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Hepatic sequestration and biliary secretion of epidermal growth factor: Evidence for a high-capacity uptake system

(hepatocytes/lobular concentration gradient/urogastrone/receptors/autoradiography)

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ABSTRACT **Epidermal growth factor (EGF) promotes hepatocyte growth and is bound in the liver by specific receptors. We have determined hepatic uptake of EGF in intact rats after an intravenous or intraportal injection of a bolus of ^{125}I-labeled EGF. Ninety-nine percent of the intraportal dose was taken up by the liver in 3 min, whereas only 58% of the intravenous dose appeared in the liver in 10 min. Uptake was inhibited by simultaneous treatment with an excess of unlabeled EGF. At time zero, uptake appeared to be complete. Disappearance from the liver followed first-order kinetics. Within 90 min of an intraportal injection, an average of 19% of the injected radioisotope appeared in bile, of which approximately one-fifth was shown to be immunoprecipitable with a specific anti-EGF antiserum. Light microscopic autoradiography demonstrated a very steep portal-to-central lobular concentration gradient consistent with a high-capacity uptake system. After intraportal injection or after incubation with cultured hepatocytes, labeled EGF was shown to be bound to its hepatic receptors. The main receptor–ligand complex had a M_r of ≈160,000–170,000, determined by NaDodSO$_4$/polyacrylamide gel electrophoresis.**

Epidermal growth factor (EGF), a single-chain polypeptide exhibiting a M_r of 6,045, is found in high concentrations in salivary and Brunner glands of humans and mice and more recently in the pituitary of goats (1–5). Although EGF has been shown to have a growth-promoting capacity in a variety of *in vivo* and *in vitro* cell systems (3, 6–14), its physiologic role and the mechanisms of homeostasis responsible for maintaining its plasma concentration are poorly understood. In adult rat hepatocytes *in vivo* or in primary culture, EGF stimulates DNA synthesis (15) and has been reported to induce hepatic hypertrophy and hyperplasia (16). In newborn rats, EGF induces thymidine incorporation into developing liver cells and enhances mitosis (15, 17). EGF receptors have been found on hepatocyte membranes (18), and an EGF–receptor complex has been isolated from hepatocytes by using a glutaraldehyde/sodium borohydride crosslinking technique (19). In HT-29, A-431, PANC-1, and other cells, EGF has been found to form a spontaneous covalent crosslinkage with its receptor (20–25). Electron microscopic evaluation of isolated hepatocytes in culture demonstrates that hepatocytes have the capacity to take up EGF *in vitro* (26).

We have investigated the role of the intact liver in EGF uptake and processing and have demonstrated: (*i*) significant hepatic uptake of ^{125}I-labeled EGF (^{125}I-EGF); (*ii*) the presence of EGF in bile; (*iii*) the existence of an EGF–protein complex consistent with an EGF receptor; and (*iv*) a portal-to-central lobular concentration gradient for ^{125}I-EGF uptake.

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METHODS

Animals. Male Sprague–Dawley rats obtained from Charles River Breeding Laboratories, weighing 300–350 g, were fed standard Purina chow ad lib and maintained on a standard wake–sleep cycle.

Materials. Mouse EGF was purified from mouse submaxillary glands as described (1, 13) and was iodinated according to the method of Hunter and Greenwood (27) as modified by Vlodavsky *et al.* (28) to a specific activity of 0.9–1.4 mCi/nmol (1 Ci = 3.7 × 10^{10} Bq). Na^{125}I was obtained from ICN. NaDodSO$_4$ was purchased from BDH.

Rabbit anti-EGF antiserum was prepared as described by Byyny *et al.* (29). When tested by quantitative precipitation tests, 0.1 ml of rabbit serum precipitated 3 mg of EGF.

