Receptor-mediated Endocytosis of Epidermal Growth Factor by Hepatocytes in the Perfused Rat Liver: Ligand and Receptor Dynamics

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ABSTRACT We have used biochemical and morphological techniques to demonstrate that hepatocytes in the perfused liver bind, internalize, and degrade substantial amounts of murine epidermal growth factor (EGF) via a receptor-mediated process. Before ligand exposure, about 300,000 high-affinity receptors were detectable per cell, displayed no latency, and co-distributed with conventional plasma membrane markers. Cytochemical localization using EGF coupled to horseradish peroxidase (EGF-HRP) revealed that the receptors were distributed along the entire sinusoidal and lateral surfaces of hepatocytes. When saturating concentrations of EGF were perfused through a liver at 35°C, ligand clearance was biphasic with a rapid primary phase of 20,000 molecules/min per cell that dramatically changed at 15-20 min to a slower secondary phase of 2,500 molecules/min per cell. During the primary phase of uptake, approximately 250,000 molecules of EGF and 80% of the total functional receptors were internalized into endocytic vesicles which could be separated from enzyme markers for plasma membranes and lysosomes on sucrose gradients. The ligand pathway was visualized cytochemically 2-25 min after EGF-HRP internalization and a rapid transport from endosomes at the periphery to those in the Golgi apparatus-lysosome region was observed (t,9 = 7 min). However, no 125I-EGF degradation was detected for at least 20 min. Within 30 min after EGF addition, a steady state was reached which lasted up to 4 h such that (a) the rate of EGF clearance equaled the rate of ligand degradation (2,500 molecules/min per cell); (b) a constant pool of undegraded ligand was maintained in endosomes; and (c) the number of accessible (i.e., cell surface) receptors remained constant at 20% of initial values. By 4 h hepatocytes had internalized and degraded 3 and 2.3 times more EGF, respectively, than the initial number of available receptors, even in the presence of cycloheximide and without substantial loss of receptors. All of these results suggest that EGF receptors are internalized and that their rate of recycling to the surface from intracellular sites is governed by the rate of entry of ligand and/or receptor into lysosomes.

Receptor-mediated endocytosis (REM) is well-recognized as a general mechanism used by many cells to take up biologically important molecules. The variety of molecules taken up by REM include polypeptide hormones (e.g., epidermal growth factor [EGF] and insulin), transport proteins carrying nutritional and regulatory substances (e.g., low density-lipoprotein (LDL)-cholesterol, transcobalamine II-vitamin B12), lysosomal enzymes, asialoglycoproteins (ASGP), and immunoglobulins (reviewed in reference 1). The destinations of these interiorized molecules vary from degradation in the lysosomal system with release of amino acids and monosaccharides (e.g., ASGP [2]) or of regulatory molecules (e.g., LDL...
and cholesterol [1] to intracellular sequestration in an 
dergraded form (e.g., yolk proteins [3]) to transport across the 
cytosol and subsequent exocytosis (e.g., immunoglobulins 
[3, 4]). The fates of the surface receptors interacting with 
specific ligands also vary from apparent reutilization during 
delay periods of activity (e.g., LDL and ASGP receptors 
[5, 6]) to loss of receptors after an initial wave of RME and 
subsequent desensitization of cells (e.g., hormonal “down-
regulation” of EGF [7–11] or insulin [12, 13]). Thus, it is 

clear that eucaryotic cells carry out diverse functions that rely 
on the selective process of RME.

The principle cell in the liver, the parenchymal cell or 
hepatocyte, takes up many different molecules from the cir-
culation via RME. These include ASGPs (2, 14), insulin (15), 
glucagon (16), prolactin (17), EGF (9, 18), transferrin II 
(19), haptoglobin-hemoglobin complexes (20), hemopexin-
glucon (21), glycoproteins with oligosaccharide chains terminat-
ing in α1,3 fucos residues (22), and immunoglobulin A (4).

All of these molecules are internalized via receptor-mediated 
processes occurring at the sinusoidal surface of the hepatocyte. 
In addition, the fates of the ligands and of their receptors 
appear to encompass all currently established fates in RME.

Therefore, the hepatocyte is particularly suitable for a com-
parative study of selected ligand–receptor systems, each hav-
ing a different fate.

In recent years, we have been studying various aspects of 
ASGP endocytosis by hepatocytes (14, 23–26). This RME 
system represents one in which the ligand is ultimately deliv-
ered to and degraded within lysosomes but the receptor is 
reused. Hepatocytes also endocytose and degrade substantial 
amounts of EGF from the circulation but, unlike the ASGP 
receptor, they appear to down-regulate the EGF receptor.

There is some evidence in cultured cells that the EGF receptor 
is delivered to lysosomes and degraded together with the 
ligand. However, no correlation has yet been made between 
the amount of EGF processed by these cells and the number of 
receptors lost.

Using the perfused liver, we have examined the kinetics of 
EGF uptake and degradation and the pathway of ligand entry 
from the cell surface to the lysosomes, both morphologically 
and biochemically. We have quantitated the amount of EGF 
processed by these cells and the number of receptors lost.

The procedure of Sell et al. (33) with modifications was used to couple the 
N-terminal amino group of EGF (containing trace amounts of 125I-EGF) to 
the carbohydrate portion of HRP. Uncoupled EGF was removed from the mixture 
by dialysis (Amicon PM-10 Amicon Corp., Danvers, MA). Using this proce-

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Preparation and Iodination of EGF

EGF was purified from male mouse submaxillary glands (Pel-Freeze, Rogers, 
AR) as described by Savage and Cohen (28). Protein concentrations were 
calculated from the absorbance at 280 nm using an extinction coefficient at 10 
mg/ml of 30.9 (29). EGF was iodinated by the chloramine T method (30) with 
resulting specific radioactivities of 20–150,000 cpm/ng.

Isolated Perfused Liver System

Livers from 24-h fasted rats (150–250 g) were surgically removed and 
perfused in a recycling system with a balanced salt solution supplemented with 
2% polyvinyl pyrrolidone-40 (26) and oxygenated with 95% O2/5% CO2.

