

Receptor-mediated Biliary Transport of Immunoglobulin A and Asialoglycoprotein: Sorting and Missorting of Ligands Revealed by Two Radiolabeling Methods

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ABSTRACT In the rat, all receptor-bindable immunoglobulin A (IgA), and 1–4% of injected asialoglycoprotein (ASG), are transported from blood to bile intact. The major fraction of the ASG is degraded in hepatic lysosomes. The study described here was designed to elucidate the sorting that occurs in hepatocytes subsequent to receptor binding of ligands not sharing the same fate. We show that conjugation of protein with the Bolton and Hunter reagent can be used as a probe for the lysosomal pathway, since 50% of the reagent is released into bile after lysosomal degradation of internalized protein. Radiolabeling by iodine monochloride was alternatively used to follow the direct pathways that deliver intact IgA and ASG to bile.

After intravenous injection of labeled proteins, first intact ASG and IgA, and then radioactive catabolites from degraded protein, were released into bile. No proteolytic intermediates were detected, and the transport of IgA or ASG directly to bile was not affected by the lysosomal protease inhibitor leupeptin. These observations indicate that divergence of the direct biliary transport pathways from the degradation pathway occurs at a stage preceding delivery to lysosomes, possibly at the cell surface. Competition studies showed that all three pathways (including the biliary transport of intact ASG) are receptor mediated, but even at supersaturating doses the uptake and processing of IgA and ASG occur independently. We propose that IgA and ASG receptors are not frequently in juxtaposition on the plasma membrane, but that ASG, after binding to its receptor, is occasionally missorted into the biliary transport pool.

Proteins endocytosed by liver parenchymal cells can be processed in one of three ways: they can be transported to bile, returned to blood, or transferred intracellularly to lysosomes, where they are degraded. Clearly, a fundamental question in cell biology is how and where the cell separates proteins with different ultimate destinations: at the cell surface before endocytosis, or in an intracellular compartment. Proteins using different pathways in the liver have not yet been studied together in the same experimental system. We have chosen to investigate the quantitative direct biliary transport of polymeric immunoglobulin A (IgA)¹ (1–3) and the uptake into the degradative pathway of asialoglycoproteins (ASG) (4), and we describe biochemical probes that permit the simultaneous

examination of both pathways. The choice of these ligands is ideal, because in the rat, short-term clearance of both proteins is the exclusive responsibility of liver hepatocytes (4–8).

Relevant aspects of these pathways previously described are as follows: IgA binds to a high-affinity receptor specific for polymeric immunoglobulins on the hepatocyte sinusoidal membrane (9, 10). This receptor is identical to the secretory component (SC) found attached to transported IgA, except that it has an additional sequence, presumably to anchor the binding site in the lipid bilayer (11, 12). The ligand-receptor complex is endocytosed into 100-nm vesicles which then migrate to the bile canaliculus (13, 14), whereupon secretory IgA is released intact into bile, leaving the cleaved hydrophobic portion of the receptor in the canalicular membrane. Transport and biliary release of the receptor is not dependent on the presence of ligand (15) and both ligand and receptor are univalent (16, 17), implying that bridging of receptors by

¹ *Abbreviations used in this paper:* ASFet, asialofetuin; ASG, asialoglycoprotein(s); ASOr, asialo-orosomucoid; BH, Bolton and Hunter (reagent); IgA, immunoglobulin A; SC, secretory component.

the ligand is not required for uptake of IgA. It has been postulated that the physiological role of this process is to salvage IgA that has been synthesized locally at the mucosa but that has escaped into lymph (18).

Mechanistically, the initial steps in the processing of ASG are similar: ASG binds to a high-affinity transmembrane receptor that recognizes terminal galactose residues, and is endocytosed through a coated pit as a ligand-receptor complex, into a small vesicle (19). Ongoing endocytosis of ASG receptors also appears to occur in the absence of ligand (20, 21). However, unlike IgA-SC complexes, the ligand dissociates from its receptor shortly after endocytosis, the receptor is recycled back to the plasma membrane, and the ligand is transferred to lysosomes for degradation (22–26). Recently, Geuze et al. (27) have shown that ASG is separated from its receptor in an intracellular compartment subjacent to the plasma membrane. Differential binding and processing of ASG depending on concentration and glycan structure suggests that bridging by ligand of receptor binding sites may play a role in the uptake and intracellular routing of receptor-bound protein (28–32). The physiological relevance of ASG processing is unknown, but it could play a role in clearing senescent plasma proteins which have lost sialic acid from their oligosaccharide chains.

In this paper, we make use of two radiolabeling methods to monitor endocytosis and subsequent intracellular processing. Labeling with the Bolton and Hunter (BH) reagent provides a novel and unique approach for the study of the lysosomal degradation pathway simultaneously with the direct biliary transport of IgA, and has facilitated a detailed analysis of the independence of the two processes. In addition, we found in the course of this study that a small proportion of injected ASG appears in bile as intact protein. This has been largely ignored in the past, mainly because morphological techniques do not lend themselves well to the study of minor pathways. We include a detailed biochemical examination of this phenomenon here, because we observed that biliary transport of ASG is also receptor mediated, and this has important implications for the level of precision in the sorting process.

MATERIALS AND METHODS

Proteins: Rat monoclonal dimeric IgA was isolated from ascites fluid of the plasmacytoma lines IR699 and IR22, kindly provided by H. Bazin of the University of Louvain, Brussels, by ammonium sulfate and octanoic acid precipitation (33). Human IgA1 was isolated from myeloma sera in a similar fashion. The polymeric fractions of all IgA preparations were recovered by subsequent chromatography on Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden; 2.6 × 90 cm). Human IgG from myeloma serum was purified using ammonium sulfate and octanoic acid and chromatographed on Sephadex G-200 (Pharmacia Fine Chemicals; 2.6 × 90 cm).

Human albumin was obtained from the Hoechst Pharmaceutical Co., Kansas City, KS. Samples of human orosomucoid (α_1 -acid glycoprotein), prepared by the method of Hao and Wickerhauser (34), were generous contributions from H. Schachter of the University of Toronto, and from the American Red Cross Blood Services Laboratory, Bethesda. Human fetuin was purchased from the Sigma Chemical Co., St. Louis. ASOr and ASFet were prepared by mild acid hydrolysis (35): Solutions of the protein in water (orosomucoid: 4 mg ml⁻¹; fetuin: 75 mg ml⁻¹) were heated to 80°C and then made 0.1 N in H₂SO₄. After 45 min, the hydrolysis was stopped by the addition of two equivalents of solid Tris; the solution was cooled and then dialysed into PBS (0.15 M NaCl, 1 mM potassium phosphate, pH 7.4). Chromatography on Sepharose 6B revealed that aggregation or fragmentation of the proteins as a result of this procedure was <5%. The remaining hydrolysable sialic acid measured by the thiobarbituric assay (36) was <1% for orosomucoid and 1–7% for fetuin. Inhibition experiments described in Fig. 7 were carried out using both ASFet prepared by acid hydrolysis and ASFet prepared with neuraminidase: 400 mg of fetuin in 5 ml of 0.1 M acetate buffer, pH 5.6, was incubated at 37°C for 20

h with 0.5 ml of agarose-linked neuraminidase, purchased from Sigma Chemical Co. Treatment with solid-phase neuraminidase resulted in the release of 54% of the total hydrolysable sialic acid. These two preparations were shown to compete in a reciprocal fashion for hepatic uptake and processing.