Uptake of ^{125}I-EGF by Intact Rats. Eighteen to 24 μCi (110–150 ng) of ^{125}I-EGF in 0.5 ml of 0.01 M phosphate-buffered saline was injected into a femoral vein of fasted rats anesthetized with pentathol. After 10 min, the animals were sacrificed by cardiac puncture and major organs (liver, gut, lungs, kidneys, thyroid, and muscle) and blood samples were quickly removed and quick-frozen in liquid nitrogen, and subsequently radioactivity in the whole organs was counted in a bulk gamma counter (Capintec CRC-5, Montvale, NJ). Livers were also perfused with 0.85% aqueous NaCl at 4°C after cardiac puncture.

Uptake of ^{125}I-EGF by Intact Rat Livers. The abdominal cavity of 12 fasted, anesthetized male rats was opened with a midline incision and the portal vein was exposed. Thirty to 50 μCi of ^{125}I-EGF dissolved in 0.5 ml of 0.01 M phosphate-buffered saline with 0.1% bovine serum albumin was infused directly into the portal vein over a 30-sec interval. In some experiments, an equivalent amount of ^{125}I-EGF with a 100-fold excess of unlabeled EGF was injected intraportally. The animals were sacrificed at 3, 15, 30, or 90 min, and their liver was perfused with 0.85% aqueous NaCl at 4°C and frozen in liquid nitrogen and subsequently radioactivity was counted in a bulk gamma counter. As an additional control, in two animals given 50 μCi of ^{125}I-EGF, an equimolar amount of insulin was substituted for the 100-fold excess of unlabeled EGF.

Autoradiography. Rats were injected intraportally with 50 μCi of ^{125}I-EGF. At 1, 3, 10, 15, 30, or 90 min after injection, the livers were perfused with 0.85% aqueous NaCl at 4°C and then perfusion-fixed for 2 min via the portal vein (30) with 2.5% glutaraldehyde/0.8% paraformaldehyde in 0.2 M sodium bicarbonate buffer (pH 7.2). Immediately after perfusion fixation, biopsy samples were taken from each lobe and the radioactivity was counted individually in a gamma counter to confirm

Abbreviation: EGF, epidermal growth factor.

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^{125}I uptake by the liver. Subsequently, a portion of the right median lobe was removed and cut into 1-mm^3 blocks. After a 2-hr immersion-fixation in fresh fixative, the tissue was rinsed overnight in 0.2 M sodium bicarbonate buffer, post-fixed in 1% osmium tetroxide containing 1.5% KCN, dehydrated in ethanol, and embedded in Epon 812.

Sections (1-μm thick) of tissue from each time point were selected for qualitative evaluation. Each section consisted of classical lobules as defined by the existence of a clearly evident portal triad and a central vein. Twenty-seven lobules from three separate animals at 1 and 10 min after injection of ^{125}I-EGF were studied quantitatively. All sections were mounted on glass slides and coated with Kodak NTB-3 nuclear emulsion. After exposure for 2–4 wk, the slides were developed in Kodak D-19 developer and stained with 0.5% toluidine blue containing 1% sodium benzoate.

Grain Quantitation. Grain quantitation of light autoradiographs was performed by using prints of hepatic lobules at ×440 magnification with a multiple grid matrix overlay (Fig. 1). Each grid square encompassed an area of tissue 4,900 μm^2 and contained approximately three hepatocytes. Grid squares were aligned between the limiting plate of the portal area and the most proximal segment of the endothelium lining of the central vein. The squares were designated with the numbers 1–10 from portal to central regions and the number of grains within each square was counted and recorded. Lobules ranged in length from 6–10 grid squares; however, most were 7–8 squares in radius.

Bile Collection After Injection of ^{125}I-EGF. In two rats, the common bile duct was cannulated with PE10 tubing and bile was collected for 90 min after an intraportal injection of ^{125}I-EGF while the rats were under pentathol anesthesia. Immunoreactive ^{125}I-EGF was detected in bile by precipitation with a specific antiserum and *Staphylococcus aureus* protein A. The radioactivity of all samples was measured in a gamma counter.

