

Transport of Epidermal Growth Factor by Rat Liver: Evidence for a Nonlysosomal Pathway

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ABSTRACT Epidermal growth factor (EGF), circulating in the blood, is taken up by rat liver hepatocytes by means of specific and saturable receptor-mediated endocytosis. These experiments were undertaken to determine (a) the transport pathway(s) of EGF taken up by rat liver and (b) the effects of lysosomal inhibition on its transport. ¹²⁵I-EGF was injected into rat portal veins, and bile samples were collected and analyzed for both total and immunoprecipitable radioactivity. In addition, the livers were examined by electron microscopic autoradiography. Some animals received injections of chloroquine before surgery, to disrupt lysosomal function.

The results indicate that most of the EGF taken up by the hepatocytes is transported to lysosomes and degraded. However, a small but significant percentage of endocytosed EGF is transported by a pathway independent of the lysosomal system, resulting in secretion of intact EGF: (a) Both degraded and immunoprecipitable EGF are secreted into bile. (b) Immunoprecipitable radioactivity peaks at 20 min after EGF injection, whereas degradation-associated radioactivity does not peak until 40 min postinjection. (c) EGF isolated from bile is specifically taken up by isolated hepatocytes in monolayer culture, indicating that it is still recognizable by the EGF receptor. (d) When the lysosomal system is inhibited with chloroquine, secretion of degraded EGF is significantly inhibited, whereas the amount of intact EGF secreted into bile is unchanged.

The utilization by liver of a dual transport process for EGF represents an unusual system of intracellular ligand processing, whose physiological significance has yet to be determined.

Epidermal growth factor (EGF),¹ a small (~6,400 mol wt) peptide originally found in salivary and Brunner's glands, has been shown to have a variety of effects on the gastrointestinal tract (1). Recent experiments in this and other laboratories have demonstrated that rat hepatocytes, by means of specific (2) and saturable (3) receptor-mediated endocytosis, take up EGF from plasma and secrete it into bile (2). By using autoradiography, we showed that endocytosed EGF formed a steep portal to central lobular concentration gradient in the liver (2). Immunoprecipitation of bile collected after EGF injection suggested, furthermore, that both intact and degraded EGF were secreted.

Degradation, via the lysosomal system, of proteins endocytosed by hepatocytes as well as other cell types has been extensively documented (4–6). Less well recognized, however,

is the transcellular route by which some proteins (e.g., dimeric immunoglobulin A [IgA]) are endocytosed from plasma and later delivered intact to bile. For this process we have proposed a second pathway, i.e., the "direct" or nonlysosomal pathway, utilizing "shuttle" vesicles passing from the sinusoidal surface of the hepatocyte to the bile canaliculus (7); we have presented morphological and cytochemical evidence for the operation of this pathway in the transport of IgA (8) and insulin (9). Further support for the direct pathway was recently reported by Schiff et al. (10), who found that inhibition of lysosomal enzymes with leupeptin had no effect on the biliary secretion of IgA.

The aim of the present experiments was twofold: (a) to delineate the transport pathway(s) and ultimate disposition of the EGF taken up by rat liver and (b) to determine the effects on EGF transport of lysosomal inhibition with chloroquine. Chloroquine was selected to perturb the lysosomal pathway because it has been shown to inhibit degradation of both

¹ *Abbreviation used in this paper.* EGF, epidermal growth factor.

exogenous and endogenous protein by isolated rat hepatocytes (11) and specifically inhibits EGF degradation in cultured hepatocytes (12) and other cells (13–16) without inhibiting uptake (14–16). Chloroquine accumulates in hepatocytic lysosomes (17, 18) and is thought to inhibit lysosomal enzymes by elevating intralysosomal pH (19).

If a direct pathway, independent of the lysosomal system, is the means by which undegraded EGF reaches bile, then inhibition of the lysosomal pathway should result in secretion of less degraded EGF without affecting the amount of immunoreactive EGF in bile.

MATERIALS AND METHODS

Preparation of ^{125}I -EGF: EGF purified from mouse submaxillary glands (20, 21) was kindly supplied by Dr. Denis Gospodarowicz, University of California, San Francisco. The EGF was radiolabeled with ^{125}I -Na (Amersham Corp., Arlington Heights, IL) by the chloramine-T method (22, 23). Unreacted ^{125}I -Na was removed by adsorption on a Sephadex G-25 column, and the ^{125}I -EGF was eluted in the void volume. The ^{125}I -EGF had a specific activity of ~ 1 mCi/nmol and was $>90\%$ immunoprecipitable with a specific antiserum (see below).

