required for any of the biological actions of polypeptide hormones and growth factors.

We have previously suggested that hormone degradation

and loss of cell surface receptors may be linked through a common sequence of events (10, 18, 19). Binding of the hormone results in internalization of the hormone-receptor complex in a membrane-bounded compartment that transfers the hormone and possibly the receptors to a lysosomal compartment. The hormone is then degraded, and the receptor may be degraded or recycled back to the plasma membrane.

The present study describes the morphological and biochemical events involved in the interaction of human growth hormone (hGH) with cultured human lymphocytes and supports the hypothesis that ligand-induced endocytosis is a major mechanism linking the binding of hormone to degradation and to the regulation of cell surface receptors.

MATERIALS AND METHODS

Cells and Reagents

Human cultured lymphocytes of the IM-9 cell line were used for all experiments (8, 15, 25). Cells were grown at 37°C in RPMI 1640 medium containing 10% fetal calf serum and 25 mM HEPES (International Biological Laboratories, Inc., Rockville, Md.). Glutamine (0.29 mg/ml) was added just before feeding. Cells were "fed" three times a week by dividing the culture 1:3 and adding fresh medium. Cells in late log phase or early stationary phase of growth were split 1:2 in fresh medium 24 h before use.

Human growth hormone (preparation 2106E, a gift of the National Pituitary Agency, National Institute of Arthritis, Metabolic, and Digestive Diseases) used for this study was iodinated at specific activity of 50 mCi/mg and purified as previously described (26).

Incubation Conditions

Cells were removed from the growth medium by centrifugation at 600 g for 5 min at 24°C and washed once by resuspension in the assay buffer (see below) (25). The washed cell pellet was then equilibrated in assay buffer at 37°C. ~10⁸ cells/ml were incubated in assay buffer (50 mM sodium HEPES, 120 mM NaCl, 1.2 mM magnesium sulfate, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 10 mg/ml bovine serum albumin, pH 7.6) with 5–20 ng/ml ¹²⁵I-hGH at 15°, 30°, and 37°C for varying periods of time in 17- × 100-mm plastic Falcon tubes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). Identical incubations were carried out in the presence of 1 μg/ml of unlabeled hGH to determine nonspecific binding. At appropriate time intervals, 1.5 ml of cells were gently transferred into conical plastic tubes and centrifuged at 200 g at 4°C for 15 min and the supernate was aspirated. 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, was then added to the cell pellet and allowed to fix for a minimum of 4 h at room temperature. The glutaraldehyde was then aspirated and replaced with 0.1 M phosphate buffer, pH 7.4, until further processing (8). Radioactivity in the cell pellet was determined by gamma counting.

Analysis of Cell-associated Radioactivity

Cells (10⁸) were diluted in 4 ml of assay buffer that contained ¹²⁵I-hGH (10 ng/ml) and incubated in a 37°C shaking water bath. At the end of specified times, 1 ml of cells was transferred to 17- × 100-mm plastic tubes and centrifuged at 400 g for 2 min at 24°C. The supernate was removed, and the cell pellet was resuspended in ice-cold assay buffer and centrifuged as before. The supernate was saved for counting radioactivity, and the cell pellet was resuspended in 1.5 ml of 1 M acetic acid. After 15 min, the acid-solubilized material was centrifuged and 1 ml of the supernate was applied to a Sephadex G-100 column (1.5 × 80 cm) equilibrated in and eluted with 1.0 M acetic acid at 4°C.

In initial experiments cells were solubilized in 0.1% Triton X-100, 6.0 M urea, and 1.0 M acetic acid as described previously for ¹²⁵I-insulin associated with rat hepatocytes (10). We found, however, that cell-associated ¹²⁵I-hGH, as well as ¹²⁵I-hGH alone when stored in Triton X-100, urea, and acetic acid at -20°C and then filtered on a Sephadex G-100 column, was recovered primarily in the void volume of the Sephadex column. A similar pattern was obtained when ¹²⁵I-hGH in 1.0 M acetic acid was stored at -20°C. Thus, all cell extracts were stored at 4°C.

Kinetics of Association and Dissociation of ¹²⁵I-hGH

¹²⁵I-hGH (10 ng/ml) was incubated with 10⁷ cells/ml in assay buffer at 37°C for 15, 30, 60, and 120 min. At intervals of association aliquots (100 μl) were taken, the cells were centrifuged at 600 g for 2 min, and the cell-associated

radioactivity was measured. To study dissociation of ¹²⁵I-hGH from the cell we took additional 100-μl aliquots and transferred them to a series of tubes that contained 10 ml of assay buffer at 37°C (we have demonstrated that this dilution prevents rebinding of the ligand). At intervals (15, 30, 50, 90, and 120 min), the tubes were centrifuged (2 min, 600 g), and the radioactivity in the cell pellet was counted.

Nature of Cell-dissociated Radioactivity

Cells (10⁸) were diluted and equilibrated in 5.0 ml of assay buffer at 37°C, ¹²⁵I-hGH (10 ng/ml) was added at time zero, and cells were incubated for 15 min at 37°C. Cells were then centrifuged at 400 g for 2 min at 24°C, the supernate was removed, and the cell pellet was resuspended in ice-cold assay buffer and recentrifuged. The cell pellet was then resuspended in 37°C assay buffer containing 1.0 μg/ml unlabeled hGH (in the presence of excess unlabeled hGH, reassociation of ¹²⁵I-hGH with receptor is prevented; thus only dissociation of ¹²⁵I-hGH is measured), and incubated for 15 min. The cells were pelleted by centrifugation at 400 g for 2 min at 24°C, and resuspended in assay buffer at 37°C with hGH (4.0 ml) for another 30 min. The cells were centrifuged as before, the supernate was saved and the cell pellet was resuspended in 37°C assay buffer with hGH (4.0 ml) for an additional 45 min. At the end of 45 min the cells were centrifuged as before, the supernate was saved, and the cell pellet was counted to determine radioactivity that had not dissociated from the cells during the total 90 min at 37°C. Each of the supernates (after centrifugation at 15, 30, and 45 min) was applied to a Sephadex G-100 column (0.9 × 50 cm) equilibrated in and eluted with 0.01 M phosphate buffer, pH 7.4. The supernatant sample from each time-point was gel filtered in reverse order (45-min supernate before 30 min before 15 min), i.e., the lowest to highest radioactive sample.