Surface Binding of EGF: We quantitated exposed EGF receptors by 
recycling a saturating dose of 125I-EGF (4.8–9.6 × 10^6 cpm/ml) at 4°C and at 
periodic intervals removing 0.2–0.5 ml samples from the perfusing medium for 
determination of the radioactive content. After 90–120 min, the bound 125I-
EGF was dissociated by addition of 5 ml of 1 M Na acetate pH 3.5, which 
lowered the medium pH to 5.0. The amount of EGF bound was quantitated 
either from the percent of radioactivity cleared from the perfusate, from the 
radioactivity present in a liver homogenate before acid treatment, or from the 
amount of bound radioactivity released when the perfusate was acidified. 
All three measurements yielded similar results. EGF binding capacity was expressed 
as micrograms of EGF bound per gram wet weight of liver and converted to 
receptors per hepatocyte using 1.38 × 10^8 hepatocytes/g wet wt of liver (31) and 
assuming a 1:1 interaction for the ligand–receptor complex.

Endocytosis and Metabolism of EGF: 10–25 μg (15–38 nm) of 
125I-EGF was recirculated at 37°C for 4–240 min, and at timed intervals acid-
soluble and insoluble radioactive was determined in 0.2–0.5 ml aliquots of 
perfusate (see below). After terminating the uptake by cooling the liver to 4°C, 
we removed surface-bound EGF by acidifying the perfusing medium as de-
scribed above and subsequently re-equilibrating the liver with cold, fresh 
perfusion. For subcellular fractionations, the liver was disconnected from the 
perfusion system before acidification, weighed, and homogenized. In vitro 
binding assays were performed on liver biopsies (0.3–0.5 g) excised before 
liver addition and after removal of surface-bound ligand.

In Vitro EGF Binding Assay

EGF binding was measured in vitro (32) on aliquots of liver homogenates 
(1.5–2 mg protein), subcellular fractions (1–2 mg protein), and fractions 
obtained from sucrose gradients. Samples were incubated with saturating doses 
(80 nM) of 125I-EGF (20–100,000 cpm/mg) for 60 min at 4°C in a total volume 
of 0.2 ml that contained 20 mM HEPES, pH 7.4, and 0.5% BSA. Since 
EGF binding was measured in vitro (32), these assay conditions 
represent steady-state. Binding was terminated by the sequential addition of 
0.5 ml of 0.1% bovine IgG in 0.1 M phosphate buffer, pH 7.4, and 0.5 ml of 
25% polyethylene glycol. The precipitated EGF-receptor complex was collected 
dergent free under gentle vacuum on Whatman GF/C filters (Whatman Laboratory Prod-
ucts Inc., Clifton, NJ). The filters were then washed with 15 ml of 10% polyethylene glycol in 0.1 M phosphate 
buffer, pH 7.4, and the radioactivity retained on the filters measured. The 
difference between 125I-EGF binding in the absence and presence of a 50-fold 
xcess of unlabeled EGF was taken as specific binding. Nonspecific 
binding ranged between 10 and 20% of total radioactivity and was <1% of 
the total radioactivity added to the incubation. With liver homogenates, 
binding capacity was expressed as micrograms of EGF bound per gram of 
liver or per milligram of protein. The number of receptors per cell was determined 
as described above or by using a calculated conversion factor of 1 × 10^8 
hepatocytes/mg protein based on the wet weight of unperfused livers.

The difference between specific 125I-EGF-binding sites measured in the 
presence (total) and absence (access) of detergent was defined as latent EGF 
receptors. As described in Results, Brij 35 was the most effective detergent in 
expressing latent receptors (detectable only after exposure of livers to EGF, see 
Results). Detergent was added along with the ligand and at several concen-
trations in order to obtain maximal EGF binding. A concentration of 0.2% (wt/ 
w) was sufficient for sucrose gradient fractions, while a range of 0.4–0.7% (wt/ 
w) was necessary when homogenates or subcellular fractions were assayed.

Preparation and Characterization of HRP 
Conjugated to EGF (EGF-HRP)

Horseradish peroxidase (HRP, type VI), 3-aminolauryl-2,4,6-triazole, 3,3'-di-
niminobenzidine (grade II), polyethylene glycol (8,000 mol wt), and mannan 
were purchased from Sigma Chemical Co. (St. Louis, MO); HEPES was from 
Research Organics Inc. (Cleveland, OH); Brij 35 was from Pierce Chemical Co. 
(Rockford, IL); BSA, fraction V, was from Miles Laboratories Inc. (Elkhart, 
IN); and ultra-pure sucrose was obtained from Schwarz/Mann Inc. (Spring 
Valley, NY). Carrier-free Na125I and uridine diporphospho-o-[6-14C]glucolactose 
were obtained from Amersham Corp. (Arlington Heights, IL); Male rats (CD strain) 
were supplied by Charles River (Wilmington, MA). All other reagents and 
compounds were of the highest purity available and were purchased from 
commercial sources.

Materials and Methods

Materials

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N-terminal amino group of EGF (containing trace amounts of 125I-EGF) to 
the carbohydrate portion of HRP. Uncoupled EGF was removed from the mixture 
by dialysis (Amicon PM-10 Amicon Corp., Danvers, MA). Using this proce-

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dure, 22% of the added 125I-EGF was coupled to the HRP, and the final product retained at least 50% of the initial peroxidase activity (34). Analysis on SDS polyacrylamide gels revealed that 82% of the radiolabel migrated with an apparent molecular weight of 45,000 which was coincident with the major stained band and similar in electrophoretic mobility to the uncoupled HRP. The remaining radioactivity was distributed between a polypeptide at 90 kdaltons (14%), which presumably represented HRP dimers plus at least one EGF, and uncoupled EGF at 6 kdaltons (4%).