Uptake of ^{125}I -EGF: Procedures for the injection of labeled EGF have been described in detail elsewhere (2). A midline abdominal incision was made in male Sprague-Dawley rats under pentobarbital anesthesia. In some rats the common bile duct was cannulated with PE-10 tubing to allow for bile collection. 30–50 μCi of ^{125}I -EGF was injected into the portal vein; at selected time points a blood sample was taken from the vena cava, and the liver was perfused with cold PBS. The liver was then removed, blotted, weighed, and homogenized in PBS, and the radioactivity of an aliquot of homogenate was counted in a gamma counter.

In studies with chloroquine, chloroquine diphosphate (Sigma Chemical Co., St. Louis, MO), was dissolved in saline and injected intraperitoneally (50 mg/kg in 0.5–0.8 ml saline) at 120 and 60 min before injection of EGF. Controls received an equal volume of saline. In some rats the bile duct was cannulated and bile was collected for 50 min; the remainder were sacrificed at 30 min postinjection and their livers were processed for autoradiography.

Autoradiography: Methods of fixation and processing of liver tissue for light microscopic autoradiography were described in an earlier study of EGF transport by rat liver (2). Thin sections (900 Å) were cut from the same tissue blocks used in that previous study. Therefore, the previously published data on specificity of uptake and clearance rates applies to these experiments as well. Thin sections were also cut from identically processed blocks of liver from three chloroquine-treated and two saline-injected rats at 30 min after ^{125}I -EGF injection. Sections were mounted on parlodion/carbon coated nickel grids, overlaid with Ilford L-4 emulsion (Ilford Ltd., Essex, UK) using the loop technique (24), exposed for 3–7 wk, developed in Kodak D-19 (Eastman Kodak Co., Rochester, NY), stained with lead citrate, and examined in a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operating at 60 kV. For quantitation of autoradiographic grains, micrographs were taken randomly and printed at a final magnification of $\times 35,000$. Grain distribution was determined by the method of concentric circle analysis (9) as described previously (25).

Cytochemistry: Tissue for cytochemical demonstration of lysosomal enzymes was immersed for 30 min in the paraformaldehyde–glutaraldehyde fixative used for perfusion. After washing overnight in bicarbonate buffer, it was chopped into 50–100 μm sections and incubated for arylsulfatase according to Goldfischer's (26) procedure, or for acid phosphatase with either the lead method of Barka and Anderson (27) or the CeCl_3 technique of Robinson and Karnovsky (28). Chloroquine-induced multilamellated structures showed reaction product with all three procedures.

Analysis of Bile: Bile from chloroquine-treated or saline-injected rats was collected into preweighed tubes at 10-min intervals. Total radioactivity was measured in a Beckman Gamma 8500 counter (Beckman Instruments, Inc., Fullerton, CA) and activity per gram bile was calculated.

For immunoprecipitation, duplicate samples (20 μl each) of bile were incubated overnight at 4°C with a 1:100 dilution of rabbit antiserum to mouse EGF (Bethesda Research Laboratories, Gaithersburg, MD) or normal rabbit serum. *S. aureus* with protein A (Zymed Laboratories, Burlingame, CA) was added to each sample and incubated for 1 h at 4°C . Total activity was counted and samples were centrifuged (Beckman Microfuge); pellets were washed three times with PBS and counted. After subtraction of the corresponding nonspecific radioactivity of each sample precipitated by normal serum (never more than

5%), the remaining radioactivity in the pellet represented immunoreactive ^{125}I -EGF.

For separation of intact EGF from degradation products, chromatography columns (1×30 cm) were prepared with Sephadex G-50 hydrated in a buffer containing 4 M urea, 1 M acetic acid, and 0.1% Triton X-100. The columns were eluted with the same buffer and 1-ml fractions were collected and counted. For isolation of biologically active EGF from bile, ^{125}I -EGF-containing bile was fractionated on identical G-50 columns, but was equilibrated and eluted with PBS rather than the urea/acetic acid buffer. The peak representing presumably intact EGF was then dialyzed for 3 h against Medium 199 and subsequently incubated with primary monolayer cultures of rat hepatocytes.

Uptake of Bile-derived ^{125}I -EGF by Hepatocyte Cultures: Primary monolayer cultures of rat hepatocytes were prepared at the Cell Culture Core Facility of the Liver Center, University of California, San Francisco, by in situ perfusion of the rat liver with collagenase followed by passage through an elutriator to yield a cell suspension of $\sim 98\%$ hepatocytes (29). Viability of the cell suspension was evaluated by trypan blue exclusion. Cells were plated on collagen-coated 35-mm petri dishes in Medium 199 OR supplemented with antibiotics, hormones, and 1% normal rat serum, and reached confluence within 24 h after plating.