Radiolabeling and Preparation of Samples for Injection: Immunoglobulin and albumin preparations were treated with 25 mM iodoacetamide for 30 min in PBS (pH 7.4) at room temperature to remove reducing activity. Proteins were labeled using iodine monochloride prepared in our laboratory (37), at a final substitution ratio of less than one atom per molecule protein. Free radioiodide was removed by exhaustive dialysis against PBS containing 0.075% NaI, and then PBS alone. Radioactivity in preparations of protein labeled using ICl was >98.5% precipitable in 15% trichloroacetic acid; the specific activity was typically 10⁹–10¹⁰ cpm mg⁻¹.

BH reagent was prepared according to published methods (38), or purchased from Amersham Corp., Arlington Heights, IL. On the day preceding a transport experiment, 10–20 μ l of reagent (2 mCi/ml benzene) was dried in a small test tube, and 0.1–0.5 mg of protein in 0.2 ml of 1 M Tris buffer, pH 8.0, was added. Labeling proceeded at 0°C for 30 min with periodic mixing. Preparations were dialysed overnight against PBS containing 0.1% glycine and then twice against PBS alone. Within 3 h of injection, the protein was passed over a Sephadex G-50 column (0.5 × 12 cm) to remove any trace of free reagent, and an aliquot was saved as a standard. The specific activity was 10⁷–10⁸ cpm mg⁻¹. IgA or ASG labeled using the BH reagent showed <20% radioactive decomposition upon incubation in plasma for 8 h at 38°C, or in bile or serum for 1 d at room temperature or 8 d at 4°C, when assessed by gel filtration. For unusual occasions where standard aliquots of stock labeled material showed >10% decomposition at the time when bile samples were to be analysed chromatographically, the clearance and transport data were excluded from this study.

Between 10⁴ and 10⁶ cpm of ¹²⁵I- and ¹³¹I-labeled proteins were mixed in 200 μ l of PBS and precounted before injection into each animal. This corresponded to 0.01–100 μ g, depending on the method of labeling employed. Data were corrected by computer for isotopic overlap and radioactive decay.

Clearance and Transport Experiments: Male Wistar rats (250–350 g) were purchased from Charles River Canada, St. Constant, Quebec, and maintained on a synthetic Basal Rat Diet, produced by Bioserv Inc., Frenchtown, NJ. Each rat was anesthetized with sodium pentobarbital and maintained in a supine position throughout the transport experiment. The femoral vein was cannulated with PE-10 Intramedic polyethylene tubing (Clay-Adams, Parsippany, NJ), and the bile duct was cannulated proximal to the pancreatic duct with 20 cm of PE-50 tubing, extending through a mid-line abdominal incision. Animals were allowed to recover from the operation for ~1 h. The mixture of radiolabeled proteins was injected through the femoral vein cannula, and bile samples were collected for regular intervals (usually 10 min) until the experiment was terminated at 4 h. The mean rate of bile production was 13 μ g min⁻¹ (range, 7–21 μ g min⁻¹). Leupeptin inhibition experiments were performed in rats weighing 200–250 g by injecting 5 mg of the drug (Sigma Chemical Co.) in 300 μ l PBS intravenously 1 h before the radioactive sample (39). For calculations of the kinetics of biliary transport, the time after injection was taken from the midpoint of the collection interval of each bile sample. This was corrected for the time required to pass through the bile cannula, but not for the time required for the bile to flow from the hepatocyte canalicular face down the ductules to the cannular opening, which requires an interval on the order of a few minutes. Total radioactivity in blood was calculated assuming a blood volume of 20 ml/300 g body wt.

For experiments in which clearance from the circulation was also monitored, PE-10 tubing was brought into the abdomen through a small incision medial to the thigh, and the left common iliac artery was cannulated just below the aortic bifurcation. Just before the sample was injected into the femoral vein, 25 U of heparin (Sigma Chemical Co.) in 300 μ l of 0.89% saline were administered through the aortic cannula. During the course of the experiment, up to 10 blood samples of ~75 μ l were withdrawn (each sample was collected within 15 s). 50 μ l was measured accurately from each aliquot, and combined with 50 μ l of heparinized saline. For all proteins except ASG, clearance half-times were calculated as the time required to reach half the level of activity observed in the blood sample taken 1 min after injection. For ASG, first clearance half-times were extrapolated from the first sample taken at 1 min, using the injected activity to calculate initial concentration. For clearance half-time calculation only, an allowance was made for a nonclearable IgA fraction (ICl-IgA: 13 ± 3% (mean ± SD); BH-IgA: 23 ± 3%), based on the fraction of injected activity in the circulation at the end of the experiment. This fraction was still precipitable in 15% trichloroacetic acid.

In inhibition studies, 100 mg of protein (75 mg/ml PBS) was infused through the femoral vein cannula during the 10 min preceding injection of the radioactive sample, which was then followed by continuous infusion of a further 50–75 mg over the next 1.5 h. V_{peak} (the peak rate of transport of radiolabel to bile) was calculated on the basis of 10-min collection intervals, and the rate at

150 min was compared by computer with V_{peak} in units of percent injected radioactivity per gram bile. Standard errors and p values shown for $\%V_{peak}$ at 150 min in Fig. 7 were calculated on the basis of the reciprocal logarithm of the relative rate. In all cases where the observed difference between inhibition experiments and autologous controls was significant by Student's t -test, the difference was also significant by Wilcoxon's test at least at the $p < 0.05$ level.

In Vitro Binding Experiments: Human secretory component (SC), prepared by affinity chromatography of colostrum whey on IgM-Sepharose (40), was coupled directly to Sepharose 4B activated with BrCN (41). Trace radio-labeled IgA in 200 μ l of PBS containing 4 mg of bovine albumin per ml was incubated overnight with 50 μ l of SC-Sepharose at room temperature before being washed and counted.

Analysis of Bile and Blood Samples: Radioactivity in bile and plasma was counted and then analysed by gel filtration, typically within 48 h of the original experiment, on Ultrogel ACA-44 (purchased from LKB, Bromma, Sweden; see Fig. 2), or on Sephadex G-200 or Sepharose 6B (1.6 \times 90 cm at 12 ml h^{-1} , PBS, 4°C; sample size <1.0 ml, fraction size 2.0 ml). Radiolabeled protein in plasma to be used for reinjection was recovered by making the sample 40% saturated in $(NH_4)_2SO_4$ and dissolving and dialysing the collected precipitate into PBS. Radiolabeled protein in bile to be used for reinjection or analysis by gel electrophoresis was recovered by passing the sample over an Ultrogel ACA-44 column equilibrated in 0.01% ammonium acetate, pooling the included peak of radioactivity, and freeze-drying. 1–10 ng of radiolabeled asialo-orosomucoid (ASOr) was recovered per pooled bile sample. Slab gel electrophoresis in SDS was carried out by the method of Laemmli (42), and autoradiograms were generated using Kodak X-omatic enhancing screens. Molecular weight standards were generously provided by R. Allore of the University of Toronto.