Isolation of ^{125}I-EGF–Receptor Complex. An EGF–receptor complex was isolated by using a modification of the method of Fox and co-workers (21, 23). Liver membranes were prepared from *in situ* perfused rat livers collected at 3 min after an intraportal injection of ^{125}I-EGF. Livers were minced, homogenized, and sonicated after the addition of phenylmethylsulfonyl fluoride (0.1 mM), *p*-hydroxymercuribenzoate (1 mM), and benzamidine (0.1 mM), and liver membranes were isolated by using differential centrifugation. The homogenized and sonicated tissue was first centrifuged at 1,000 × *g* at 4°C for 30 min. A membrane pellet was prepared by centrifugation of the resultant supernatant at 100,000 × *g* at 4°C for 60 min, followed by repeated washes and recentrifugation at 100,000 × *g*. The washed membranes were solubilized with Nonidet P-40, diluted to 0.5% Nonidet P-40 by the addition of phosphate-buffered saline, and then centrifuged at 100,000 × *g* for 1 hr. The supernatant was incubated overnight at 4°C with rabbit anti-mouse EGF and the IgG was precipitated by using *S. aureus* protein A. The washed pellet was dissolved in $NaDodSO_4$ sample buffer with 5% 2-mercaptoethanol and boiled at 100°C for 3 min. The *S. aureus* protein A was separated by centrifugation and the supernatant was prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. The EGF receptor was also isolated from cultured rat hepatocytes (31) that had been incubated with ^{125}I-EGF, with or without the addition of an excess of unlabeled EGF. $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography were performed on sonicates of the incubated cells as well as on solubilized, immunoprecipitated membrane proteins from the same cells.

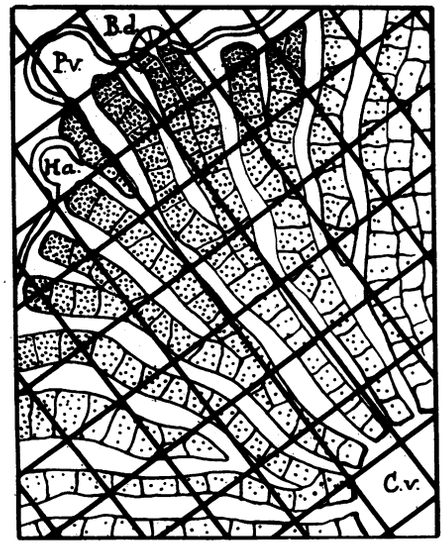

FIG. 1. Diagram illustrating orientation of grid squares used for quantitation of autoradiographic grains (black dots) within the liver lobule as seen by light microscopy. P.v., portal vein; H.a., hepatic artery; C.v., central vein; B.d., bile duct.

RESULTS

Total Uptake. Ten minutes after injection of ^{125}I-EGF into the femoral vein of three rats, radioactivity (mean ± SEM) was found predominantly in the liver (58.4 ± 5.5%), kidney (15.8 ± 1.0%), and gut (10.7 ± 1.3%), whereas the blood contained 6.2 ± 1.5% and the remainder of the body contained 9.0 ± 3.3% of the injected dose (Fig. 2).

Hepatic Uptake. Three minutes after the injection of ^{125}I-EGF into the portal vein, 99.0 ± 7.0% (mean ± SEM) of the injected EGF radioactivity was found in the liver. Uptake was decreased to 24% when the ^{125}I-EGF was injected with a 100-fold excess of unlabeled EGF, demonstrating the saturability of the system. Injected radioactivity (mean ± SEM) found in the liver after 15 min was 71.1 ± 0.8%, after 30 min was 57.4 ± 3.1%, and after 90 min was 9.9 ± 2.0%. This disappearance of EGF radioactivity from the liver followed first-order kinetics, and a logarithmic linear disappearance plot extrapolated to time zero estimated a 100% uptake.

In the two rats studied, 19% of intraportally injected EGF radioactivity was found in the bile during the 90-min collection period. When using the EGF-specific antiserum, 3.5% was found to be immunoprecipitable, and 15.5% was found to be non-immunoprecipitable.