For uptake studies, the medium was removed, and cultures were incubated for 1 h at 37°C with fresh medium containing: (a) ^{125}I -EGF used for intraportal injection, (b) ^{125}I -EGF isolated from bile, or (c) ^{125}I -EGF isolated from a mixture of the original ^{125}I -EGF and unlabeled bile. A 500-fold excess of unlabeled EGF was added to some cultures. The cultures were rinsed three times with ice-cold PBS and scraped, and their radioactivity was measured. Uptake was expressed as the percentage of initial radioactivity that was retained by the cells.

RESULTS

Electron Microscopic Autoradiography

Analysis of autoradiographic grains on electron micrographs demonstrated a sequential change in association of grains with the plasma membrane, endocytic vesicles, multivesicular bodies, and lysosomes (Table I). Although 40–45% of the grains were associated with other organelles, these values did not change with time, and their significance is at present unknown.

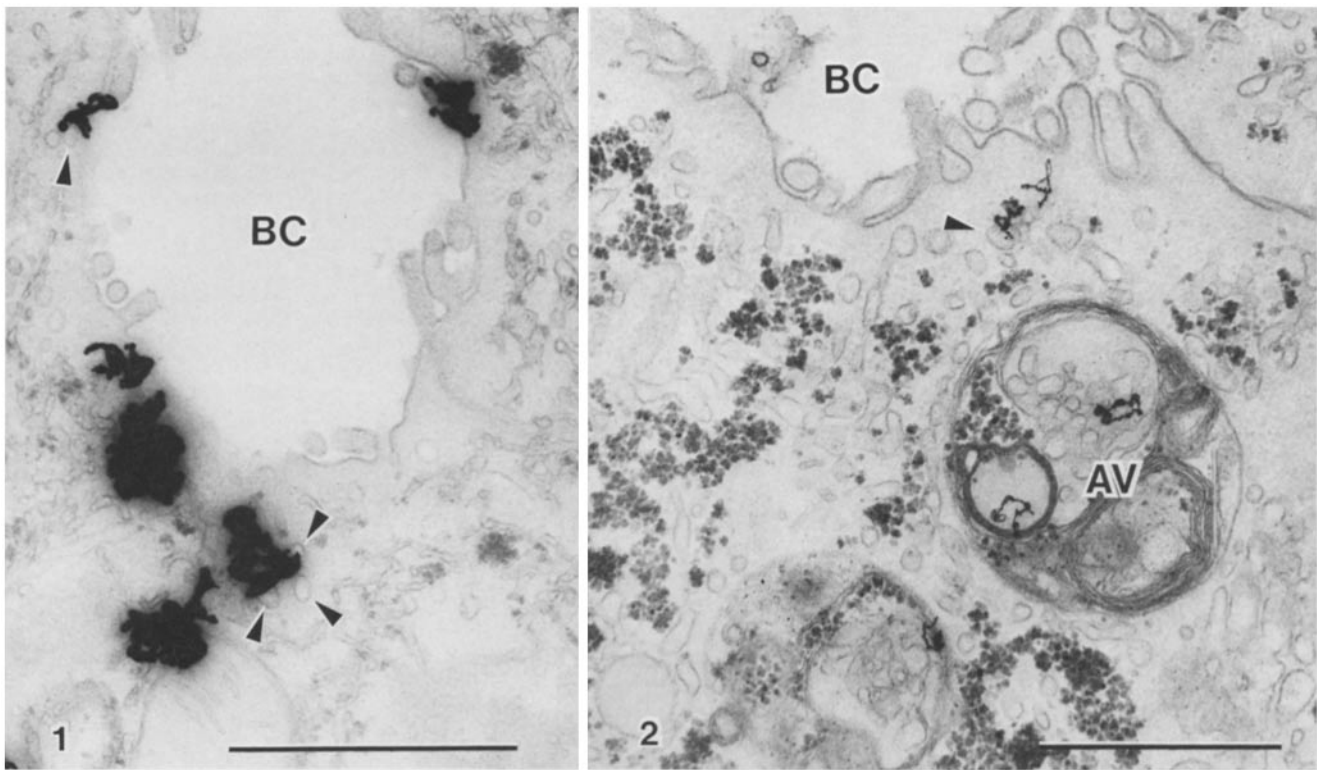
At 3 min grains were primarily associated with endocytic

TABLE I
Percentage Distribution of Autoradiographic Grains in Hepatocytes after Intraportal Injection of ^{125}I -EGF*