RESULTS

Uptake and Processing of IgA and ASG

Asialo-orosomucoid (ASOr) and asialofetuin (ASFet) were

radioiodinated either directly on tyrosine residues using iodine monochloride (ICI) (37), or by conjugating primary amino groups (mainly ϵ -amino groups of lysine residues) with 3-(4-hydroxy 5-[^{125}I]iodophenyl) propionate by using its N -hydroxysuccinimide ester, the BH reagent (38). ICI- or BH-ASG was injected with ICI-IgA into rats intravenously, and the kinetics of clearance from blood and transport to bile were monitored. All preparations of IgA and ASG were cleared rapidly from the circulation (Fig. 1, Table I). 67% of injected ICI-IgA was transported to bile within 150 min of injection. This corresponded (within experimental confidence limits) to the fraction of the IgA preparations able to bind to SC-Sepharose (data not shown). The amount of radioactivity originally associated with ICI-ASG detected in bile after intravenous injection represented only 2–6% of the injected amount, and peaked in bile \sim 10 min before IgA (Fig. 1, C and E). In contrast, \sim 50% of the radioactivity originally associated with BH-ASG was transported to bile, and reached a maximum transport rate \sim 7 min later than IgA (Fig. 1, D and F).

Fractionation of Radioactivity Recovered in Bile

Radiolabel from BH-ASG evidently was being processed differently than radiolabel from ICI-ASG. It was therefore important to investigate whether this reflected differential processing of radioactive catabolites after ASG was degraded, or whether the processing of ASG was itself affected by the method of labeling. To address this issue, we analyzed bile samples from transport experiments by gel filtration chro-

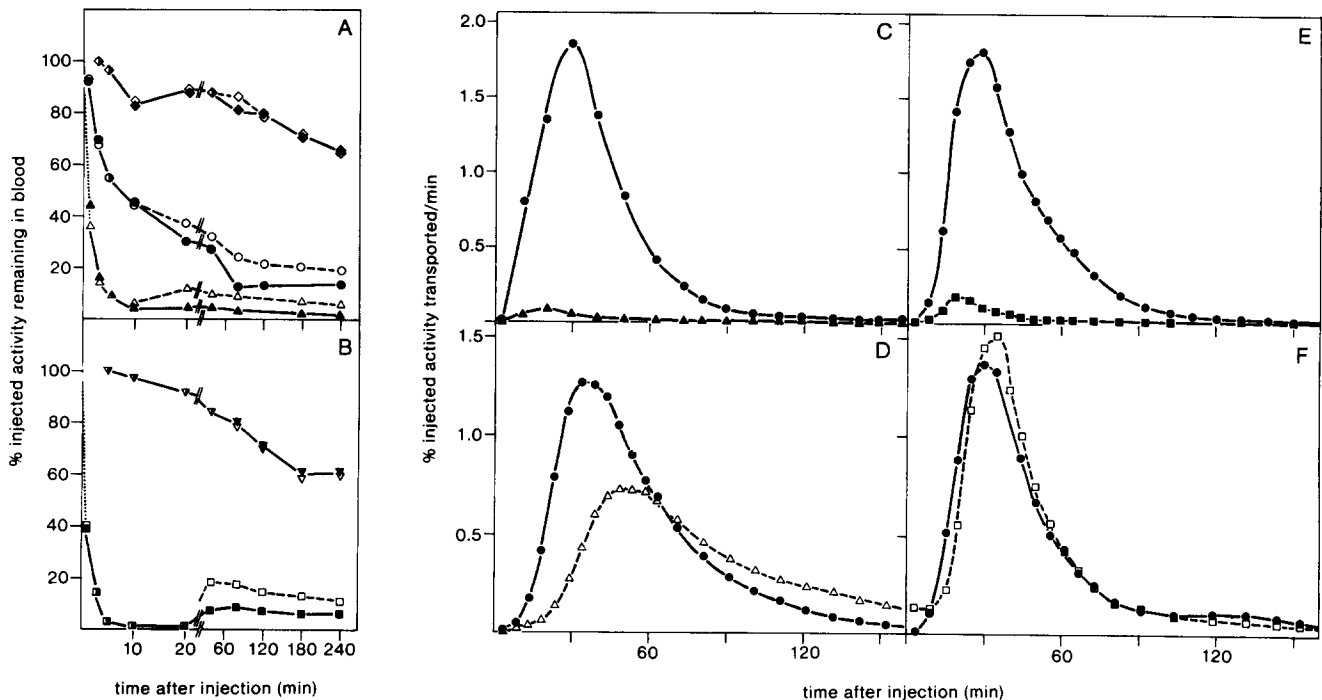


FIGURE 1 Clearance from blood and transport to bile of radioactivity from labeled proteins. A and B show the kinetics of disappearance of radioactivity from blood for several representative experiments in which two aliquots of a single protein were alternatively labeled with [^{125}I]BH reagent or [^{131}I]ICI and injected together. C–F show the results of individual transport experiments, in which IgA and asialoglycoprotein were labeled with alternative isotopes and injected together: C and E show the kinetics of appearance of radioactivity in bile when ICI-IgA was injected with ICI-ASFet (C) or ICI-ASOr (E); in D and F, the respective asialoglycoproteins were labeled with the BH reagent. % injected dose transported to bile/min is plotted against the elapsed time at the midpoint of the collection interval for each bile sample, which has been corrected for the time required for the sample to pass through the bile cannula. Symbols used in all figures: (●, ○) rat dimeric IgA; (◆, ◇) human IgG; (▲, △) ASFet; (▼, ▽) native fetuin; (■, □) ASOr. Closed symbols are used to indicate results from protein labeled with ICI; open symbols denote proteins labeled with the BH reagent.

matography (Fig. 2). The elution volume of ICI-IgA in bile indicated that it had been transported >98% intact. However, radioactivity recovered from BH-ASG eluted in two positions: a small fraction appeared at an elution volume corresponding to that of the injected material, and the bulk of the radioactivity eluted near the void volume. In fact, the radioactivity still bound to ASG in bile represented about the same percentage of the injected dose regardless of which method had

been used for radiolabeling (Fig. 3). This suggested that the protein was being processed identically in both cases, but that subsequent to lysosomal degradation of ASG labeled using the BH reagent, the radioactive tag (or a derivative) was being excreted through the biliary tract.