125I-EGF-HP binding measured in vitro with liver homogenates was specific and had an apparent dissociation constant of 15 nM, which is within the range determined for unconjugated 125I-EGF (Fig. 1). Inclusion of mannan had no effect, indicating that there was no binding in homogenates via the HRP moiety.

**Localization of EGF-HRP in the Perfused Liver and Quantitation of HRP-containing Structures**

EGF-HRP (4 μg of EGF) was perfused through a liver at 4°C for 120 min. 5 mg of mannan was included as a precaution to inhibit binding of the conjugate via the HRP moiety to mannosyl/N-acetyl-galactosamine receptors on nonparenchymal cells (14). Unbound ligand was removed and the liver was either immediately fixed by perfusion with 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, or warmed to >30°C for 2-25 min and then fixed. Peroxidase activity in the fixed liver was visualized using the method of Wall et al. (25). The presence of EGF-HRP in specific regions of the hepatocyte cytoplasm (as defined in reference 25) was quantitated either at the microscope or on electron micrographs by counting the number of structures (regardless of size) containing HRP reaction product.

**Subcellular Fractionation of Liver Homogenates**

Livers were weighed and homogenized in 5 vol of 0.25 M sucrose in 3 mM imidazole buffer, pH 7.4 (SI), and the following fractions were prepared (Beckman centrifuge, 60 Ti rotor, Beckman Instruments Inc., Palo Alto, CA): a 12,000 g (10 min) pellet (12-K fraction), a 145,000 g (90 min) pellet (microsomal fraction), and a 145,000 g (90 min) supernate (cytosolic fraction) (35). The 12-K pellet was resuspended in SI (5 ml/g liver) using a loose Dounce homogenizer and 5 ml (65-85 mg of protein) was applied to the top of a linear 1.11-1.25 g/cc sucrose gradient (32 ml). The microsomal pellet was resuspended in 15 ml of SI with a Potter-Elvejhem homogenizer, and 5 ml (50-60 mg of protein) was applied to a 1.06-1.20 g/cc linear sucrose gradient (32 ml) that was made above a 4 ml cushion of 1.22 g/cc sucrose. After centrifugation of both gradients for 12-15 h at 83,000g, -inan SW28 Beckman rotor, fractionation of both gradients for 12-15 h at 83,000g was further analyzed by column chromatography. Sephadex G-25 medium (PD-10 columns, Pharmacia Inc., Piscataway, NJ) equilibrated with 1 M acetic acid was used to separate free iodide from iodotyrosine derivatives (36).

Protein was determined in triplicate following the procedure of Bradford (37) using BSA as the standard. 5'-nucleotidase (5'-NUC) was assayed by measuring the release of phosphate from adenosinemonophosphate (38). Alkaline phosphodiesterase (APDE) and β-N-acetyl-glucosaminidase (6-NAG) activities were determined following published procedures (38). Galactosyltransferase (Gal-Trans) was assayed by monitoring the incorporation of [6H]galactose into ovalbumin (39).

**Analytical Procedures**

Percusate and liver homogenates (0.3-0.5 ml) were precipitated with 10% trichloroacetic acid (TCA) in the presence of 0.2-1% BSA at 4°C for 60 min. The samples were centrifuged (3,000 rpm for 15 min), supernate and pellet were separated, and the radioactivity in each was determined in a Beckman Gamma 4000 counter. Acid-soluble radiolabeled products released by the liver into the perfusion medium were further analyzed by column chromatography. Sephadex G-25 medium (PD-10 columns, Pharmacia Inc., Piscataway, NJ) equilibrated with 1 M acetic acid was used to separate free iodide from iodotyrosine derivatives (36).

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**RESULTS**

**Properties of EGF Binding**

We first examined EGF binding in the isolated perfused liver at 4°C (Fig. 1). Binding of EGF was both saturable and reversible. Saturation occurred at EGF concentrations of 6-8 nM and yielded a maximum of 150,000 receptors per hepatocyte (see below for cell type identification). The apparent dissociation constant was 1-2 nM. When a 50-fold excess of unlabeled EGF was added at 4°C, 90% of the 125I-EGF previously bound was displaced with a t1/2 of 60 min. Dissociation of bound ligand was also pH dependent and occurred rapidly (t1/2 = 2-3 min) in the absence of excess EGF or calcium ions. At pH 6, 50% of the prebound ligand was released, while 90% was removed at pH 4.5-5.5 (Fig. 2A). Therefore, acid treatment (pH 5.0) for 10 min at 4°C was used as a measure of surface-bound EGF. This treatment had no significant effect on cell morphology (data not shown) nor on subsequent EGF binding (Fig. 2B) or uptake.

We next characterized EGF binding in liver homogenates (Fig. 1) and found 300,000 high-affinity sites of KD = 8-15 nM by Scatchard analysis with no low-affinity sites (Fig. 1, inset). In addition, we found that 10-20% of the available binding sites in a liver homogenate were unstable to storage.
on ice for 4–6 h or to freeze/thaw. For this reason binding assays were performed, when possible, on fresh homogenates.

In an effort to assay active receptors in a latent (intracellular) compartment, various detergents were screened for their ability to maintain EGF binding in liver homogenates (Fig. 3). The following detergents at final concentrations of 0.2–0.6% resulted in an almost total inhibition of EGF binding in the liver homogenate and were considered inappropriate for measuring intracellular receptors: digitonin, Triton X-100, 2-deoxycholate, Lubrol WX, and 3-[3-cholamidopropyl] dimethylammonio]-1-propane-sulfonate (CHAPS). Only the nonionic detergent, Brij 35, at concentrations of 0.3–0.7%, had no effect on receptor number in homogenates of freshly-isolated livers, but decreased receptor affinity (Kd = 25 nM). The finding that Brij 35 did not increase the specific binding of 125I-EGF in freshly prepared liver homogenates, even at concentrations as low as 0.05%, suggested that all 300,000 receptors per cell were in an accessible compartment (i.e., at the cell surface).