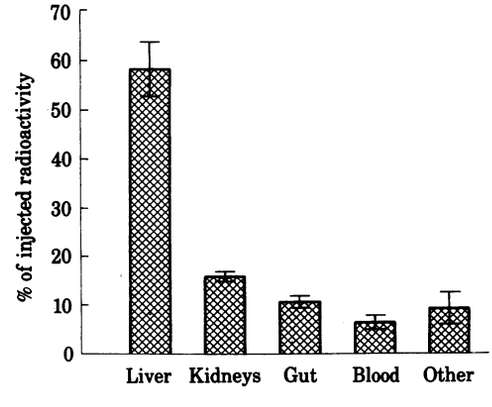

FIG. 2. Uptake of ^{125}I at 10 min after an intrafemoral injection of ^{125}I-EGF. Each bar represents the mean ± SEM. Other, remainder of the body.

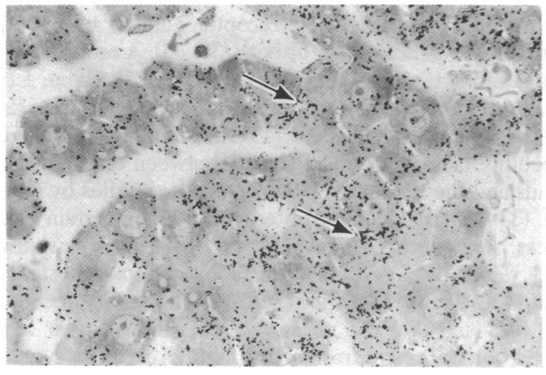

FIG. 3. Light microscopic autoradiograph illustrating numerous grains (black dots) associated with hepatic parenchymal cells at 10 min. Note the grains near the bile canaliculi (arrows).

Light Microscopic Autoradiography. Autoradiographic grains representing ^{125}I-EGF were observed almost exclusively over or in parenchymal cells at all time points evaluated (Fig. 3). Little evidence of Kupffer cell uptake was observed. Although the autoradiographic grains were seen near the sinusoidal cell surface at the early time periods (1–10 min), at the later time periods, concentrations of grains surrounding the bile canaliculi were readily apparent (Fig. 3). Because essentially 100% of the intraportally injected ^{125}I-EGF was taken up between 1 and 3 min, the 1-min and 10-min time points were considered to be suitable time points at which to quantitate the lobular distribution of autoradiographic grains. Based on qualitative evaluation, no differences in grain distribution were noted between any of the later time points.

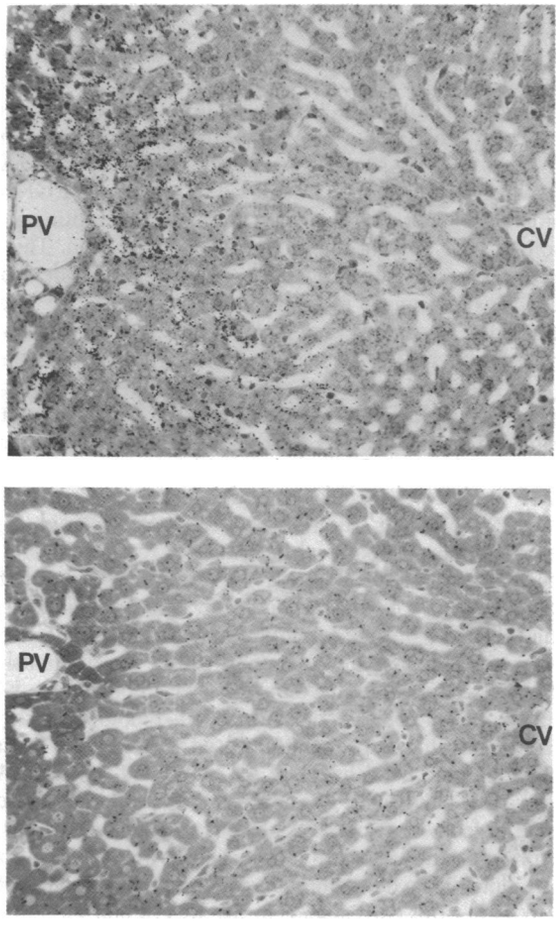

FIG. 4. Light microscopic autoradiographs of a portion of a hepatic lobule at 1 min after an intraportal bolus injection of ^{125}I-EGF (*Upper*) and injection as in *Upper* with a 100-fold excess of unlabeled EGF (*Lower*). *Upper* illustrates a distinct concentration gradient of grains from portal to central regions, whereas *Lower* illustrates the abolishment of the grain concentration gradient. PV, portal vein; CV, central vein.