TABLE I
Kinetics of Uptake and Biliary Transport of Radiolabeled Proteins

	Time of maximal appearance of radiolabel in bile <i>min</i>	Clearance half-time of radiolabel from blood <i>min</i>	Number of clearance experiments
ICI-IgA	34.9 ± 1.5	5.1 ± 0.4	6
BH-IgA	33.2 ± 2.5	5.0 ± 1.4	2
ICI-IgG	—	>240	3
BH-IgG	—	>240	2
ICI-ASFet	22.6 ± 2.4	0.8 ± 0.1	3
BH-ASFet	41.2 ± 3.9	0.9 ± 0.1	2
ICI-Fet	—	>240	3
BH-Fet	—	>240	2
ICI-ASOr	24.8 ± 2.3	0.7 ± 0.1	2
BH-ASOr	40.4 ± 4.0	0.7 ± 0.1	3
ICI-HSA	—	>240	3
BH-HSA	—	>240	3

The time of peak transport rate of radiolabel to bile is calculated for the experiments shown in Fig. 3. For each experiment, this time was taken as the midpoint of the 10-min interval when the transport rate (in % injected radiolabel/g bile) was highest. First half-time of clearance of radiolabeled protein from blood was measured via a cannula of the iliac artery in the number of experiments shown at right. Uncertainty shown is mean ± SE when $n > 2$, or mean ± range where $n = 2$.

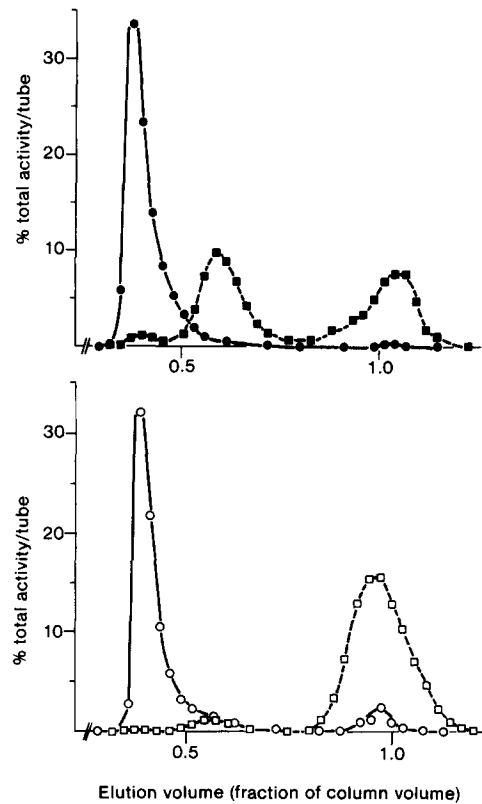


FIGURE 2 Gel filtration analysis of radioactivity transported to bile from labeled IgA and ASOr. Elution position from Ultrogel ACA-44 columns is shown as a fraction of the total column volume: (●) ICI-IgA; (■) BH-IgA; (○) BH-ASOr; (□) ICI-ASOr. Column conditions: 1.6 × 35 cm at 20 ml h⁻¹ in 0.15 M NaCl, 20 mM Tris, pH 8.0, 0.02% NaN₃ at 4°C; sample size <0.5 ml, fraction size 2.0 ml.

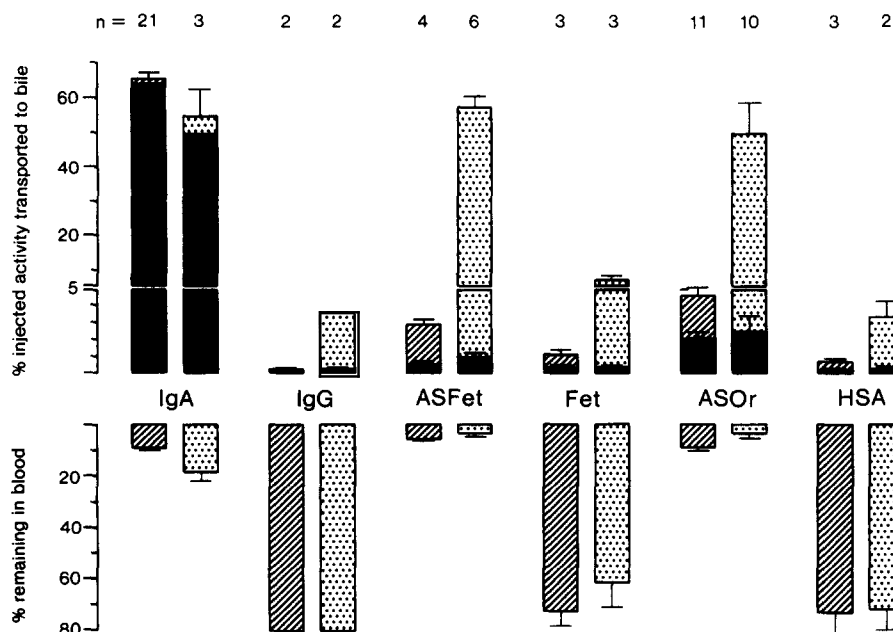


FIGURE 3. Biliary transport of total radioactivity from labeled proteins. The labeling reagent used was ICI (hatched bars) or the BH reagent (speckled bars). *Top*: accumulated radioactivity in bile at 150 min after injection (mean ± SE, or mean ± range where $n = 2$). Samples were analyzed by gel filtration as in Fig. 2 for the fraction of radioactivity attached to intact protein; this is indicated within each bar by solid shading. The remaining area corresponds to radioactivity that eluted after 70% of the column volume, representing low molecular weight catabolites. The scale of percentage transport to bile has been expanded in the 0–5% range to display the amount of protein-associated radioactivity transported to bile from labeled ASG and control proteins. *Bottom*: radiolabel remaining in blood at the end of the experiments (total radioactivity; the subtraction that is protein-bound is not indicated). IgA, rat dimeric IgA from plasmacytoma line IR699; HSA, human serum albumin.

remaining in blood at the end of the experiments (total radioactivity; the subtraction that is protein-bound is not indicated). IgA, rat dimeric IgA from plasmacytoma line IR699; HSA, human serum albumin.

To verify that the biliary appearance of low molecular weight radioactive material from BH-ASG was reflective of an event subsequent to receptor-mediated endocytosis, and not due to spontaneous dissociation of the label in blood, we then used the BH reagent to label a number of proteins that are known not to be removed from the circulation. In the subsequent transport experiments (Fig. 3), injection of labeled IgG, native fetuin, and human albumin resulted in <6% of the radioactivity appearing in bile; as expected, most of the injected radioactivity was recovered in blood. When analyzed, the activity remaining in blood was found to be at least 95% protein bound (data not shown).