**Cytochemical Localization of EGF in the Perfused Liver**

EGF covalently coupled to HRP (EGF-HRP) was used to visualize the distribution of EGF binding sites at the cell surface (i.e., 4°C binding) and to investigate the route of ligand entry. At 4°C peroxidase reaction product was distributed along microvilli of the sinusoidal front and the lateral surface membrane of hepatocytes with some concentration in coated pit regions (Fig. 4). Although we did observe EGF-HRP binding to nonparenchymal cells, the reaction product was light and variable. When a 50-fold molar excess of EGF was perfused with EGF-HRP, the conjugate was not bound (as detected by the 125I-EGF moiety) and no HRP reaction product was observed on parenchymal or nonparenchymal cells.

To investigate the route of ligand entry into the cell, EGF-HRP was first perfused through a liver at 4°C. Unbound ligand was then washed away and the medium was rapidly warmed to 35°C for 2, 4, or 10–25 min. The HRP reaction product was almost exclusively associated with hepatocytes, demonstrating that liver parenchymal cells were responsible for the endocytosis of EGF. The HRP-positive structures were characterized morphologically (Fig. 5) and quantitated at selected times after warming (Table I). At 2 min, HRP product was predominantly localized in structures similar in size (50–100 nm) and shape to coated pits and vesicles (80% of the positive structures counted) (Table I and Fig. 5a). However, clathrin coats were not visible under the conditions of fixation and HRP visualization. Reaction product was absent from both the microvilli and lateral surfaces. By 4 min, HRP-positive structures included larger vesicles and tubules (100–200 nm diam) in addition to coated pits and vesicles (Fig. 5, b and c and Table I). These larger structures, which accounted for ~50% of the positive vesicles observed, were located at the cell periphery and were morphologically identical to type I endosomes as previously described for the endocytosis of ASGPs (41, 42). In fact, when EGF-HRP was perfused in the presence of asialoorosomucoid adsorbed to colloidal gold, 60–75% of the HRP-positive vesicles contained the gold tracer (A. L. Hubbard and W. A. Dunn, unpublished results). After 10–25 min of perfusion at 35°C, 66–85% of the vesicles containing HRP product were located in the Golgi apparatus -lysosome region of the cell (Fig. 5, d–f). These vesicles included small and large vesicles and tubules (type II endosomes, see references 41 and 42) and structures containing either lipoproteins or small vesicles (type III endosomes,
FIGURE 4 Surface distribution and specificity of EGF-HRP binding in the perfused liver. The distribution of EGF-HRP binding at 4°C to the cell surface was visualized as described in Materials and Methods (a and b). The reaction product was localized along microvilli of the sinusoidal front (SF), in coated pits (arrowheads), and along lateral surfaces (LS) of hepatocytes. Endothelial cells lining the sinusoidal lumen (SL) were found unlabeled. (c) When a 50-fold excess of unlabeled EGF was perfused along with EGF-HRP, no labeling of the hepatocyte plasma membrane was observed. Bar, 0.5 μm. (a–c) × 25,000.

Kinetics and Extent of EGF Uptake

125I-EGF concentrations from 0.5–40 nM were continuously perfused through livers for up to 4 h and the disappearance of acid-insoluble radioactivity from the medium was monitored over time. The clearance curves of 125I-EGF at three representative concentrations are plotted in Fig. 6. At subsaturating levels (0.5 nM), >90% of the added 125I-EGF was cleared by 20 min. At intermediate and saturating EGF concentrations (5 and 20 nM) the clearance was biphasic, with a fast primary phase that abruptly changed at 15–20 min to a slower secondary phase. When the primary clearance phase (0–15 min) at these three concentrations was plotted semilogarithmically, straight lines were obtained (Fig. 6, inset). The rates of this initial uptake were calculated from the slopes and are presented in Fig. 7. From 0.06–1.5 μg EGF/g liver, the rate of uptake increased concentrations of ligand. A double-reciprocal plot of the data indicated that the half-maximal clearance rate was obtained at 5 nM EGF. A maximal primary rate of 20,000 molecules/cell per min occurred at ~1.5 μg EGF added per gram liver (15 nM) and remained unchanged up to 4.0 μg/g (40 nM).

When the added levels of EGF were ≥0.5 μg/g (5 nM), the primary uptake was followed by a slower secondary clearance that was linear from ~30 min to at least 240 min (Fig. 6). The rate of uptake increased with increasing ligand input to a maximal level of 2,500 molecules of EGF/min per cell at an EGF dose of ~1.4 μg/g liver (Fig. 7). This rate was 12–13% of that observed for the primary clearance and, as will be discussed below, equalled the rate of release of acid-soluble radioactivity from the liver. ²

The amount of ligand internalized (i.e., inaccessible to acid release) as a function of time was quantitated using saturating doses of EGF (>1.4 μg/g) and expressed on a per cell basis (Fig. 8). A rapid accumulation of 250,000 molecules/cell occurred during the first 20 min of EGF exposure. This was followed by a slower uptake of ligand which was linear for up to 4 h. The amount of EGF internalized at 4 h was three

² The secondary clearance phase was not due to EGF uptake by other cell types, since only hepatocytes possessed the ability to endocytose EGF. In addition, the secondary rate of uptake, although slower than the primary clearance, was rapid and saturable, two characteristics of receptor-mediated endocytosis, not fluid-phase pinocytosis (43). Finally, the difference between the primary and secondary rates of ligand uptake was not due to changes in EGF concentrations in the perfusate nor in receptor K₄ values, but the result of a decrease in hepatocyte cell surface receptors.
Figure 5  Intracellular localization of EGF-HRP in hepatocytes at 2 min (a), 4 min (b and c), and 10-25 min (d-f) after ligand internalization. An experimental protocol was used such that a single wave of EGF-HRP movement from the cell surface to the lysosomes could be visualized with time (see Materials and Methods). (a) 2 min after warming, EGF-HRP was near the plasma membrane in structures similar in dimensions to coated pits and vesicles (arrowheads). (b and c) By 4 min, HRP-positive structures included larger vesicles and tubules (type I endosomes, En I) in addition to coated pits and vesicles (arrowheads). (d-f) When the liver was warmed for 10-25 min, the HRP product was located in the Golgi-lysosome region of the cell in small and large vesicles and tubules designated endosomes type II (En II) and in structures resembling either lipoprotein-containing vesicles or multivesicular bodies, designated endosomes type III (En III). GO, Golgi apparatus; AV, autophagic vacuoles. Bar, 0.5 μm. (a-f) x 25,000.

times the total number of EGF binding sites in liver homogenates (Fig. 1). Cycloheximide (0.2-1 mM) at concentrations shown by others to inhibit protein synthesis in the perfused rat liver (44) had no effect on the amount of EGF internalized over a 4-h period.