Preparation for Electron Microscopy and Radioautography

After three successive washings in phosphate buffer, the cell pellet was postfixed in 0.1 M osmium tetroxide, pH 7.4, for 2 h at room temperature, dehydrated in graded ethanol, and embedded in Epon. Grids that contained the sections were coated with Ilford L4 emulsion (Ilford Limited, Basildon, England) as previously described (8, 10). After 3–5 wk, the grids were developed in Microdol X. Samples were then examined in a Philips EM 300 electron microscope, and grains were photographed only on cells that were judged to be well preserved (8). Photographs were taken at a magnification (× 11,000) calibrated with a reference grid (2,160 lines/mm) (7, 9).

Morphometry

The volume density of the main intracytoplasmic structures that could be detected at the magnification chosen (i.e., endoplasmic reticulum, nucleus, lysosomal structures, and Golgi apparatus) was determined on cells incubated for 120 and 180 min at 30°C. For each condition, four different Epon blocks were cut and a total of 48 pictures were analyzed (12 pictures per block section). For each section, random sampling was ensured by photographing the closest lymphocyte cytoplasm to each corner of three consecutive squares of the supporting grid. Pictures were taken at an initial magnification of × 11,000. Morphometric determinations were made on prints enlarged three times (final magnification, × 33,000) with a test screen in the form of a double square lattice. The volume density of organelles was determined as previously described (10).

The percentage of grains beyond 250 nm of the plasma membrane, which were related to each of the intracellular organelles previously characterized by morphometry, was evaluated by a probability circle method (10).

Cytochemical Studies

Cytochemical reactions for acid phosphatase using β-glycerophosphate as substrate (60 min of incubation at 37°C) were carried out on "chopper" sections of lymphocyte pellets as described by Barka and Anderson (5). Control incubations were carried out either in the absence of β-glycerophosphate or in the presence of an inhibitor of the reaction (NaF: 0.01 M). Radioautography was carried out on the cytochemically reacted tissue as described above.

RESULTS

Quantitative EM Radioautographic Localization of ¹²⁵I-hGH to the Cell Surface

For these and previous studies (8, 10, 19), we have used the earliest time-points at the lowest temperature studied as the

most accurate reflection of initial binding at the cell surface. Under these conditions, using the line-source model of Salpeter (30), we have shown that labeled insulin initially localizes to the plasma membrane of cultured human lymphocytes and the cell-associated radioactivity is analyzed by EM radioautography, grains distribute predominantly in a symmetrical fashion around the plasma membrane (Fig. 1) (for further data on the morphologic integrity of these cells, see references 8, 21, and 28). These data are consistent with predominant localization of the ligand to surface membrane receptors.

Quantitative EM Radioautographic Studies of ^{125}I -hGH Binding to Cultured Human Lymphocytes as a Function of Time and Temperature

Because ^{125}I -hGH localizes predominantly to the plasma membrane of the cultured human lymphocyte at 15°C , we wished next to determine the fate of the labeled ligand at higher temperatures. When ^{125}I -hGH is incubated with cultured human lymphocytes at 30°C , binding comes to an apparent steady state by 30–60 min of incubation and is maintained up to at least 180 min (Fig. 2). When cells are examined by radioautography at various time-points of incubation at 30°C , it can be seen that after only 2 min of incubation there is a progressive internalization of the labeled ligand (Fig. 3).

At 37°C the time-course of binding (data not shown) is similar to that shown for 30°C in Fig. 2. When the grain distribution of the labeled ligand is examined for 180 min of

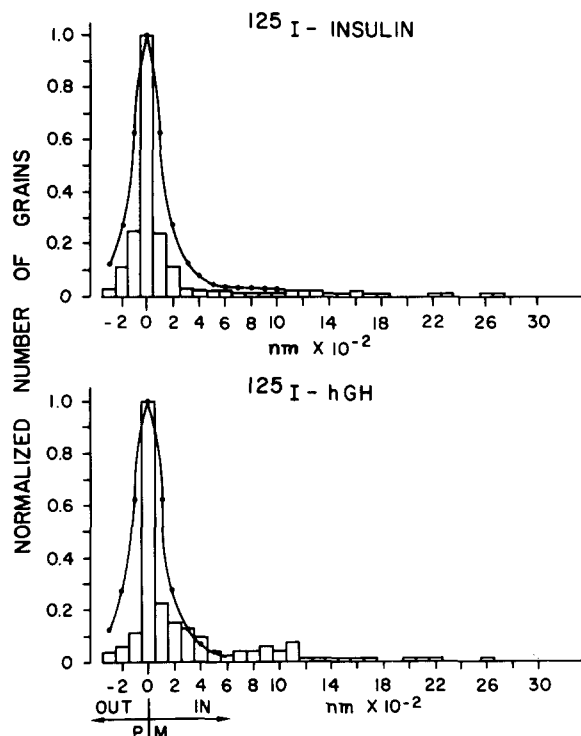


FIGURE 1 Grain distribution histogram of initial localization of ^{125}I -insulin (upper; 2 min, 15°C) and ^{125}I -hGH (lower; 5 min, 15°C) to cultured human lymphocytes. The normalized number of grains (vertical axis) is plotted as a function of the distance of the grain center from the plasma membrane (30) (horizontal axis). The zero point refers to the plasma membrane (PM). The solid line is the universal curve of ^{125}I adapted from Salpeter et al. (30) and represents the distribution of ^{125}I around a defined line source of irradiation. Number of grains analyzed: upper, 229; lower, 160.