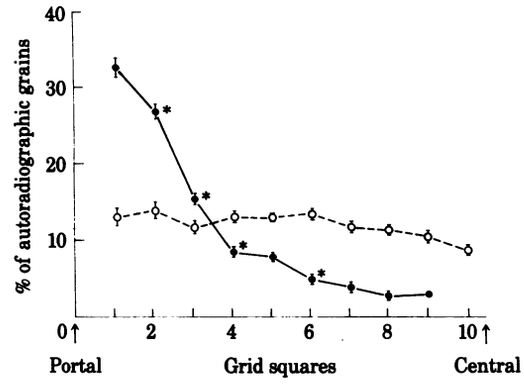

FIG. 5. Percentage of autoradiographic grains per grid square (see Fig. 1) 1 min after injection of ^{125}I-EGF. Quantitation of 27 lobules after an injection of ^{125}I-EGF alone (●) and after injection of ^{125}I-EGF and unlabeled EGF (○). Major differences in hepatocyte uptake of ^{125}I-EGF were noted between grid squares 1 and 2, 2 and 3, and 3 and 4 ($P < 0.001$), whereas no differences were noted between any grid squares when ^{125}I-EGF and unlabeled EGF were injected. Vertical bars represent SEM between the lobules. * notes a significant difference from the previous grid square.

Fig. 4 *Upper* illustrates the autoradiographic grain distribution along a typical hepatic lobule at 1 min after an intraportal injection of ^{125}I-EGF alone, and Fig. 4 *Lower* demonstrates for comparison a similar lobule at 1 min after a portal injection of an equal amount of ^{125}I-EGF with a 100-fold excess of unlabeled EGF. A concentration gradient of grains from this periportal to the pericentral region of the lobule was clearly evident. This gradient was almost totally abolished when an excess of unlabeled EGF, but not insulin, was injected with ^{125}I-EGF.

At the 1-min time point, ≈60% of the grains fell within the first two grid squares—i.e., within the first six layers of cells, closest to the portal region of the lobule when evaluated quantitatively (Fig. 5). The number of grains per grid square dropped significantly between each of the first four squares ($P < 0.001$). When an excess of unlabeled EGF was injected with ^{125}I-EGF, only 25% of the grains were found within the first two grid squares. No significant statistical difference in grain numbers per grid was noted between adjacent grid squares. The distribution of grains at 10 min after injection with ^{125}I-EGF was virtually superimposable on the 1-min gradient.

Receptor Complex Isolation. Polyacrylamide gel electrophoresis and autoradiography were carried out on immunoprecipitated membrane proteins isolated from liver cells after ^{125}I-EGF incubation with cultured hepatocytes or intraportal ^{125}I-EGF injection into rats (Fig. 6). Several membrane-associated autoradiographic bands with high molecular weights were seen. Two bands with the highest molecular weights were found to be significantly greater than M_r 200,000, by using 7% $NaDodSO_4$/polyacrylamide gel electrophoresis. The main band had a M_r of ≈160,000–170,000, similar to that of the EGF–receptor complex found in other cell systems (20, 24, 25). When isolated hepatocytes were incubated with ^{125}I-EGF and a 500-fold excess of unlabeled EGF, the above bands were decreased significantly in intensity, suggesting that these EGF–protein complexes have saturable binding characteristics (Fig. 6).