We then wanted to show that hepatocellular endocytosis of BH-labeled protein did not by itself result in release of most of the injected radioactivity into bile as low molecular weight material. Thus, in the next experiment we tested the appearance in bile of radiolabel from BH-IgA, since IgA is endocytosed and transported without degradation. Radiolabel recovered from bile from these experiments was found by gel filtration chromatography to be >90% protein bound, as predicted (Fig. 2, lower graph). However, it was necessary to explain why BH-IgA was cleared from blood and transported to bile slightly less well than ICI-IgA (Fig. 3). Previous experience in our laboratory has indicated that the binding of polymeric immunoglobulins to SC (the putative transport receptor binding region) is acutely sensitive to perturbations in immunoglobulin conformation, and we suspected that BH labeling was functionally inactivating a small proportion of the IgA molecules that otherwise would be able to bind to the receptor. To test this possibility, identical samples of IgA were concurrently labeled using [¹²⁵I]BH reagent or [¹³¹I]. The fraction of these preparations that could bind to human-SC Sepharose was 53% and 73%, respectively. The preparations were then injected together into rats, and radioactivity still in blood at 4 h was recovered and reinjected into a new animal. The reinjected BH-IgA and ICI-IgA were not cleared from the circulation of the second animal, and only ~6% of either isotope was transported to bile. This demonstrated that IgA remaining in the circulation in the initial experiment was unable to bind to receptors in the liver, and thus represented functionally inactive protein. The small percentage difference in the clearance from blood and subsequent biliary transport of BH-IgA and ICI-IgA is therefore an artifact of the labeling technique; once bound by SC on the hepatocyte, either preparation is transported quantitatively to bile.

We next turned our attention to the kinetics of appearance of the small fraction of intact ASG in bile. [¹²⁵I]BH-ASG and [¹³¹I]ICI-ASG were injected together into the same animal, and timed aliquots of bile were analyzed individually by gel filtration (Fig. 4). This revealed that the protein-bound radioactivity transported was the same proportion of the injected dose for both preparations, demonstrating that the transport of intact ASG to bile was independent of the method of labeling. The total radioactivity from ICI-ASG peaked at the same time as protein-bound radioactivity from either preparation, and established ICI-ASG as a useful direct probe for kinetic studies on the transport of intact protein to bile. For BH-ASG, early bile samples were relatively enriched in protein-bound radioactivity, but low molecular weight catabolites peaked later, reflecting the protein processed by the degradative pathway.

It was therefore clear that BH-labeled protein behaved like ICI-labeled protein up to the point where ligands transported to bile intact are separated from ligands destined for lyso-

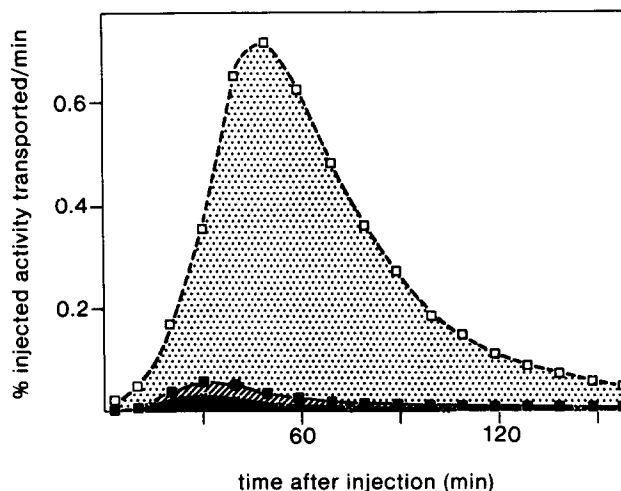


FIGURE 4 Kinetics of transport of protein-associated and unassociated radioisotope to bile after injection of labeled ASG. [¹²⁵I]BH-ASOr and [¹³¹I]ICI-ASOr were injected together and timed samples of bile were analyzed on Ultragel ACA-44 as in Fig. 2 for the proportion of radiolabel associated with intact protein (radioactivity eluting at <70% of the column volume) or present as low molecular weight catabolites (eluting at >70% of the column volume). (□) Total transported radioactivity from BH-ASOr; (■) total transported radioactivity from ICI-ASOr; (X) protein-associated radioactivity from BH-ASOr or ICI-ASOr (superimposable).

somes. To gain more direct evidence that biliary release of low molecular weight radioactivity from BH-ASG reflected differential handling of radioactive catabolites subsequent to lysosomal degradation of the protein, we made use of leupeptin, a thiol protease inhibitor that affects the activity of cathepsins (43), thus reducing lysosomal degradation of ASG (39, 44). As shown in Fig. 5, prior administration of leupeptin resulted in the accumulation of radiolabel from injected ASG in the liver, and a decrease in the release of radioactive catabolites from BH-ASG into bile. Thus, release of these catabolites requires lysosomal degradation of the protein. In contrast, there was only a slight increase in the total biliary transport of intact IgA and ASG (not statistically significant), showing that direct transport of proteins to bile is quantitatively unaffected by the modulation of lysosomal function.

Analysis of Labeled Protein Recovered from Bile

To characterize the material that was transported to bile, we recovered the protein-bound radioactivity by gel filtration on Ultragel ACA-44 and then analyzed it by SDS PAGE (Fig. 6). Prolonged exposure of the autoradiograms generated no evidence for proteolytic intermediates large enough to be included in the column and fixed in the gel. Calibration of the Ultragel columns showed that insulin was just barely excluded from the peak area pooled for SDS gel analysis; this established a maximum size for proteolytic fragments of IgA or ASG that could be present in bile. When ASOr transported to bile was passed over a 1.6 × 90 cm Sephadex G-200 column, its elution volume was identical (within 1% of the column volume) to a simultaneously run ASOr standard. IgA and ASOr found in bile therefore showed no evidence of degradation.

To demonstrate that the ASG transferred to bile was not a particular subfraction of the preparation predestined for biliary transport, we recovered intact ICI-ASOr transported to

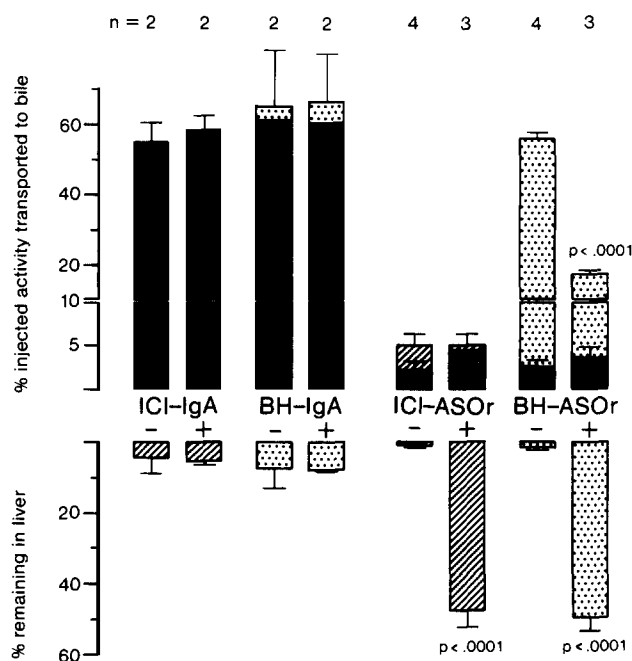


FIGURE 5 Effect of leupeptin on the transport of radiolabeled material through the liver into bile. Transport through the liver of proteins with (+) or without (-) pretreatment of the animals by administering 5 mg of leupeptin intravenously, 1 h before injection of the radioactive sample. Radioactivity in bile was analyzed by gel filtration to determine the fraction still associated with intact protein; this is shown within the bars on the top line of the figure by solid shading. The bottom line shows total radiolabel remaining in liver: the liver was perfused in situ through the portal vein with ice-cold saline, and then the whole organ was removed for counting. IgA, rat dimeric IgA from plasmacytoma line IR22; *p*, probability of equal means for control and leupeptin experiments with the same ligand, calculated by the two-sided Student's *t*-test.

bile from one experiment to use it in a subsequent study: If the ASOr recovered from bile was indeed a special subfraction of the original preparation, then it should transport almost completely to bile when reinjected into a second animal. When this experiment was performed, the reinjected ASOr was cleared from the circulation of the second animal, but again only a small fraction (4.8%) of the dose was transported to bile intact.