Kinetics and Extent of EGF Degradation

No acid-soluble radioactivity appeared in the perfusate until 20 min, after which it increased linearly for up to 240 min (Fig. 6). Analysis by column chromatography revealed that the released radioactivity was free iodide. Since no significant accumulation of acid-soluble radioactivity was evident in the liver, we used the rate of increase of acid-soluble radioactivity in the perfusate to estimate the rate of hydrolysis of EGF by hepatocytes (Fig. 7).

As shown in Fig. 7, the rate of hydrolysis increased with EGF concentration up to ~1.4 μg/g liver (14 nM) and thereafter remained unchanged at a maximum of 2,500 molecules of EGF degraded/min per cell. By analyzing this data on a double-reciprocal plot (not shown), the EGF concentration necessary for a half-maximal degradative rate was 7 nM, which was comparable to that observed for the primary phase of uptake (i.e., 5 nM).

The maximal rate of release of acid-soluble radioactivity from the liver was identical to the maximal rate observed for the secondary phase of EGF uptake, 2,500 molecules/cell per min, suggesting establishment of a steady state. Indeed, as shown in Fig. 8, after 20 min of perfusion with saturating levels of EGF (>1.4 μg/g), a steady state was reached such that the amount of EGF internalized paralleled that which was degraded. The difference between the amount of EGF internalized and degraded remained constant from 20 to 240 min and represented an intracellular pool of ~200,000 ligand molecules/cell, which was comparable to the amount of EGF internalized in the initial uptake phase, from 0-20 min.

Finally, we measured the amount of EGF hydrolyzed under saturating conditions. These results are presented in Fig. 8. After the initial 20-min lag, the amounts of EGF degraded increased linearly to a total of 690,000 molecules/cell at 4 h. This represents 2.3 times more ligand than the initial number of total available receptors measured in the liver homogenate. In addition, the amount of EGF degraded within 4 h was unaltered when protein synthesis was inhibited by 0.2-1 mM cycloheximide.
When the primary rapid clearance phase at each concentration was reached and the acid-insoluble radioactivity (open symbols) was plotted and precipitated with TCA as described in Materials and Methods, the number of structures containing EGF-HRP in the indicated regions of the hepatocyte was determined without regard to size, and the percent distribution was calculated.

This category contains structures of size (50-100 nm) and shape similar to those of coated pits and vesicles. However, clathrin coats were not visible under the conditions of fixation and HRP visualization; therefore, some type I endosomes, En I, may have been included in this category.

Endosomes were classified as type I (peripheral vesicles and tubules <200 nm diam); type II, En II, (small vesicles and tubules in the Golgi-lysosome region; and type III, En III, (large, >200 nm diam) vesicles in the Golgi-lysosome region usually containing distinguishable smaller vesicles or other inclusions). Refer to Fig. 5.

The number of structures containing EGF-HRP in the indicated regions of the hepatocyte was determined without regard to size, and the percent distribution was calculated.

The rate of the primary clearance phase at each concentration was determined by multiplying the ligand input by the slope of a semilog plot of the acid-insoluble radioactivity in the perfusate at 0-15 min (Fig. 6, inset). The rates of the secondary clearance phase and of degradation were calculated from the specific radioactivities of the ligand and the slopes of the linear plots of the acid-insoluble and soluble radioactivity, respectively, in the perfusate from 30-240 min (Fig. 6).

as described in Materials and Methods. These results are presented in Fig. 8. Prior to EGF exposure the number of accessible and total receptors was the same, indicating the absence of a substantial intracellular pool. However, in the presence of EGF, a rapid decrease in accessible binding activity was observed which was coincident with an increase in latent binding. Total binding activity (accessible plus latent) remained constant for up to 2 h and then declined to 70% of initial values by 4 h.

Between 4 and 20 min after EGF addition, when ligand was being rapidly internalized but not yet degraded, there was a progressive and rapid loss of up to 80% of the accessible binding activity (Fig. 8). This decrease in accessible receptors coincided in time and amount with both an increase in latent binding and with the internalization of EGF. For example, when 250,000 molecules of EGF had been internalized per cell by 20 min, the number of accessible receptors decreased and that of latent receptors increased by approximately 200,000 per cell (65-70% of total).

At times longer than 20 min, when the rates of internalization and degradation of EGF had reached steady state, the number of accessible receptors remained constant at 50-60,000 receptors per cell, which represented 20% of the receptors initially accessible (Fig. 8). This 80% reduction coincided with a similar reduction in the rate of EGF uptake observed

**Table I**

| Time of warming to 31-32°C | Total structures counted | Peripheral Cytoplasm | Golgi-lysosome region |
|--------------------------|-------------------------|----------------------|----------------------|
| min                      |                         | En I                  | En II                | En III               |
|                          |                         | %                     | %                    | %                    |
| 2                        | 173                     | 81                    | 19                   | 5                    |
| 4                        | 527                     | 28                    | 49                   | 18 (23)              |
| 10                       | 539                     | 9                     | 25                   | 54 (66)              |
| 15                       | 459                     | 12                    | 16                   | 56 (72)              |
| 25                       | 344                     | 4                     | 11                   | 50 (85)              |

*The number of structures containing EGF-HRP in the indicated regions of the hepatocyte was determined without regard to size, and the percent distribution was calculated.