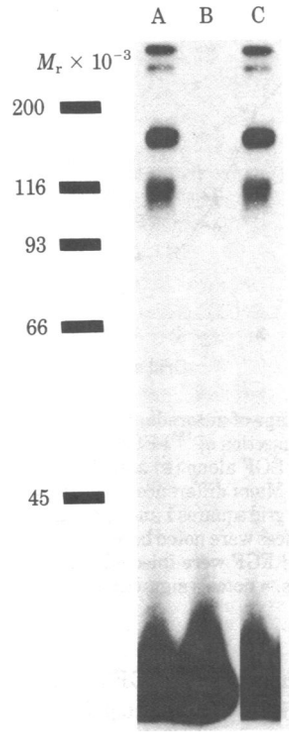

FIG. 6. $NaDodSO_4$ autoradiograph of an ^{125}I-EGF–protein complex after incubation of cultured hepatocytes with ^{125}I-EGF (lane A), with ^{125}I-EGF and unlabeled EGF (lane B), or when hepatic membrane proteins were prepared and solubilized 3 min after an intraportal injection of ^{125}I-EGF (lane C). ^{125}I-EGF itself is found at the dye front. The cathode is at the bottom of the gel autoradiograph.

DISCUSSION

The light microscopic autoradiographic and biochemical data presented here demonstrate that EGF is avidly sequestered by liver parenchymal cells in the rat when injected either intraportally or intravenously. Even though liver membranes have been shown to possess EGF receptors (18), this remarkably high-capacity uptake system in liver was not anticipated because only small quantities of EGF were found in liver homogenates (32).

The extraordinary efficiency of this liver EGF uptake system is demonstrated by the fact that 98% of injected EGF radioactivity was taken up by 3 min after supraphysiologic doses of ^{125}I-EGF. Furthermore, extrapolation of the disappearance curve of EGF radioactivity from liver to time zero yielded a theoretical 100% uptake at $t = 0$. The efficiency of the uptake system for EGF is demonstrated additionally by the steep portal-to-central lobular sequestration gradient even in the presence of supraphysiologic concentrations of ^{125}I-EGF.

A portal-to-central lobular concentration gradient for the uptake of plasma-derived substances by liver has been demonstrated previously, specifically for galactose (33–35) and the modified bile salt cholylglycylhistamine (36). However, when looked for, no such concentration gradient has been found to exist for the uptake of any plasma-derived protein thus far studied, including insulin (37), IgA (38), and apoprotein B (39). This pattern of uptake may be especially significant for a protein with a known biological effect on hepatocytes.

The underlying mechanism for the intralobular gradients is not well understood. Zonal differences in lobules have been well documented morphologically (40, 41) and also functionally (30, 42–44). Several theories have been suggested to explain these intralobular differences, including relative differences in blood oxygen level (45), hepatocyte maturity (42, 46), blood flow patterns (47), and hepatocyte receptor number or affinity for plasma-derived substances, or both (33, 36). A recent study using quantitative morphology has shown that the zone 1 (periportal) sinusoidal surface-to-volume ratio is greater than that of zone 3 (pericentral) sinusoids (48), thereby favoring a greater probability of interaction in zone 1 between circulating compounds and the fenestrae of the sinusoids. Studies by Goresky *et al.* (33–35) have demonstrated that, under certain circumstances, hepatocytes remove substances from the sinusoidal blood, depending on the concentration of those substances at various locations within the hepatic lobule. Evidence has also been provided that the periportal and centrolobular hepatocytes differ in their ultrastructure (40). It is unclear whether intralobular differences in hepatocytes are solely a function of their location within the hepatic lobule or reflect a more fundamental functional difference between these cell groups. An alternate explanation for the lobular gradient is the presence of a different number of receptors, a different receptor affinity, or a difference in receptor activity in periportal compared with pericentral cells. However, these explanations are unlikely due to the homogeneous uptake seen along the lobule after coinjection of an excess of unlabeled EGF with ^{125}I-EGF (Fig. 5) and the fact that essentially 100% of labeled EGF was taken up in the first pass ($t = 0$).