IgA and ASG Uptake and Processing Are Not Cross-inhibitable

To determine whether IgA and ASG receptors are sufficiently close on the plasma membrane of the hepatocyte for cross-modulation of endocytosis to occur, we performed a series of inhibition experiments. Labeled protein was injected intravenously and bile was monitored for radioactivity, using the following probes: ICI-IgA for the IgA transport pathway; BH-labeled glycoprotein for the lysosomal degradation pathway; and ICI-labeled glycoprotein for the minor (direct biliary) pathway of ASG transport. 10–15 min before injection of labeled material, 100 mg of one of several potential competing proteins was infused per 300 gram body weight. This dose was selected to ensure the presence of a large excess of inhibitor, so that even low-affinity and less accessible receptors would be as saturated as possible. 100 mg in 10 ml of plasma is equivalent to $\sim 2 \times 10^{-4}$ M for ASFet and 3×10^{-5} M for

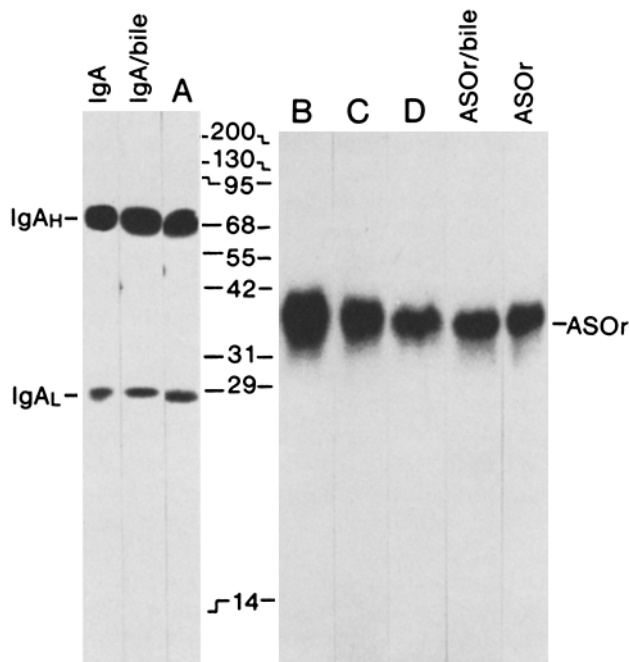


FIGURE 6 SDS gel autoradiograms of radiolabeled IgA and ASOr recovered from bile. Samples were reduced in 2% β -mercaptoethanol and run on gels 12.5% in acrylamide. Tracks in which labeled IgA or ASOr was run in the presence or absence of endogenous protein from rat bile are shown, alongside protein recovered from the bile of animals injected with BH-IgA (experiment A) or ICI-ASOr (experiments B–D). Indicated at the sides are the mobilities of unlabeled IgA and ASOr, and the apparent molecular weights of proteins in a standard marker mix, which were run on the same gels and detected by staining with Coomassie Blue R-250. Molecular weight standards ($\times 10^{-3}$, in order of decreasing molecular weight) were: myosin heavy chain, β -galactosidase, phosphorylase a, bovine albumin, isolated heavy chain from human IgG1, actin, bovine pancreatic DNase I, carbonic anhydrase, and cytochrome c. IgAH and IgAL, heavy and light chains from rat dimeric IgA.

IgA. To maintain a high concentration, infusion of the competitor was continued at ~ 40 mg h^{-1} as the experiment progressed.

In experiments where arterial blood was monitored for uptake of labeled proteins, neither IgA nor ASG showed significant increase in clearance half-time when the animals were preinfused with the alternative ligand, when compared with control infusions with bovine albumin. This indicated that these two proteins did not compete at the level of receptor binding. We then investigated whether there was any cross-inhibition after the molecules had bound at the cell surface. Data from bile transport experiments were analyzed in terms of both the cumulative percent of injected dose transferred to bile and the shape of the transport profile (Fig. 7): When a trace dose of radiolabeled IgA or ASG is processed by the liver, the rate of transport to bile peaks rapidly and recovers to a few percent of the peak value within a few hours of injection. A competing protein that partially inhibited processing could cause a decrease in the maximum transport rate (V_{peak}) and delay this recovery, without ultimately reducing the size of the fraction transported. The recovery from peak transport rate at a predefined timepoint can therefore be used as a sensitive kinetic indicator of inhibition.

Results of this analysis (Fig. 7, right) show that prior infusion of unlabeled ASFet was able to inhibit the processing of