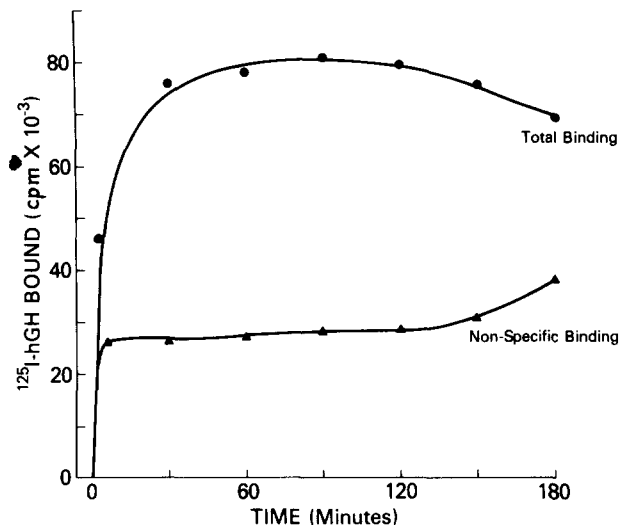


FIGURE 2 Time-course of binding of ^{125}I -hGH with cultured human lymphocytes. ^{125}I -hGH (15 ng/ml) was incubated with cultured human lymphocytes (10^7 cells/ml) in assay buffer at 30°C . At each time-point shown (solid line), cells were separated from the medium by centrifugation, and the radioactivity in the cell pellet was determined by gamma counting. A parallel incubation was carried out in exactly the same way except that $1\ \mu\text{g/ml}$ of unlabeled hGH was added at the beginning of the incubation (nonspecific binding). Percent specific binding: 2 min, 2.6; 30 min, 6.4; 60 min, 6.4; 90 min, 6.6; 120 min, 6.5; 150 min, 5.6; and 180 min, 3.9.

incubation at 37°C , the labeled ligand is internalized in a fashion similar to the 30-minute incubation (data not shown). These data suggest that labeled hGH initially localizes to the cell surface but is progressively internalized by the cell as a function of time and temperature.

Because radioautography measures total cell-associated radioactivity, we wished next to determine how nonspecific binding (cell-associated radioactivity in the presence of a large excess of unlabeled hormone) relates to this process. Nonspecific binding reaches maximum by 2 min of incubation at 30°C and changes only slightly over the remainder of the incubation (Fig. 2). With a higher concentration of labeled hormone than in our previous experiments (25), there is higher nonspecific binding. When grains from a number of grids are pooled and the nonspecific binding is quantitatively analyzed, it has a distribution essentially the same as that of total binding (Fig. 4). Note also that nonspecific binding represents the highest percentage of total binding at 2 min of incubation (Fig. 2). To the extent that some portion of nonspecific binding relates to trapping of the ligand in the pellet, this radioactivity, not fixed by glutaraldehyde, is lost from the pellet during processing and not seen radioautographically. Previous studies have demonstrated that inactive growth hormones do not bind to these cells (25) and, similarly, ^{125}I and insulin degradation products do not bind to hepatocytes (10). Thus, nonspecific binding in these experiments cannot explain the progressive internalization of labeled hGH by the cultured human lymphocyte.

Intracellular Localization of ^{125}I -hGH in Cultured Lymphocytes

Because labeled hGH is progressively internalized by the cultured human lymphocytes, we wished next to determine whether the internalized ligand (i.e., grains $>250\ \text{nm}$ intracellularly) localizes to specific regions of the cell. $>70\%$ of the

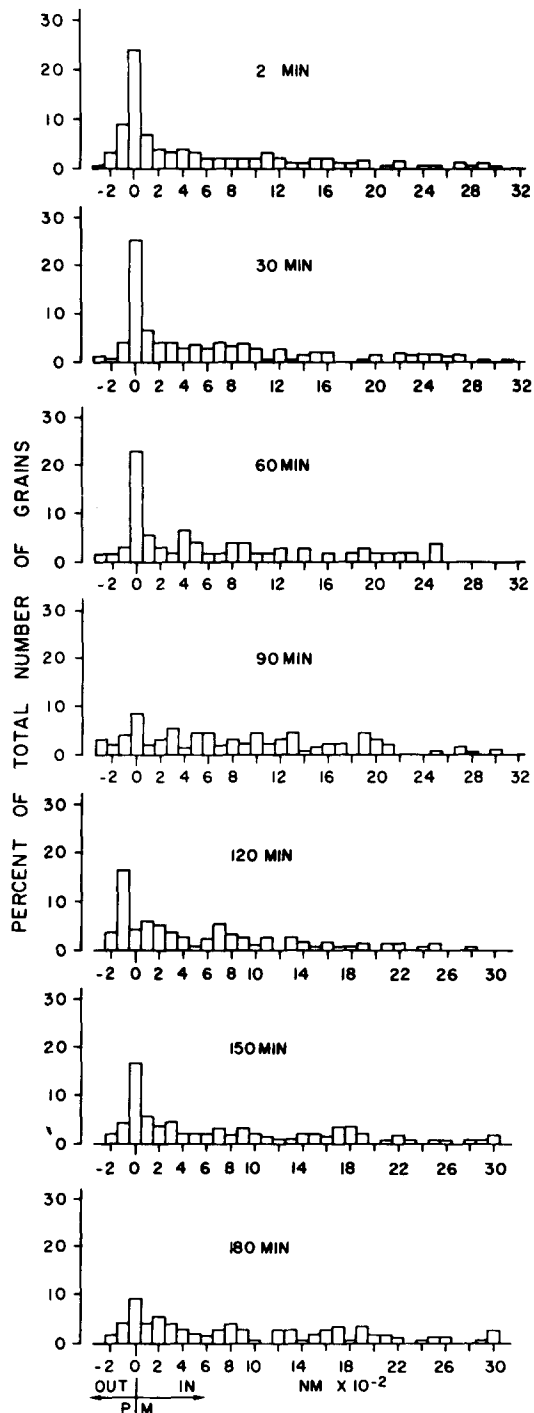


FIGURE 3 Grain distribution histogram from each time-point of incubation shown in Fig. 2. The percent of total grains (vertical axis) is plotted as a function of the distance of the grain center from the plasma membrane (horizontal axis). Note the progressive decline in the percentage of grains over the plasma membrane and spreading of radioactivity intracellularly as a function of incubation time. Grains analyzed at: 2 min, 143; 30 min, 154; 60 min, 73; 90 min, 123; 120 min, 117; 150 min, 127; and 180 min, 195. A second incubation at 30°C (data not shown) was carried out for each time-point shown here, with essentially identical results.

internalized grains localize in the Golgi pole of the cell. This region is always located in the indentation of the nucleus and is easily recognizable (Fig. 5).