Organelle category	Controls				Chloroquine treated	
	Time after injection (min)					
	3	15	30	90		30
	%					
Plasma membrane	19.0	8.6	6.8	5.6	4.5	
Endocytic vesicles	14.1	5.8	3.1	2.1	0.7	
Multivesicular bodies	22.1	30.1	34.0	12.7	30.0	
Secondary lysosomes	2.0	3.5	10.2	28.7	2.5	
Multilamellated structures	—	—	—	—	12.4	
Golgi-rich area	1.9	6.7	4.2	5.6	0.9	
Smooth endoplasmic reticulum	26.5	23.9	30.8	30.0	32.3	
Rough endoplasmic reticulum	3.2	3.4	2.0	3.0	4.6	
Mitochondria	7.6	10.1	6.5	9.1	7.5	
Nuclei	0.4	0.5	0.4	0.5	1.3	
Other [†]	3.0	2.0	1.9	2.5	3.2	

* Values are means of percentages based on $\sim 9,000$ grains from over 1,000 micrographs (fields), representing two control rats for each time point and three chloroquine-treated rats.

[†] Endothelial cells, Kupffer cells, extracellular space, and cytoplasm with no identifiable organelles.



FIGURES 1 and 2 Fig. 1: 10 min after intraportal injection of ^{125}I -EGF, autoradiographic grains are sometimes found at the bile canaliculus surface of the hepatocyte. Several small vesicles are also apparent in this micrograph; grains are associated with at least four of them (arrowheads). BC, bile canaliculus. Bar, 1 μm . $\times 37,500$. Fig. 2: Chloroquine treatment; 30 min after ^{125}I -EGF administration. In addition to their association with multilamellated structures (autophagic vacuoles, AV), grains are occasionally seen over small vesicles near the bile canaliculus (arrowhead). BC, bile canaliculus. Bar, 1 μm . $\times 37,800$.

pits and/or vesicles at the sinusoidal surface of the hepatocyte. 15 min postinjection, grains were associated with multivesicular bodies usually located near Golgi-rich areas of hepatocytes, whereas by 30 min, labeled multivesicular bodies were found in the pericanalicular area of the hepatocyte. The association of grains with multivesicular bodies peaked at 30 min postinjection, then declined as the association of grains with secondary lysosomes increased steadily through 90 min (Table I).

These data provide evidence for the usual pathway of entry of endocytosed EGF into a lysosomal compartment. However, at 10 min postinjection, grains were also found clustered around the bile canaliculus (Fig. 1), suggesting a more rapid nonlysosomal transport pathway. Nevertheless, endocytic "shuttle" vesicles, analogous to insulin/horseradish peroxidase- or IgA-containing vesicles (8, 9) could only occasionally be resolved (Fig. 1). In an attempt to enhance EGF transport by this pathway, the lysosomotropic agent, chloroquine, was administered to rats prior to injection of EGF; autoradiography of the liver at 30 min postinjection and analysis of bile from these animals were performed.

In hepatocytes from chloroquine-treated rats, many large, multilamellated structures containing cytoplasmic inclusions were present (Fig. 2). The lysosomal nature of these multilamellated structures was demonstrated by cytochemical localization of arylsulfatase and acid phosphatase within them (data not shown). The presence of these lysosomal enzymes in chloroquine-induced multilamellated structures has also been demonstrated by other methods (30, 31). At 30 min after the intraportal injection of ^{125}I -EGF, autoradiographic

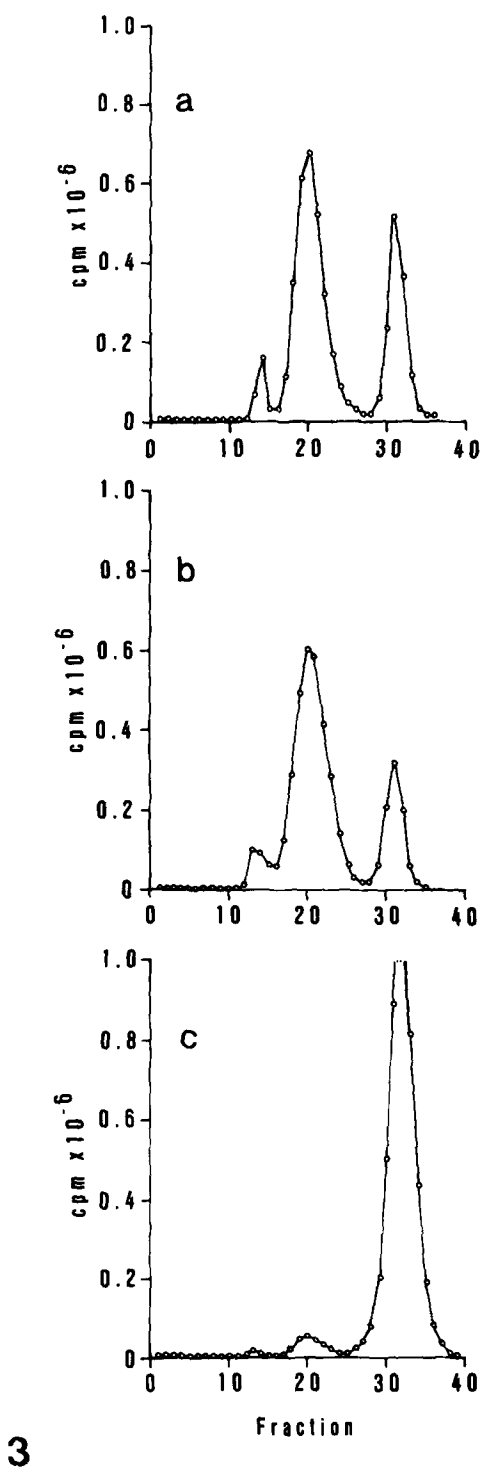
TABLE II
Morphometry of Chloroquine-treated Rat Liver*