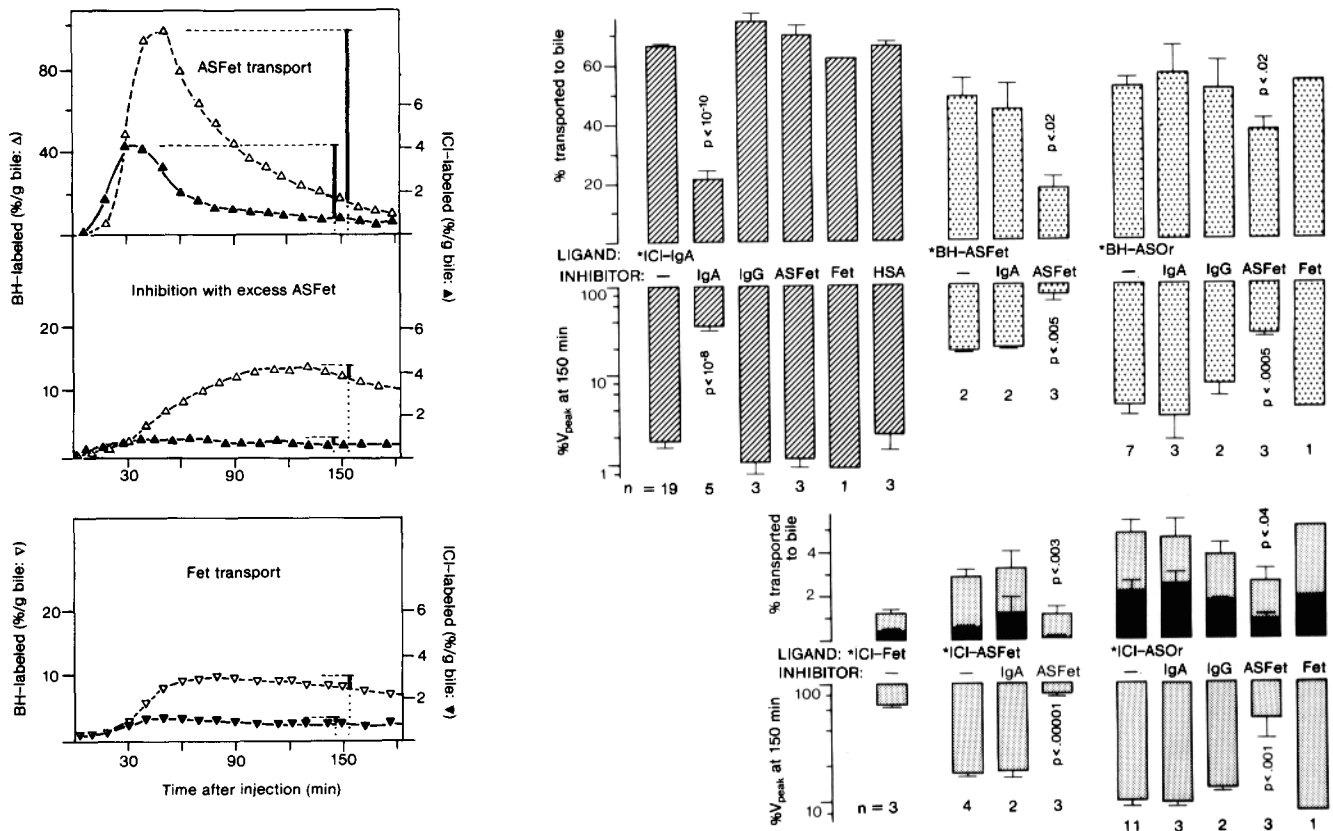


FIGURE 7 Ligand inhibition experiments. *Left*: biliary transport of radiolabel from a trace dose of labeled ASFet, compared with transport of ASFet in the presence of excess competing ligand and transport of the nondesialylated molecule (*Fet*). Radioactivity from ICI-labeled protein reflects direct transport; activity from BH-labeled protein principally reflects the material processed by the degradative pathway. The vertical bars illustrate the use of recovery from peak transport rate (V_{peak}) as a kinetic indicator of specific inhibition. *Right*: cumulative radiolabeled in bile, and the relative recovery from V_{peak} at 150 min, calculated for inhibition studies conducted on the three hepatocellular transport pathways: (1) The biliary transport of IgA (using ICI-IgA as probe, hatched bars); (2) the lysosomal degradation pathway (using BH-labeled glycoprotein, speckled bars); and (3) the biliary transport of intact ASG (using ICI-labeled glycoprotein, tinted bars). Control experiments are compared with experiments conducted after infusion of 100 mg of unlabeled protein. Bile from experiments on the third pathway (tinted bars) was further analyzed by gel filtration chromatography for protein-bound radioactivity; this fraction is shown within the bars of transported radiolabel by solid shading. ICI-IgA (ligand), ICI-labeled rat dimeric IgA from the plasmacytoma line IR699; IgG and IgA (inhibitors), human monoclonal IgG and polymeric IgA; HSA, human serum albumin; p , probability of equal means when compared with control experiments with the same ligand, using the one-sided Student's t -test (see Materials and Methods). Where p values are not shown, comparisons with autologous control experiments were not significant at the $p < 0.05$ level by either the one-sided or two-sided test.

ASFet and ASOr, whether these proteins were labeled to reflect lysosomal degradation of ASG (speckled bars) or the transport of ASG intact to bile (tinted bars). This demonstrates that both pathways are mediated by a receptor specific for asialoglycoprotein. Inhibition of ASG processing by excess unlabeled ASFet was accompanied by a 21- to 64-fold increase in the circulating half-time of the labeled protein. Human polymeric IgA was able to inhibit the biliary transport of rat IgA dimer (hatched bars). In marked contrast, there was no quantitative or kinetic evidence for cross-inhibition between IgA and ASG processing, in terms of the degradation of ASG, or the transport of either protein to bile.

DISCUSSION

The Bolton and Hunter Reagent Is a Probe for Lysosomal Processing

All of the most frequently used methods of protein radioiodination, such as iodine monochloride, chloramine-T, and

lactoperoxidase, involve oxidation of iodide and direct substitution onto tyrosine residues. Labadie, Chapman, and Aronson (45) have demonstrated that lysosomal degradation of ASG in the hepatocyte leads to deiodination of iodotyrosine and subsequent return of the radioisotope to blood as iodide. The BH reagent contains a phenol group presubstituted with iodine, and conjugates to lysine residues by nucleophilic substitution. It was developed as a more gentle and versatile labeling method but results in the modification of the protein structure with a larger substituent. We have shown in this paper that in contrast to directly iodinated material, the lysosomal degradation of BH-ASG results in the release of half of the injected radioisotope into bile, mostly as material of <6,000 mol wt. The exact chemical nature of this catabolite is presently under investigation.

It is clear that BH labeling does not in itself affect the early events in protein processing, and that regardless of the labeling method employed most ASG is ultimately degraded in lysosomes: (a) BH-labeled IgA and ASG are cleared from blood at the same rate as protein labeled on tyrosine residues; (b)

uptake and biliary release of radiolabel from BH-ASG can be inhibited by excess unlabeled ASG; (c) IgA and a small proportion of injected ASG are transported to bile intact, regardless of the labeling method used; and (d) leupeptin, a lysosomal enzyme inhibitor, causes accumulation of the radiolabel from ASG in the liver, and (for BH-ASG) inhibits the release of radioactive catabolites into bile. The ability of the liver to recover iodide from iodotyrosine but not BH-lysine indicates a high degree of specificity for the microsomal enzymes described by Labadie et al. (45). Data that we will publish elsewhere show that hepatic uptake of BH-hemoglobin also results in the release of radioactive catabolites into bile: hence the release of catabolites apparently requires delivery to lysosomes but otherwise is not restricted to mediation by any particular receptor. Thus, the BH reagent provides an experimental probe for studying uptake and lysosomal processing of proteins by the hepatocyte.

The Major Metabolic Pathways of IgA and ASG Diverge Early

The experiments described in this paper were designed to examine the interdependence of IgA and ASG processing, in terms of both the receptors and the intracellular organelles involved in ligand sorting. Separate receptors have previously been described for the binding of these proteins to isolated hepatocytes, but recent *in vitro* binding data have been interpreted by Stockert et al. (46) to imply that the receptor for ASG participates in the uptake of IgA for biliary transport. The work described here is the first to demonstrate that when the ASG receptor is saturated there is no quantitative or kinetic effect on the transport of IgA to bile. The role of a separate receptor for IgA transport is therefore now conclusively established.