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The number of structures containing EGF-HRP in the indicated regions of the hepatocyte was determined without regard to size, and the percent distribution was calculated.

The rates of the secondary clearance phase and of degradation were calculated from the specific radioactivities of the ligand and the slopes of the linear plots of the acid-insoluble and soluble radioactivity, respectively, in the perfusate from 30-240 min (Fig. 6).

Fate of EGF Receptor during Endocytosis

At selected times after addition of saturating doses of EGF, livers were homogenized and homogenates were assayed for accessible (minus Brij 35) and latent receptors (plus Brij 35)

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EGF binding activities were determined in the three subcellular fractions of bound or sequestered EGF and of accessible and/or total EGF, isolated on continuous sucrose gradients. The distribution of bound or sequestered EGF and of accessible and/or total EGF binding activities were determined in the three subcellular fractions as well as in the sucrose gradients and compared with those of conventional enzyme markers for the plasma membrane (5'-NUC and APDE), Golgi apparatus (Gal-Trans), and lysosomes (β-NAG) (Figs. 9 and 10). Enzyme markers for plasma membrane and Golgi apparatus were equally distributed between the 12-K and microsomal fractions, while lysosomes were predominantly located in the 12-K fraction (80% of β-NAG activity). Less than 5% of any of the enzyme activities remained in the cytosolic fraction.

**Subcellular Distribution of EGF and Its Receptor**

Livers were perfused with [125I]-EGF either at 4°C for 90 min (to label the cell surface), at 35°C for 10-20 min when ligand degradation was not yet evident (to label the contents of endosomes I-III), or at 35°C for 120 min when ligand degradation was occurring (to possibly label the contents of lysosomes). Livers were then homogenized and three fractions were prepared: material sedimenting at 12,000 g (the 12-K fraction) or at 145,000 g (the microsomal fraction), and material remaining in the 145,000 g supernatant (the cytosolic fraction). The 12-K and microsomal fractions were fractionated on continuous sucrose gradients. The distributions of bound or sequestered EGF and of accessible and/or total EGF binding activities were determined in the three subcellular fractions as well as in the sucrose gradients and compared with those of conventional enzyme markers for the plasma membrane (5'-NUC and APDE), Golgi apparatus (Gal-Trans), and lysosomes (β-NAG) (Figs. 9 and 10). Enzyme markers for plasma membrane and Golgi apparatus were equally distributed between the 12-K and microsomal fractions, while lysosomes were predominantly located in the 12-K fraction (80% of β-NAG activity). Less than 5% of any of the enzyme activities remained in the cytosolic fraction.

**DISTRIBUTION OF LIGAND AT 0-20 MIN AFTER EXPOSURE TO EGF:** When fractions from livers exposed to [125I]-EGF at 4°C (surface-bound) were examined, ~20% of the ligand did not sediment, suggesting release. However, the remaining 80% was equally divided between the two sedimentable fractions. As described in the morphology section, 10-25 min after exposure to EGF-HRP, many endosomes (II + III) containing ligand were located within the peribiliary region (Table I and Fig. 5). Fractionation of homogenates from comparable livers exposed to [125I]-EGF for 10-20 min revealed that 78-96% of the homogenate radioactivity was associated with vesicles that sedimented at 12,000 g (20-51%) or 145,000 g (45-58%). The equal distribution of bound or sequestered ligand between the 12-K and microsomal fractions was not coincident with β-NAG activities but did resemble that seen for Gal-Trans, 5'-NUC, and APDE activities.

The heterogeneous composition of the two sedimentable fractions prompted us to resolve the different subpopulations (i.e., plasma membrane, lysosomes, Golgi apparatus, and endosomes) by isopycnic centrifugation on sucrose gradients (Figs. 9 and 10). The distribution of surface-bound EGF (i.e., 90 min, 4°C) was examined first. [125I]-EGF that sedimented with the microsomal fraction equilibrated as a single band on sucrose gradients at a peak density of 1.15 g/ml (Fig. 10). This
FIGURE 9 Distribution on sucrose gradients of sequestered EGF and of EGF receptors in the 12-K fraction. Livers were either perfused with saline (0 min, control) or perfused at 37°C with 20 nM 125I-EGF for 10–20 min or 120 min. 12-K fractions were obtained and further fractionated on continuous sucrose gradients as described in Materials and Methods. EGF-binding activity in 0.1-ml aliquots was measured in the absence or presence of detergent. Owing to the lower protein content in these samples as compared with liver homogenates, 0.2% Brij 35 was sufficient for measuring maximal EGF binding.

FIGURE 10 Distribution on sucrose gradients of sequestered EGF and of EGF receptors in the microsomal fraction. Livers were either perfused with saline (0 min, control), perfused at 4°C with 8 nM 125I-EGF for 90 min (0 min), or perfused at 37°C with 20 nM 125I-EGF for 10–20 min or 120 min. Microsomal fractions were obtained and further fractionated on continuous sucrose gradients as described in Materials and Methods. EGF-binding activity in 0.1-ml aliquots was measured in the absence or presence of 0.2% Brij 35.

peak was coincident with that of the plasma membrane markers, 5'-NUC and APDE (latter enzyme not shown). We then examined the behavior of EGF-containing vesicles or endosomes (i.e., 10–20 min, 37°C) on sucrose gradients. 90% of the radioactivity applied entered the gradients and none coincided with the lysosomes (1.18–1.25 g/cc) (Figs. 9 and 10). Only ~20% of the radioactivity distributed at densities corresponding to the plasma membrane (1.14–1.17 g/cc, Figs. 9 and 10), which was consistent with the amount of residual 125I-EGF bound to the surface. At least 70% of the ligand-containing vesicles migrated at densities between 1.08 and 1.13 g/cc. These vesicles were well-separated from the bulk of the protein (80%) and from at least 70% of the 5'-NUC and APDE activities. The behavior of Gal-Trans activity was similar but not identical to that of endosomes. That is, Gal-Trans distributed at higher densities (1.11–1.16 g/cc) than those seen for the 125I-EGF-labeled endosomes. Although still contaminated with other organelles, a 10–20-fold purification of the endosomes was accomplished with this procedure.