To determine whether grains preferentially localize to specific morphologically detectable organelles, the volume density

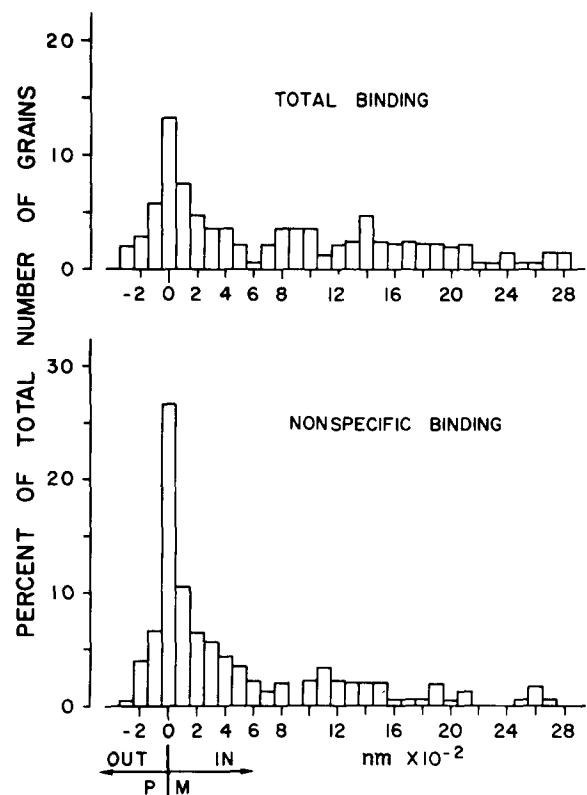


FIGURE 4 Grain distribution histogram of 120-min time-point incubated at 30°C. Total associated radioactivity (upper) and nonspecific binding (lower); cell-associated radioactivity in the presence of 1 $\mu\text{g}/\text{ml}$ of unlabeled hGH. It can be seen in Fig. 2 that the nonspecific binding at 120 min of incubation represents 30% of the total binding. Background or spontaneous photochemical events ≈ 0.006 grains/ nm^2 or $\approx 1-2$ grains per histogram column. For further discussion of this type of analyses, see Fig. 7 of reference 8. Number of grains analyzed: upper, 141; lower, 165.

of the various organelles was determined (22), and the grains associated with these organelles were identified by a probability circle method. When this is done, a five-fold preferential association of grains with lysosomal structures is observed (Fig. 6). By contrast, there is no preferential localization of grains to mitochondria, Golgi elements, rough endoplasmic reticulum, or nucleus (Fig. 6).

Morphology and Cytochemistry of Structures to Which Grains Localize

We consider as lysosomes the membrane-bounded structures shown in Fig. 7. In other studies we have included as lysosomes structures that can be described morphologically as multivesicular bodies, dense bodies, autophagosomes, and heterophagosomes (4, 10, 11, 18, 21). To characterize these structures further, we have revealed cytochemically the lysosomal enzyme acid phosphatase and studied the relationship of radioautographic grains related to membrane-bounded structures positive for acid phosphatase. When this is done, it can be seen that grains progressively associate with the acid phosphatase-positive structures as a function of time. By 120 min of incubation at 30°C, up to 50% of grains are related to structures positive for this single lysosomal marker (Table I).

To be certain that the cytochemical reaction was specific for acid phosphatase, control experiments were carried out in either the absence of substrate or the presence of an inhibitor

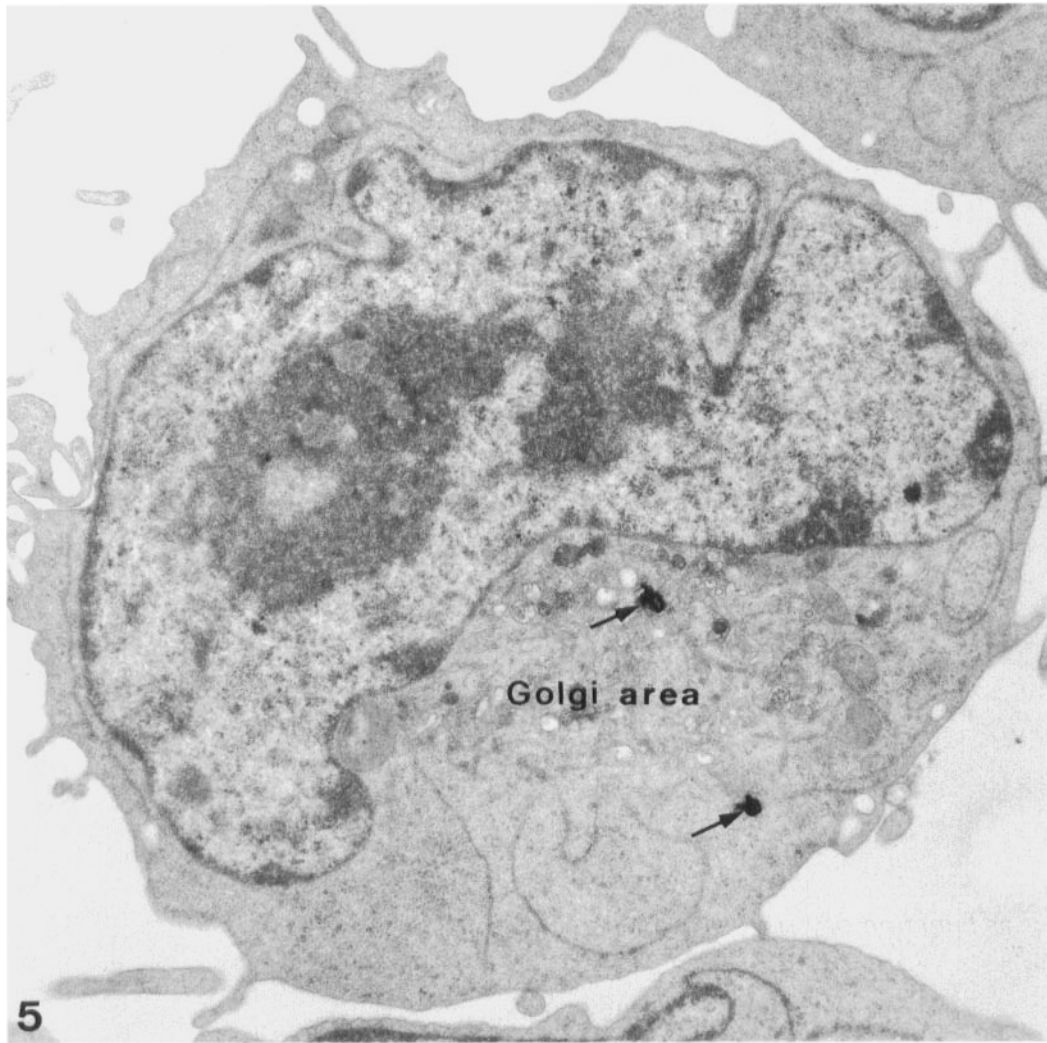


FIGURE 5 General view at low magnification of an IM-9 lymphocyte incubated for 180 min at 37°C in the presence of ^{125}I -hGH. Two radioautographic grains (arrows) are seen in the Golgi region of the cell. $\times 25,000$.

of the reaction. Under these circumstances there was no morphologically detectable reaction.