Component	Chloroquine	Control
	%	
Multilamellated structures	3.5 \pm 0.4	0.0
Secondary lysosomes	0.5 \pm 0.1	0.6 \pm 0.1
Multivesicular bodies	0.6 \pm 0.1	0.7 \pm 0.1
Mitochondria	19.9 \pm 0.6	20.7 \pm 0.6
Cytoplasm	62.7 \pm 1.8	68.1 \pm 1.5

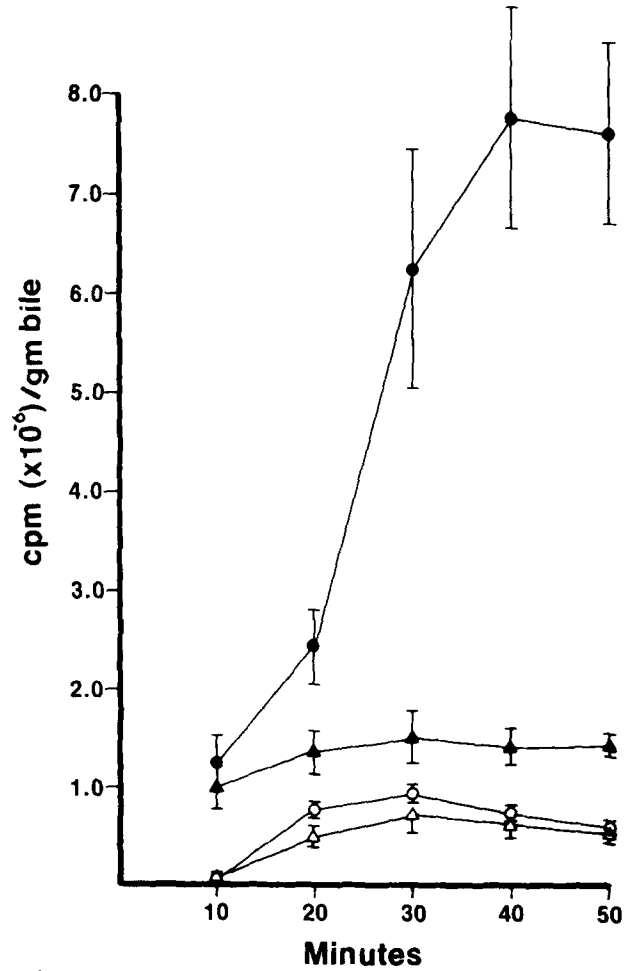
Morphometric analysis of livers from chloroquine-treated and control rats were performed according to the method of Weibel et al. (32). 90 micrographs (fields), representing three rats from each group, were used in this analysis.

* Values expressed as percentage of volume of component per unit volume total hepatic tissue (mean \pm SEM).