DISTRIBUTION OF LIGAND AT LATER TIMES: When the subcellular distribution of sequestered 125I-EGF from livers exposed to the ligand for 120 min was examined, 30% of the homogenate radioactivity sedimented at 12,000 g and 48% at 145,000 g. Subfractionation on sucrose gradients revealed that the bulk of the sequestered 125I-EGF at these later times was still in endosomes, not in lysosomes. Of the radioactivity present in the 12-K fractions, 40% equilibrated at densities of 1.08–1.13 g/cc, 29% at 1.14–1.17 g/cc (i.e., plasma membrane), and only 21% (6% of liver radioactivity)
The increased level of EGF present in this latter fraction was probably due to breakage during homogenization at 1.18–1.25 g/cc (i.e., lysosomes). The $^{125}$I-EGF in the microsomal fraction was distributed between the endosome-rich peak (70%) and the plasma membrane peak (20%).

**Subcellular Distribution of EGF-Binding Activity Before and After EGF Exposure:** We first examined EGF receptor distribution in livers that had not been exposed to ligand. 85–90% of the EGF receptor was sedimentable and accessible to ligand binding (i.e., EGF binding ± detergent was the same). The membrane-associated receptor was equally distributed between the 12-K and microsomal fractions. Analysis of these fractions on sucrose gradients (Figs. 9 and 10) revealed in both cases that EGF-binding activity coincided with the plasma membrane marker enzymes (5'-Nuc and APDE). Over 80% of the total binding activity applied to the sucrose gradient could be recovered and only 5% of this activity failed to enter the gradient.

After exposure of livers to EGF for only 10–20 min, as much as 80% of the total receptors were localized to a latent pool (Fig. 8). When total EGF binding (i.e., nonlatent and latent) was measured in fractions from liver homogenates prepared 10–20 or 120 min after EGF addition, 20–50% sedimanted at 12,000 g, 45–58% sedimented at 145,000 g, and <5% was nonsedimentable. When the 12-K and microsomal fractions were analyzed on sucrose gradients, we found that the receptors had shifted to a lighter density (1.08–1.13 g/cc, Figs. 9 and 10). As much as 70% of the total EGF-binding activity recovered now co-localized with the EGF-containing vesicles, not with the plasma membrane. Of the receptors that equilibrated in this region of the gradient (i.e., endosomes), 40–80% displayed latency. In contrast, the bulk of the accessible receptors (20% of the total receptors) equilibrated with the plasma membrane markers and presumably represented those receptors still at the cell surface.

At 120 min but not 10–20 min after EGF addition, a small fraction of the EGF-binding activity was detected at densities corresponding to lysosomes (1.18–1.25 g/cc, Fig. 9). However, due to the limitations of the binding assay, samples with a low capacity to bind EGF (<3 ng EGF bound/ml) were difficult to measure accurately. For this reason the presence of EGF receptors in the lysosomes could not be demonstrated conclusively.

**Effects of Leupeptin on the Metabolism of $^{125}$I-EGF**

We found little EGF accumulating in hepatic lysosomes at any time despite extensive hydrolysis of the ligand. Although a similar result for asialofetuin has been reported (23), indicating a very efficient lysosomal system in hepatocytes, we wanted to determine whether lysosomes were responsible for the degradation of EGF. Therefore, we used leupeptin, a proteinase inhibitor that has been shown to inhibit the degradation of asialofetuin resulting in its accumulation in lysosomes (23). We found that 0.4 mM leupeptin had no effect on EGF uptake but inhibited the rate of release of acid-soluble radioactivity by as much as 80%. In the presence of leupeptin, there were 340,000 molecules of EGF per hepatocyte at 2 h and 440,000 molecules per hepatocyte at 4 h, as compared with 200,000 molecules in the untreated cell at steady-state. Subcellular fractionation of leupeptin-treated livers revealed an increase in EGF content in the 12-K and cytosolic fractions (Table II). The increased level of EGF present in this latter fraction was probably due to breakage during homogenization of the enlarged lysosomes in leupeptin-treated livers (45).

Analysis of the 12-K fraction on a sucrose gradient revealed that 50% of the $^{125}$I-EGF migrated in the density range of lysosomes (1.18–1.25 g/cc, Fig. 9). Furthermore, the amount of $^{125}$I-EGF co-migrating with lysosomes in leupeptin-treated livers was increased sevenfold over that of controls (Table II).

**DISCUSSION**

In this study, we have demonstrated that rat hepatocytes possess many EGF receptors that mediate the endocytosis of substantial amounts of circulating EGF. We found that EGF is internalized and transported to lysosomes via the same pathway as that used by other ligands destined for hydrolysis in hepatocytes. Concomitant with the initial wave of EGF internalization there is the redistribution of an equivalent number of receptors from the cell surface to endosomes but apparently not to lysosomes. However, unlike the ligand, which is degraded, our results suggest that a majority of the internalized receptors are reutilized. Furthermore, indirect evidence presented here suggests that at steady state the rates of uptake and degradation of EGF are dependent on the rate of receptor insertion into the plasma membrane and the rate of endosome/lysosome fusion, respectively.

**Exposure to EGF Induces a Rapid Disappearance of Cell Surface EGF Receptors**

We measured 150,000 receptors per cell in the perfused liver and 300,000 receptors per cell in vitro. At present we don't understand the basis for this two-fold difference, but we believe, for several reasons, that the increased number found in liver homogenates represents receptors at the plasma membrane and not those in an intracellular pool. First, the binding in naive cells displays no latency, whereas we can detect latent receptors in cells actively endocytosing EGF. Second, all the binding activity in naive cells migrates on sucrose gradients with conventional plasma membrane markers, not internal...
membrane markers. Finally, the initial rapid wave of EGF clearance results in the uptake of about 300,000 ligand molecules, suggesting that all the receptors detected in vitro (300,000/cell) are in a functionally equivalent pool or site.