Nature of Cell-associated Radioactivity

At the end of specified times of incubation of ^{125}I -hGH with cultured lymphocytes at 37°C, cells were centrifuged and the pellet was washed and extracted in 1 M acetic acid. From 75 to 83% of the radioactivity was extractable from the cell. The cell extract was then applied to a Sephadex G-100 column equilibrated in and eluted with 1 M acetic acid. Under these circumstances, 73% of the radioactivity eluted as intact hGH after 15 min of incubation, and this decreased to 58% by 120 min of incubation (Fig. 8).

Analysis of Dissociation of Cell-associated Radioactivity

To determine whether the time of association of ^{125}I -hGH with the cell affected the dissociability of the labeled ligand, ^{125}I -hGH was incubated at 37°C with cultured lymphocytes, and at specified times cells were diluted 100-fold, and the cell-bound radioactivity was allowed to dissociate for 120 min (Fig. 9). Under these conditions, the absolute amount of radioactivity

dissociable in 120 min was progressively decreased as a function of increased association time (Fig. 9).

Nature of Radioactivity Dissociated from Cultured Human Lymphocytes

To analyze the radioactivity released by the cell, it was first necessary to show that the incubation medium per se did not contain degrading activity. Cells were incubated in buffer for 90 min at 37°C. The cells were then separated from the medium by centrifugation, and the supernatant buffer was incubated with ^{125}I -hGH for 90 min at 37°C and filtered on a Sephadex G-100 column. Under these conditions, there was no alteration of the ^{125}I -hGH, and the resultant gel-filtration pattern was indistinguishable from that seen with fresh nonincubated ^{125}I -hGH (data not shown). We conclude, therefore, that any change in the labeled material released into the medium results from the cell itself.

^{125}I -hGH was then incubated with cultured lymphocytes at 37°C for 15 min. Cells were then pelleted by centrifugation, washed, and resuspended in fresh buffer supplemented with 1 $\mu\text{g}/\text{ml}$ unlabeled hGH to prevent reassociation to cells of labeled material released into the incubation medium. At the times specified, aliquots of the incubation medium were filtered

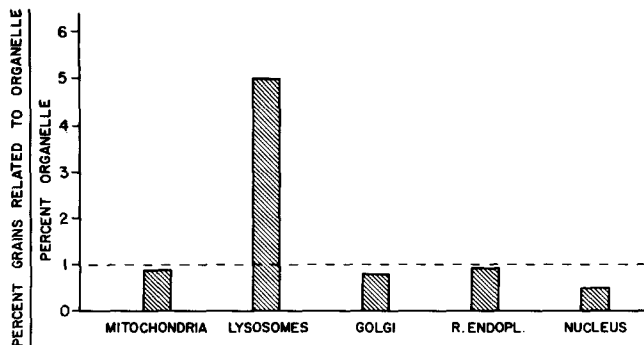


FIGURE 6 Relationship of developed radioautographic grains to cytoplasmic structures (120 and 180 min of incubation at 30°C). The percent grains related to organelles was determined by a probability circle as previously described (10), and the volume density of the cytoplasmic organelles was determined by standard morphometric techniques (see Materials and Methods). The dashed line represents a ratio of one, which indicates no preferential localization to a given structure. Structures considered as lysosomes are illustrated in Fig. 7.

on a Sephadex G-100 column equilibrated in and eluted with 0.1 M phosphate buffer, pH 7.4 (Fig. 10). During the first 15 min of dissociation, 64% of the recovered radioactivity elutes in agreement with intact hGH. With longer periods of dissociation, less of the labeled material elutes as intact hGH and more appears as low molecular weight degradation products. Radioactivity eluting in the void region is relatively constant with time.

Distribution of ^{125}I -hGH on Cultured Human Lymphocytes as Function of Association and Dissociation Time

We wished next to determine the distribution of cell-associated radioactivity as a function of dissociation time after both 30 and 120 min of association at 37°C.

After 30 min of association at 37°C, the grain distribution is similar to that seen in Fig. 3 for 30°C incubation (Fig. 11 A). As a function of dissociation time, a higher percentage of grains is internalized, as can be seen for the 30- and 120-min periods of dissociation (Fig. 11 B-C). A similar pattern is seen for the 120-min association period, except that the percentage of internalized grains is less at the 30-min dissociation point (Fig. 11 D-F).

The interpretation of these data is complex because at the more prolonged times of association less of the total cell-associated radioactivity is dissociable. For further analysis of the data with respect to radioactivity in the incubation medium, on the plasma membrane, or internalized by the cell, see Discussion.