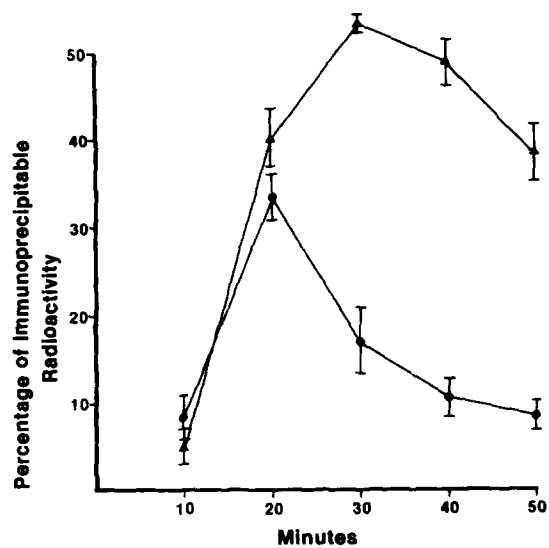
grains in chloroquine-treated rats were associated with both multivesicular bodies and multilamellated structures (Table I, Fig. 2). Morphometric analysis (32) indicated that, as a result of chloroquine treatment, the volume density of the total lysosomal compartment (secondary lysosomes plus multilamellated structures) increased by 3.5% (Table II). This increase in volume density was accompanied by a concomitant decrease in cytoplasmic volume. Even though the volume density of the total lysosomal compartment increased, the chloroquine-treated rats had approximately the same percentage of autoradiographic grains associated with this compartment as the controls; however, of these grains, 80% were associated with multilamellated structures rather than secondary lysosomes (Table I). The distribution of grains among



3



4



5

FIGURES 3-5 Fig. 3: Elution profile of ¹²⁵I-EGF fractionated on a Sephadex G-50 column in 4 M urea, 1 M acetic acid, and 0.1% Triton X-100. (a) Starting material, i.e., freshly iodinated EGF. The batch used in this particular experiment was only ~70% immunoprecipitable with anti-EGF. (b) Starting material incubated with unlabeled bile at 37°C for 40 min. (c) Bile collected between 30 and 40 min after intraportal injection of ¹²⁵I-EGF. In each case the middle peak represents intact EGF. Fig. 4: Total and immunoprecipitable radioactivity in bile collected over a period of 50 min. ●, total activity—control (n = 4); ▲, total activity—chloroquine-treated (n = 5); ○, immunoprecipitable activity—control (n = 4); △, immunoprecipitable activity, chloroquine-treated

other organelle compartments was also approximately the same in control and chloroquine-treated rats.

Analysis of Bile

The amount and form of radioactivity secreted into bile was used as an indicator of the metabolism of ^{125}I -EGF by liver. Therefore, it was necessary to establish that there was no degradation of ^{125}I -EGF as a result of its contact with bile, and that any alteration of its form could only result from its transport through the liver.

Labeled bile from control rats, collected between 30 and 40 min after the intraportal injection of ^{125}I -EGF, was fractionated on a Sephadex G-50 column in 4 M urea, 1 M acetic acid, and 0.1% Triton X-100. Its elution profile was compared with those of ^{125}I -EGF which had been incubated with unlabeled bile for 40 min at 37°C and of ^{125}I -EGF that had never been in contact with liver or bile. The elution profiles of the ^{125}I -EGF starting material and the ^{125}I -EGF incubated with unlabeled bile were virtually identical (Fig. 3, *a* and *b*). The middle peak, representing intact EGF, accounted for most of the radioactivity (61–68%) eluted from the column. The void volume peak, containing larger molecules (>10,000 mol wt) had ~5% of the eluted radioactivity whereas the total volume peak, consisting of lower-molecular-weight breakdown products, tyrosine residues, or free ^{125}I , accounted for 20–28% of the eluted radioactivity. In contrast, the elution profile of the labeled bile (Fig. 3 *c*) showed that >90% of the eluted radioactivity was associated with the total volume peak, indicating that most of the ^{125}I -EGF secreted into bile at this time had been degraded as a result of its transhepatocyte transport. These results demonstrate that even prolonged contact with bile does not cause degradation of ^{125}I -EGF, and that therefore, alterations in the structure of the ^{125}I -EGF secreted into bile are due to the metabolic activity of hepatocytes.

Bile collected over 10-min intervals after the intraportal injection of ^{125}I -EGF was analyzed for its radioactive content by measurement of both total and immunoprecipitable radioactivity. Total radioactivity secreted per gram bile was significantly reduced in chloroquine-treated rats; at 40 min it was <20% that secreted by controls (Fig. 4), although total bile output did not significantly decrease (0.868 ± 0.05 g/50 min in controls vs. 0.716 ± 0.27 g/50 min in chloroquine-treated rats). The percentage of immunoprecipitable radioactivity in each bile sample was compared for chloroquine-treated versus control rats (Fig. 5). In chloroquine-treated rats, immunoprecipitable radioactivity peaked at 30 min postinjection, and accounted for 55% of the total radioactivity secreted, whereas in controls, immunoprecipitable radioactivity peaked at 20 min and represented only 35% of the total. However, when the amount of immunoprecipitable radioactivity (the percentage immunoprecipitable times the total counts per minute/gram) was calculated, it was apparent that this value was virtually the same in both groups of rats (Fig. 4). Thus, in control rats, degradation products accounted for the great majority of the radioactivity secreted into bile, especially at later time points.