The physiological significance of the extremely efficient EGF uptake mechanism in liver is not yet understood. Obviously, the growth-promoting capabilities of EGF are not expressed in normal liver because liver cells divide at an exceedingly slow rate. However, EGF treatment has been shown under special circumstances to induce hepatic hypertrophy and hyperplasia *in vivo* (16), to induce DNA synthesis in rat hepatocytes *in vitro* (15, 17), and to increase neonatal hepatocyte numbers *in vitro* (17). Based on these studies as well as those of Earp and O'Keefe (49), which demonstrated a decrease in binding of EGF to receptors in regenerating liver membrane, it is tempting to propose that EGF plays a role in hepatic regeneration. This suggested role is especially intriguing because the rapidly developing liver acinus in neonatal rats (50) as well as hepatic regeneration begin in the periportal regions (51, 52), zones which may be enriched with EGF. The rapid and efficient sequestration of EGF by liver also suggests that the liver may play a major role in maintaining EGF homeostasis in plasma. This periportal sequestration of radiolabeled EGF may be used as a marker for separating periportal from pericentral hepatocytes.

EGF forms covalent linkages with its receptor in several cell systems (20–25). Our studies demonstrate that a rapid, "spontaneous" covalent linkage also forms between EGF and hepatocyte membrane protein thought to be the receptor both *in vivo* and *in vitro*. Of concern, however, is recent evidence suggesting that this linkage formation may be the result of chloramine-T used in labeling EGF (53). Unlike the previously reported cell systems that have been shown to possess an EGF–receptor protein complex of M_r 170,000–180,000 (20, 21, 24, 25), in the hepatocyte preparations used in this study, the main autoradiographic band had a M_r of ≈160,000–170,000. Also, several other bands with M_rs >200,000 were found. These results differ from those of Sahyoun *et al.* who found a M_r 100,000 glycoprotein subunit for the EGF receptor in liver membranes using a glutaraldehyde/sodium borohydride crosslinking method (19). Whether this corresponds to the M_r 112,000 subunit seen in this study is unclear. The apparent difference in subunit molecular weight might be explained by linkage to receptor-adjacent proteins when using the glutaraldehyde method. Further, a bifunctional reagent like glutaraldehyde could potentially link the receptor to many other macromolecules in the membrane as well as forming polymers of the ligand. Therefore, it

is understandable that there might be differences in several EGF–protein complexes when comparing glutaraldehyde with spontaneous crosslinking.

The multiple high-molecular-weight subunit species demonstrated in this study might represent more than one population of receptor proteins with which EGF forms spontaneous crosslinking. The presence of a receptor crosslinking of multiple subunits of varying molecular weights of the same receptor would be an alternate explanation.

The demonstration of immunoprecipitable and radiolabeled EGF in the bile suggests that the transport of a small fraction of EGF is similar to that for IgA because IgA is secreted almost totally intact (38). The presence of intact EGF in the bile might explain the finding of bile duct proliferations seen with cholestasis. However, EGF was also secreted in a nonimmunoactive and most likely a degraded form. This suggests that EGF may be transported by an alternative route, the lysosomal pathway, such as that used for the processing of apoprotein B as demonstrated by Chao *et al.* (39). Therefore, these data suggest that EGF might be translocated across the hepatocyte via two routes—specifically, a direct vesicular pathway from the plasma membrane to the bile canaliculus as well as an indirect pathway involving lysosomal degradation (37).

Although EGF appears to have a growth-promoting effect on hepatocytes (17) and hepatocytes have been shown to contain saturable EGF receptors (18), a high-capacity uptake system with the ability to translocate immunoactive EGF across the hepatocyte has, to the best of our knowledge, not been demonstrated previously.

EGF has been shown to have growth-promoting effects on the rapidly proliferating large and small intestinal mucosa (54, 55) and may be one of the humoral factors involved in the maintenance of gut mucosal integrity and in stimulating hepatic growth and regeneration. It is enticing to speculate that uptake, translocation, and secretion of immunoprecipitated EGF by the hepatocyte from plasma and secretion into bile might be part of a system that not only provides the liver with adequate amounts of growth factor for normal growth and regeneration but also further increases gut luminal EGF concentrations. The liver itself may play a major role in regulating plasma concentration of EGF and in its transfer from plasma to gut lumen. The exact physiological role of these findings has yet to be determined.

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