DISCUSSION

The data presented are consistent with a sequence of events in which ^{125}I -hGH in the incubation medium binds to specific sites on the surface of cultured human lymphocytes. The specificity of binding has been previously demonstrated (25), and the present experiments show quantitatively the localization of the ligand to the cell surface, after a brief incubation at 15°C. At both 30°C and 37°C, a large proportion of the ligand is internalized as the hormone-cell interaction approaches an apparent steady state. Although we have not quantitatively demonstrated the mechanism of internalization, we have shown

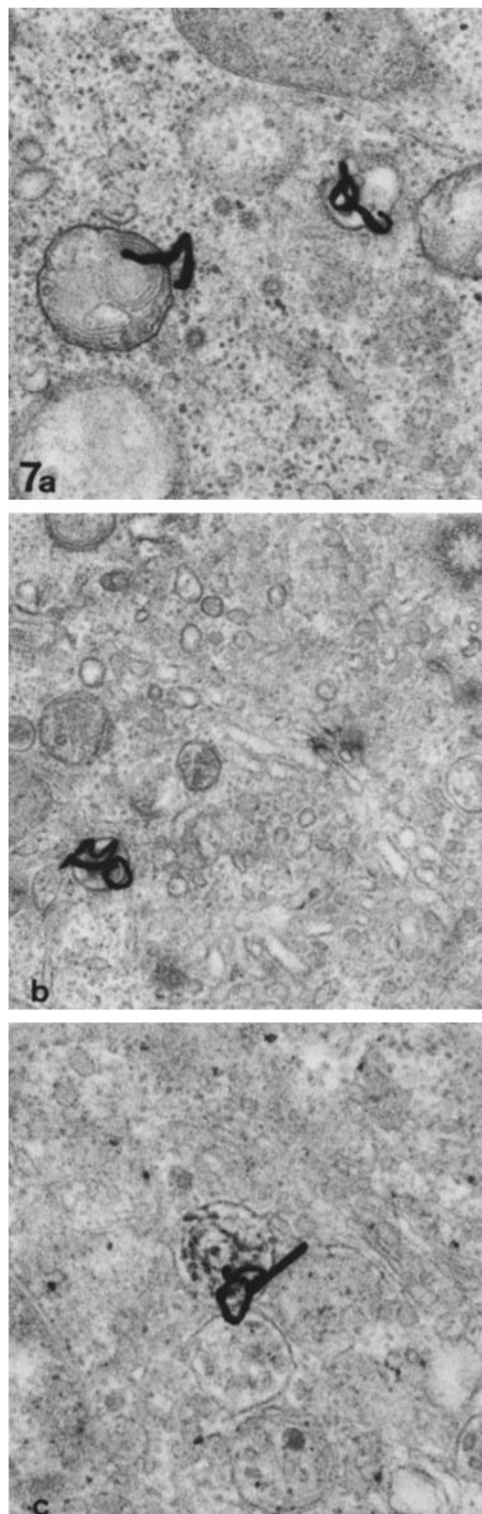


FIGURE 7 Selected images of developed radioautographic grains associated with vesicles. In a and b, grains are situated over three representative examples of those structures that are considered as lysosome-like. In c, a grain is associated with a membrane-bounded structure close to a Golgi complex and shows an positive reaction for acid phosphatase. $\times 33,000$.

that the methods used for this study give results similar to those found with ferritin-labeled low-density lipoproteins (2, 23) and epidermal growth factor (7) in other systems, in which endocytosis presumably mediates internalization of the ligand.

TABLE I
Relationship of Radioautographic Grains to Lysosome-like Structures

Time	Lysosome-like structures		Total
	Acid phosphatase +	Acid phosphatase -	
5	5 (31%)	11 (69%)	16
60	15 (28%)	38 (72%)	53
120	34 (59%)	24 (41%)	58
Total	54 (43%)	73 (57%)	127

Incubation of IM-9 lymphocytes with 10^{-9} M 125 I-hGH.

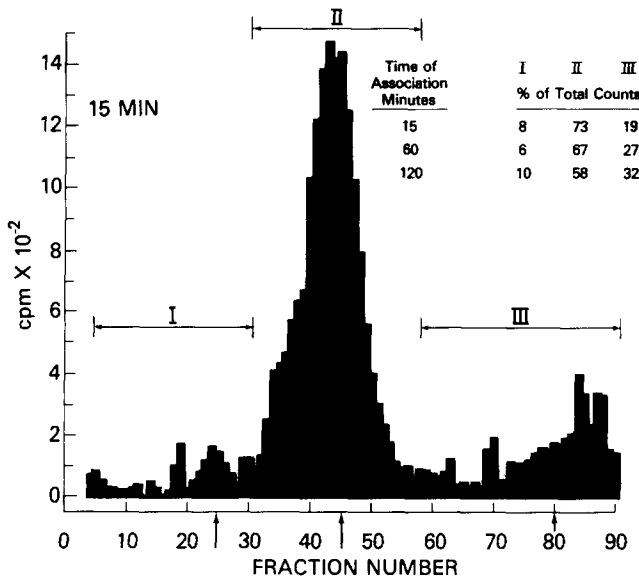


FIGURE 8 Sephadex G-100 elution profile of cell-associated radioactivity. Cells were incubated with 125 I-hGH in assay buffer for the times shown in the inset and at each time-point were extracted in 1 M acetic acid. The radioactivity was then applied to a Sephadex G-100 column equilibrated in and eluted with 1 M acetic acid. The arrows from left to right refer to the peak elution of 125 I-porcine thyroglobulin, 125 I-hGH, and 125 I, respectively. The brackets designated I, II, and III represent the fractions included under each region shown in the inset. When 125 I-hGH was exposed to these extracting conditions in the absence of cells, 95% of the radioactivity eluted in peak II and ~1 and 4% eluted in peaks I and III, respectively.

The data demonstrate by both standard morphological and cytochemical criteria that the labeled ligand preferentially localizes in lysosomes, as would be expected from an endocytotic process (34). These events are qualitatively similar to those for other ligands labeled with iodine (1, 10-13, 18, 20, 21) or ferritin (2, 23) in other cell types.

Our data differ, to some extent, from those of other studies that have used subcellular separation techniques and radioautography. Goldfine et al. (17) have reported that 125 I-insulin is localized in nuclei and endoplasmic reticulum in cultured human lymphocytes. We find that the internalization of insulin in cultured lymphocytes is quantitatively small, and we have never found preferential localization of the ligand to nuclei or endoplasmic reticulum (8, 21). The explanation for these differences is unclear.