Upon addition of EGF to a perfused liver, we observed a rapid and dramatic disappearance of cell surface EGF receptors that closely paralleled the internalization of ligand in both time and extent. The number of surface receptors decreased by 80% within 20 min and remained at this low level for up to 4 h. Furthermore, the observed down-regulation of EGF receptors at the surface coincided with the appearance of an internal pool of receptors having the biochemical characteristics of endosomes. Thus, one wave of EGF internalization was sufficient to induce a massive redistribution of receptors and to establish a new equilibrium such that only 20% of the EGF receptors remained at the cell surface and 80% were within endosomes.

Receptor down-regulation has been described previously for EGF as well as for hormones (e.g., insulin [12, 13]) in hepatocytes and other cells (7–11). However, the mechanism(s) of EGF receptor down-regulation (redistribution and/or degradation) have not yet been resolved. Das and Fox (8) and Krupp et al. (10) concluded that receptor degradation accounted for down-regulation in 3T3 and A431 cells, respectively. In contrast, Wiley and Cunningham (11) used a mathematical modeling approach to analyze EGF endocytosis at steady state in 3T3 cells and concluded that down-regulation was the result of an increased rate of receptor internalization. We were able to follow the EGF receptor into the cell and demonstrate that down-regulation in hepatocytes was due to a rapid and substantial redistribution, not degradation, of receptors.

Pre-existing EGF Receptors Are Reutilized

We found that hepatocytes were able to internalize and degrade EGF well in excess of total functional receptors. Since this occurred in the absence of new receptor synthesis, reutilization of pre-existing receptors must have occurred. Receptor reutilization has been implicated in the endocytosis of several other ligands, such as α2-macroglobulin–protease complexes and mannose glycoconjugates in alveolar macrophages (46, 47), LDLs lipoproteins in fibroblasts (1, 5), and ASGPs and insulin in liver (6, 48). However, the evidence that receptors actually disappear from the cell surface in the process of internalizing ligand and then reappear (i.e., recycle) for further rounds of internalization is still indirect. Our observations that surface EGF receptors are initially internalized into vesicles similar to endosomes and continue to be found in these vesicles during the steady state that follows, together with our evidence for reutilization, suggest strongly that EGF receptors are recycled between surface and intracellular sites.

The processes required for return of internalized receptors to the plasma membrane are (a) dissociation of the ligand–receptor complex; (b) separation of the receptor from ligand-containing vesicles; (c) transport to the plasma membrane; and (d) insertion. We have shown that dissociation of the EGF receptor complex can occur at low pH (Fig. 2A), an environment that exists in endosomes of other cells (49, 50) and lysosomes of liver (51). The observation of Fitzgerald et al. (52) that EGF was released into the cytosol when adenovirus induced endosome lysis suggests that the ligand had dissociated from its receptor while in a prelysosomal compartment. In contrast, on the basis of a close association of internalized EGF-ferritin with the membrane, McKanna et al. (53) suggested that EGF remained bound to its receptor until entry into lysosomes. We have found that the amount of EGF internalized by hepatocytes at 16°C, a temperature that inhibits endosome–lysosome fusion in liver (24), is limited and equals the total complement of EGF receptors, that surface receptors are depleted by >90% at 16°C, and that an equivalent number of latent receptors appears in endosomes at this temperature (W. A. Dunn and A. L. Hubbard, unpublished observations). These results suggest that entry of the EGF receptor into lysosomes (whether bound to ligand or free) may be a prerequisite for recycling. Furthermore, the existence of large amounts of undegraded EGF, apparently together with receptor in endosomes at steady state at 37°C, again suggests that entry into lysosomes may be the rate-limiting step for both EGF degradation and for return of receptor to the plasma membrane. An alternate possibility which might account for the excess of EGF internalized over receptors measured would be activation of previously inactive receptors in response to EGF exposure. However, until immunological reagents are obtained and the existence of an inactive pool of receptors in hepatocytes can be evaluated, we believe receptor reutilization and recycling adequately explain our data.

After the initial wave of receptor and EGF internalization, we found that the number of surface receptors in hepatocytes remained constant despite continued EGF endocytosis. Thus, the rate of insertion of recycling receptors from the internal pool must have equaled the rate of EGF uptake, which was 2,500 molecules/min per cell. Since the clearance rate of EGF at steady state appeared to be zero-order, either insertion of unoccupied receptor or internalization of the ligand–receptor complex must have been rate limiting, not binding of EGF to receptor, at least at the saturating EGF concentrations we used. Wiley and Cunningham (11) estimated that 320 EGF receptors were inserted into the plasma membrane per min per cell at steady state in fibroblasts, but the origin of these receptors (recycled vs. newly synthesized) was not determined. From our studies with the perfused liver we conclude that receptor recycling is occurring during the continual uptake of EGF and propose that the rate of ligand clearance at steady state is governed by the rate of insertion of receptors into the plasma membrane. Furthermore, this rate must be 2,500/min per cell, a value 10 times higher than that calculated for EGF receptor insertion in fibroblasts (11).

Degradation of EGF by Hepatocytes

What is the evidence that EGF is degraded within lysosomes? Since this ligand was hydrolyzed rapidly by hepatocytes once it reached a degradative compartment, it was difficult to detect significant amounts of intact or even partially degraded 125I-EGF within these structures. Similar observations were reported for asialofetuin (23). However, we found that leupeptin, a potent inhibitor of lysosomal cathepsins B and L (54), inhibited EGF degradation by 80% resulting in the accumulation of ligand in lysosomes. In addition, other studies have reported EGF entry into structures identified morphologically as lysosomes (18, 53). Thus, we believe EGF hydrolysis occurs within lysosomes subsequent to their fusion with endosomes.

Our cytochemical experiments with EGF-HRP revealed that ligand was transported rapidly from the surface to endosomes in the Golgi apparatus–lysosome region of hepatocytes.
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