The radioactivity in blood and liver of chloroquine-treated versus control rats was compared at 50 min postinjection

TABLE III
Radioactivity in Liver and Blood

	Control (n = 4)	Chloroquine (n = 6)
Liver (cpm $\times 10^{-6}$ /g)	1.75 ± 0.32	3.90 ± 0.10
Blood (cpm $\times 10^{-6}$ /ml of whole blood)	0.42 ± 0.10	0.20 ± 0.07

Blood samples were collected 50 min after intraportal injection of ^{125}I -EGF. The entire liver was removed, blotted, weighed, and homogenized, and an aliquot was counted. Blood was allowed to clot and an aliquot of serum was counted.

(Table III). Radioactivity per gram of liver more than doubled, whereas radioactivity in blood decreased relative to controls, as a result of chloroquine treatment. All of these data taken together suggest that chloroquine inhibits degradation of ^{125}I -EGF; this results in accumulation of radioactivity in liver with a concomitant decrease in release of radioactive degradation products into both the blood and bile, whereas the secretion of undegraded ^{125}I -EGF is unaffected.

Uptake of ^{125}I -EGF Derived from Bile

Although some of the ^{125}I -EGF in bile was immunoprecipitable, implying an intact molecule, this in itself does not indicate biological activity. To further characterize the presumably unaltered EGF secreted in bile, it was isolated and incubated with primary cultures of rat hepatocytes.

For this purpose ^{125}I -EGF-containing bile samples collected from control rats were pooled (composite immunoprecipitability $\approx 10\%$) and fractionated on a Sephadex G-50 column in PBS. The middle peak, corresponding to intact EGF, was dialyzed against Medium 199, using dialysis tubing with a molecular weight exclusion limit of 2,000. <4% of the radioactivity was lost through the dialysis membrane. ^{125}I -EGF mixed with unlabeled bile was similarly fractionated and dialyzed.

Primary cultures of rat hepatocytes were incubated with the above column-derived preparations of ^{125}I -EGF, as well as with ^{125}I -EGF starting material. A 500-fold excess of unlabeled EGF was also added to some of the cultures. After 1 h at 37°C, the cultures were rinsed, the cells were scraped free, and the radioactivity retained by the cells was measured (Table IV).

In order to take into account the variable concentrations of ^{125}I -EGF in column-derived material, some of the ^{125}I -EGF starting material and some of the ^{125}I -EGF mixed with unlabeled bile were further diluted with Medium 199 to a concentration of radioactivity equal to that of the ^{125}I -EGF isolated from bile. There was no difference in the percentage of radioactivity taken up by hepatocytes incubated with the diluted versus undiluted preparations. Therefore, these results have been combined under each category of labeled material in Table IV.

The results of this experiment indicate that ^{125}I -EGF isolated from bile was specifically taken up by cultured hepatocytes with an efficiency slightly less than that shown for the starting material either alone or mixed with unlabeled bile.

(n = 5). Error bars = standard error of the mean. Total radioactivity in control bile continued to increase for 40 min whereas in chloroquine-treated rats it remained relatively constant at a much reduced level. Except for a small difference at 20 min, immunoprecipitable activity in the two groups was identical. Fig. 5: Immunoprecipitable radioactivity secreted in bile, expressed as a percentage of the total. ▲, chloroquine-treated (n = 5); ●, control (n = 4). Error bars = standard error of the mean.

TABLE IV
Uptake of Bile-derived EGF by Cultured Hepatocytes

Labeled material	% Bound	% Specific binding
^{125}I -EGF in Medium 199 ($n = 9$)	12.2 ± 0.6	92.6
^{125}I -EGF in Medium 199 + $\times 500$ cold EGF ($n = 9$)	0.9 ± 0.2	
^{125}I -EGF from bile ($n = 6$)	10.0 ± 0.8	92.0
^{125}I -EGF from bile + $\times 500$ cold EGF ($n = 6$)	0.8 ± 0.2	
^{125}I -EGF mixed with bile ($n = 3$)	13.7 ± 0.7	85.7
^{125}I -EGF mixed with bile + $\times 500$ cold EGF ($n = 3$)	2.1 ± 0.2	

Bile from ^{125}I -EGF-injected rats or ^{125}I -EGF mixed with unlabeled bile was eluted from a Sephadex G-50 column with PBS. The middle peak was dialyzed against Medium 199. In addition, ^{125}I -EGF that had not been in contact with bile was added to Medium 199. Some of each of these latter two preparations were further diluted with Medium 199 to a concentration of radioactivity equal to that of the ^{125}I -EGF isolated from bile. 1.0 ml of each of these preparations was added to 35-mm culture dishes containing 48-h primary cultures of rat hepatocytes. After incubation at 37°C for 1 h, the cultures were washed three times in ice-cold PBS, and the cells were scraped from the dishes and counted. Values represent mean \pm SEM.