Using EM radioautography, Bergeron et al. (6) have reported that 125 I-insulin is localized in Golgi elements as well as in lysosome-like structures in rat livers. Although we find that

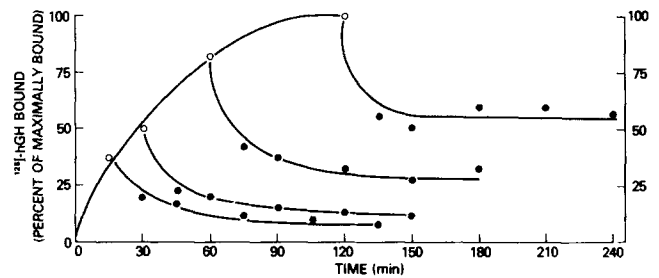


FIGURE 9 Dissociation of cell-associated radioactivity. 125 I-hGH (5 ng/ml) was incubated for varying periods of time with cultured human lymphocytes in assay buffer at 37°C. At the end of specified periods of time, an aliquot of cells was diluted 100-fold, and cell-associated radioactivity was allowed to dissociate for 120 min. Note that, as a function of time of association, there was a decrease in the radioactivity dissociable from the cell. Open circles, time-points of association; solid circles, time-points of dissociation.

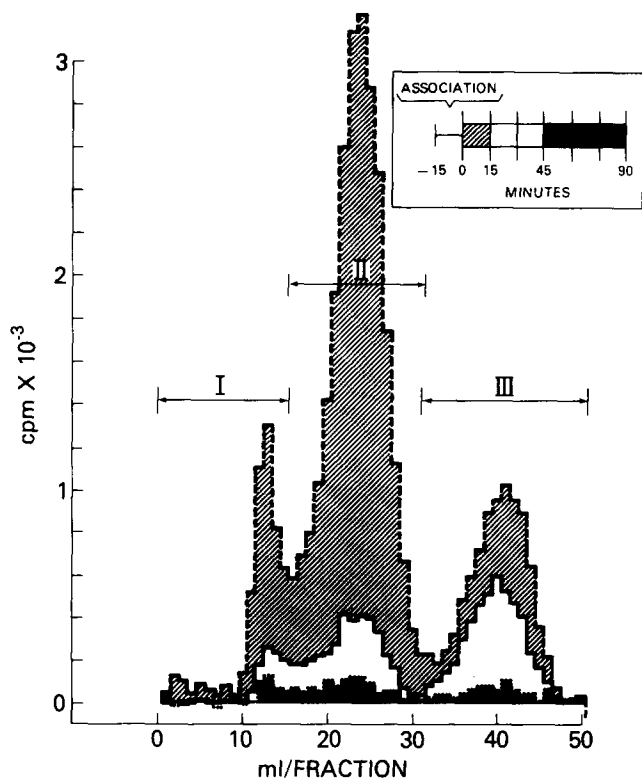


FIGURE 10 Sephadex G-100 gel filtration of radioactivity recovered in the incubation medium. Cultured human lymphocytes were incubated with 125 I-hGH in assay buffer at 37°C for 15 min. At the end of this period, the radioactivity was allowed to dissociate from the cell in the presence of 1 μ g/ml of unlabeled hGH to prevent reassociation. The key in the upper right of the figure designates the time period over which the radioactivity was collected in the medium. The gel-filtration patterns correspond to the patterns shown in the key with respect to the time periods of collection. The enclosed brackets include the fractions designated as regions I, II, and III, respectively.

labeled hGH present in the cultured lymphocyte as well as insulin present in the cultured lymphocyte (21) and hepatocyte (9) are localized in the Golgi region of the cell, we do not find them localized to morphologically identifiable Golgi structures.

These are the first studies to examine the morphological and biochemical events that occur during the dissociation of a

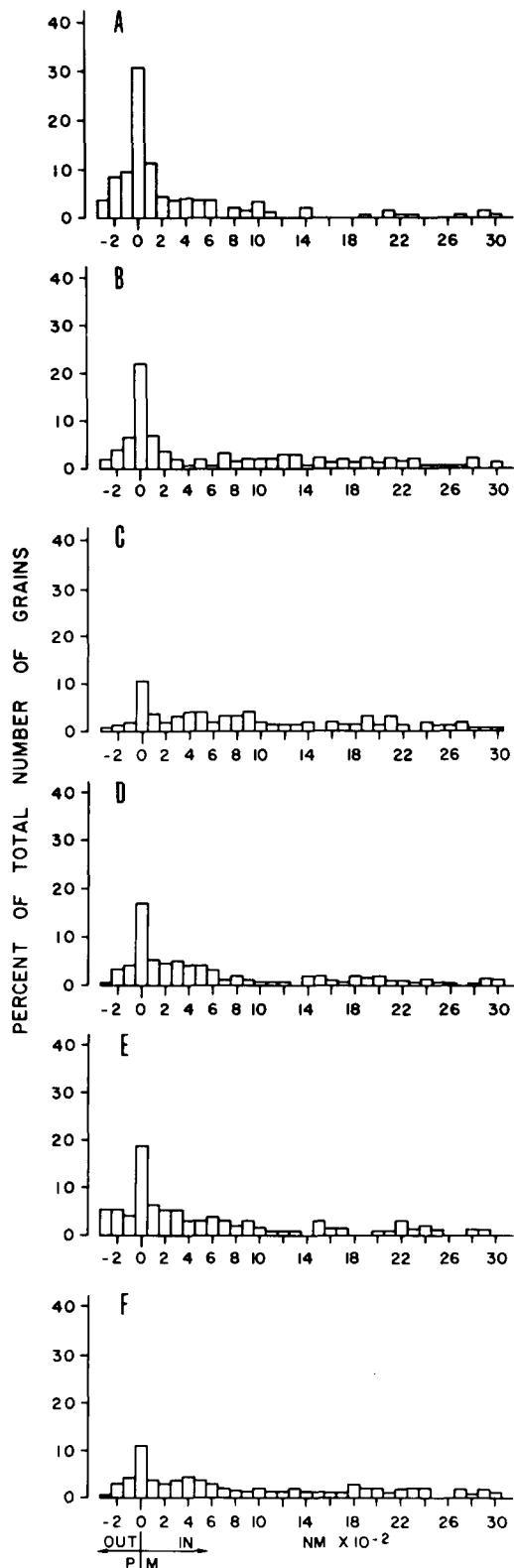


FIGURE 11 Grain distribution histogram of ^{125}I -hGH association with cultured human lymphocytes incubated in assay buffer at 37°C . Cells were incubated for 30 min at 37°C with ^{125}I -hGH (A), and radioactivity was allowed to dissociate for 30 and 120 min at 37°C after a 100-fold dilution of the cells (B and C). In addition, cells were incubated for 120 min (D), and radioactivity was allowed to dissociate for 30 min and 120 min at 37°C after a 100-fold dilution of the cells (E and F). In each histogram the percent of total grains is plotted as a function of the distance of the grain center to the plasma membrane. Number of grains analyzed at: (A) 30-min as-