Neither the presence of incidental biliary components in column-derived material, nor variable concentrations of ^{125}I -EGF, could account for this less efficient uptake.

DISCUSSION

The experiments reported here provide further support for the hypothesis that some proteins traverse the hepatocyte by a pathway independent of the lysosomal system and are thus secreted intact into bile. When ^{125}I -EGF is injected intraperitoneally into rats, both degraded and intact protein is demonstrable in the bile. Although immunoprecipitable radioactivity peaks at 20 min, that associated with degradation products does not reach a peak until 40 min postinjection. Furthermore, the intact EGF isolated from bile is specifically taken up by cultured hepatocytes, indicating that it is still recognizable by the EGF receptor. When the lysosomal system is inhibited with chloroquine, secretion of degraded EGF is significantly decreased, whereas the amount of intact EGF appearing in bile remains unchanged.

The actual amount of intact EGF that reaches the bile is ~4% of the injected dose (20% of the injected radioactivity is secreted into bile over 90 min, of which 20% is immunoprecipitable [2]). This percentage is slightly higher than that reported for asialoglycoproteins secreted intact into bile over a 2-h period (33). Because this percentage is not quantitatively significant, the nonlysosomal pathway has been overlooked as having any physiological significance for plasma-derived proteins normally degraded by liver.

Because the amount of intact EGF in bile is small relative to the injected dose, the possibility exists that a nonspecific uptake and transport process (fluid-phase endocytosis) might account for the appearance of intact EGF or other proteins in bile. However, Schiff et al. (10) have reported that, in the case of two proteins that are taken up nonspecifically by rat liver (fetuin and human serum albumin), <1% of the injected dose of these proteins appears intact in bile over a period of 150 min. Therefore, it does not appear likely that nonspecific fluid-phase endocytosis can account for the majority of the intact EGF in bile.

The operation of the nonlysosomal pathway most likely involves specific, receptor-mediated endocytosis, as does the operation of the lysosomal pathway. Because there is no evidence for more than one class of EGF receptor on liver membranes (34), the sorting of EGF into the two separate pathways probably takes place subsequent to the formation of the EGF-receptor complex. The mechanisms which regulate the selection of transport pathway are at present unknown.

The best characterized nonlysosomal transport pathway in the rat hepatocyte is that used for the transport of rat dimeric IgA (8, 10). However, the kinetics of its transhepatocyte transport appear to be different from those of EGF. Its peak secretion into bile occurs between 30 and 40 min postinjection, and ~67% of the injected radioactivity is eventually secreted into bile (150 min) (10), whereas the peak secretion of intact EGF occurs at 20 min postinjection, and only 20% of the injected radioactivity is secreted into bile over 90 min (2). However, the apparent discrepancy in the kinetics of transport of these two proteins probably does not involve the operation of the shuttle-vesicle transport system, but rather is due to differences in the rate and efficiency of their uptake by liver and other organs. The uptake of even supraphysiological doses of EGF appears to be virtually complete on a single pass through the liver (2); for IgA it is not (10). Also, whereas IgA is taken up from plasma mainly by the liver (10), EGF is sequestered by other organs as well (2).

The use of supraphysiological doses of EGF in these studies does not affect the percentage of intact EGF reaching the bile, as long as the transport system is not saturated (the highest dose used in the present experiments [70 ng], was well below the level of saturation [3, 35]). In other experiments, we compared the transport of physiological with supraphysiological doses of EGF and found the same relative proportions of immunoreactive and degraded EGF in bile (35).

Up to now, morphological demonstration of the "direct," nonlysosomal transport pathway in hepatocytes has been inconclusive (8, 9). This is because, as was recently pointed out (10), morphological methods lack sufficient sensitivity to detect nonlysosomal transport. However, analysis of bile as to the amount and form of secreted radioactivity gives much better sensitivity than morphological methods alone, and indicates how transhepatocyte processing affects the molecule being transported. Using similar methods, the kinetics of the nonlysosomal transport of IgA by rat liver has recently been demonstrated (10). The evidence presented here strongly supports the existence of a "direct," nonlysosomal transport pathway for EGF, as well as for IgA. Furthermore, EGF, unlike IgA, utilizes a dual transport process. The physiological significance of this unusual system of intracellular processing is not yet clear. However, the liver's dual role in both conserving and destroying EGF may provide a homeostatic mechanism for maintaining EGF levels in liver, bile, gut, and plasma.

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