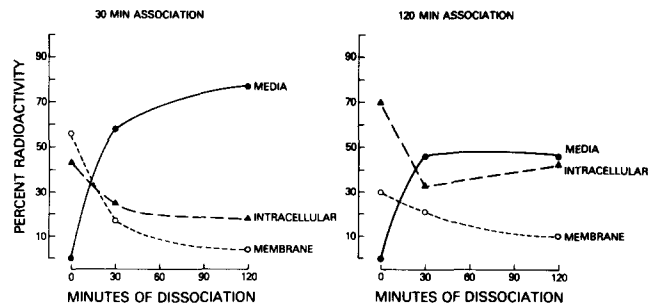


FIGURE 12 Distribution of radioactivity bound to cultured human lymphocytes. The data shown here were derived from Fig. 9 and 11 (left panel). ^{125}I -hGH was incubated for 30 min at 37°C in assay buffer. At the end of this incubation period, cells were washed, diluted 100-fold, and further incubated for 120 min at 37°C . The data shown represent the distribution of radioactivity in the above labeled compartments. 0 min = 30-min association, and minutes of dissociation refers to time after dilution. The right panel has exactly the same format as the left panel, except that cells were incubated for 120 min at 37°C before dilution. 0 min = 120 min of association, and minutes of dissociation refers to time after dilution.

polypeptide hormone from a cell. These studies are summarized in Fig. 12. Combining the biochemical studies with the radioautographic studies, it is possible to estimate both the nature and amount of labeled hGH in the incubation medium on the plasma membrane, and internalized by the cell. When cells are incubated for 30 min at 37°C with ^{125}I -hGH and washed, the radioactivity dissociates from the cell and accumulates in the incubation medium. Initially, most of the radioactivity represents intact hGH but, with time, most of the label consists of low molecular weight products (Figs. 10 and 12). Initially, a higher proportion of the membrane-associated radioactivity is lost, and this could happen through dissociation, internalization, or, more likely, simultaneous occurrence of both. Except for the zero time-point, the internalized labeled material represents more of the cell-associated radioactivity. Because after the first 30 min of dissociation most of the radioactivity appearing in the medium is degraded, it would appear that most of the membrane-bound ligand is being internalized and that the radioactivity appearing in the incubation medium is from the intracellular compartment.

After the longer incubation period of 120 min, another set of conditions exists (Fig. 12, right); over the first 30 min of dissociation the process is similar to that seen in the 30-min incubation (Fig. 12, left), except that most of the radioactivity is lost from the intracellular compartment. After the first 30 min of dissociation there is no further loss of radioactivity from the cell. In this situation, essentially all the radioactivity from the membrane must be internalized. What is most remarkable, however, is that not only is the membrane-bound material nondissociable from the cell, but so is the intracellular labeled material. Thus, irreversible binding initially shown for growth hormone association to hepatocytes (14) may reside at the level of the plasma membrane or at an intracellular site.

There are a number of implications of these data that suggest a physiological role for the internalization process. The degradative process described requires the presence of cells (i.e., there is no soluble degradative activity in the incubation me-

sociation, 166; (B) 30-min association + 30-min dissociation, 181; (C) 30-min association + 120-min dissociation, 149; (D) 120-min association, 202; (E) 120-min association + 30-min dissociation, 113; (F) 120-min association + 120-min dissociation, 167.

dium) and further appears to require binding of the ligand to the cell. Most, if not all, polypeptide hormones and growth factors are internalized by cells after binding (1, 4, 6, 8, 10, 13, 17-19, 22, 23, 31). Further, there is considerable morphological (1, 4, 10, 13, 21, 22, 24) and biochemical (3, 7, 20, 24, 27, 29, 34, 35, 37-39; see also footnote 1) evidence that the internalized ligand is localized in and degraded by lysosomes.

In the system described here and in other systems where continuous binding of the labeled hormone occurs, most of the cell-associated radioactivity represents intact hormone (7, 10). However, when further binding is prevented, cell-associated radioactivity is rapidly degraded. Thus, intact ligand is constantly being internalized and processed. We have suggested that this is primarily a degradative pathway, but other investigators have suggested that intracellular receptors may be involved in hormone action (6, 17). Szego et al (36, 37) have suggested that the lysosome may contain information that can be expressed as a component of estrogenic action in certain tissues. Our data provide no new information on the possible role of intracellular receptors or lysosomes in hormone action.

The process of hormone-induced receptor loss represents a state in which exposure of the cell to the hormone, under certain conditions, results in a functional loss of receptors. This process must involve mechanisms whereby receptors are inserted into the membrane as well as mechanisms whereby receptors are lost from the membrane. It has been demonstrated that both insulin and hGH will induce the loss of their specific cell surface receptors on cultured human lymphocytes (17, 25). For insulin to induce the loss of its receptor in the cultured lymphocyte, high receptor occupancy is required. It is of note that the extent of internalization of insulin by the cell is small and that all of the ligand is dissociable from the cell, regardless of the time of association (8). By contrast, lower occupancy of hGH receptor is necessary to induce receptor loss, more of the ligand is internalized, and less is dissociable as a function of association time.

Our data suggest at least three mechanisms that may act singly or in concert to control cell surface receptor concentration: (a) irreversible binding to the plasma membrane, (b) internalization, and (c) irreversible association with the intracellular compartment.

Finally, recent studies have suggested that antibodies bound to plasma membrane components may be used as direct probes of membrane recycling (32, 33). Similarly, the hormone-cell interaction may provide a useful model for studying directly the turnover of plasma membrane and its components.

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A preliminary report of these studies has appeared in abstract form (Gorden et al. 1980. *Clin. Res.* 28:556A).

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¹ Preliminary studies indicate that the lysosomal stabilizing agent NH₄Cl inhibits the degradation of ¹²⁵I-hGH in cultured human lymphocytes (N. Hizuka, M. A. Lesniak, and P. Gorden, manuscript submitted for publication).

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