We know from other work that hepatocytes near the portal triad can participate in metabolism of both IgA and ASG. Individual cells must therefore sort the ligands according to one of three models (Fig. 8). First, both proteins may be delivered to lysosomes through a common route, but IgA may survive digestion and be subsequently transported to bile (47). Second, IgA and ASG may be endocytosed together and then sorted in a prelysosomal compartment: Material entering the degradative pathway is delivered shortly after endocytosis to intermediate vesicles of 200–500 nm (48–52); intermediate vesicles may also participate in the IgA pathway (53), and thus IgA-ASG sorting could occur at this stage. Third, IgA and ASG may be endocytosed from the plasma membrane directly into separate compartments.

The data presented in this paper eliminate the possibility that both proteins are delivered to lysosomes before sorting (Fig. 8, model 1): essentially all transportable IgA can be recovered in bile, with no detectable proteolytic intermediates. IgA is taken up more slowly but is excreted into bile more rapidly than most of the radiolabel from BH-ASG, presumably reflecting an additional time requirement for delivery to and from lysosomes of ASG and its catabolites. The most direct evidence against lysosomal involvement in the IgA pathway is provided by the failure of leupeptin to decrease the amount of IgA reaching bile. Leupeptin clearly affected lysosomal function in our experiments, since it prevented release of degradation products from BH-ASG and caused the accumulation of ASG in the liver. The ensuing conclusion is that lysosome-canalicular shuttling of intact protein does not occur.

Therefore two alternatives remain: that IgA-ASG sorting occurs in a prelysosomal vesicle, or that the two ligands are endocytosed separately from the plasma membrane (Fig. 8, models 2 and 3). Possible precedents for these models include

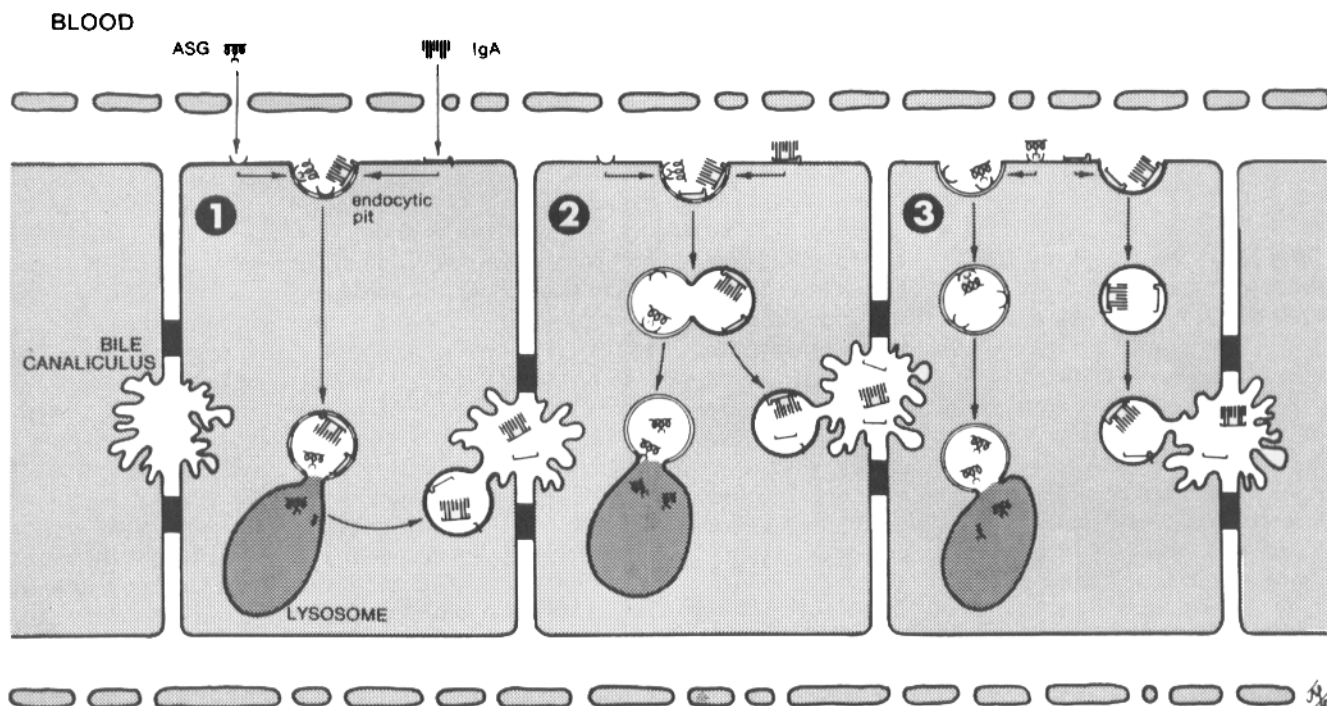


FIGURE 8 Models for the partitioning of IgA and ASG. Shown are the transport pathway of IgA from blood to bile, and the major ASG pathway which leads to ligand degradation. (1) Common transport through lysosomes. ASG is specifically degraded; IgA survives digestion and continues on to the canaliculus. (2) Endocytosis through common vesicles with subsequent prelysosomal sorting. (3) Endocytosis of different ligands into specific vesicles. Our data exclude the first model.

the following: Double-label studies using α_2 -macroglobulin, insulin, epidermal growth factor, iodothyronine, or low density lipoprotein have shown that certain ligands cluster together in common endocytic pits and can subsequently share intracellular vesicles (54–56). Intracellular sorting of endocytosed protein precedes return of integral membrane components back to the plasma membrane of the fibroblast (57). Receptor-bound IgG in transit from the intestinal lumen to blood, and other proteins endocytosed nonspecifically for lysosomal degradation, are taken up by intestinal epithelial cells into common vesicles and sorted intracellularly (58). On the other hand, some membrane antigens such as Thy-1 are excluded from coated pits (59). In placental epithelial cells, IgG designated for placental transport is internalized separately from IgG that is pinocytosed nonspecifically and degraded in lysosomes (60). The ASG receptor on liver cells can be induced to internalize without affecting transferrin or insulin receptors on the cell surface (61). Thus, there appear to be biological precedents for ligand sorting both before and shortly following endocytosis.

The cross-inhibition studies described in this paper can assist in the evaluation of these two alternatives for the partitioning of IgA and ASG in the liver. If the two ligands are endocytosed together through common coated pits, it is reasonable to expect that the binding of one ligand would interfere with the processing of the other. This is because (a) IgA (320,000 mol wt) is larger than its receptor (SC, 90,000 mol wt) and probably overlaps it sterically, thereby limiting interactions with membrane proteins directly adjacent; (b) binding of ASG to its receptor induces receptor aggregation (62); (c) covalent association of aggregated receptors is a necessary requirement for ligand uptake by other cells (63), implying that ligand-receptor aggregates undergoing endocytosis through a single pit are tightly packed; (d) endocytosis is slower and saturable at lower ligand concentration than the binding of ASG to its receptor (64); and (e) on the basis of the high surface density of SC (9), we estimate that potentially 5% of the sinusoidal surface area is covered with IgA at saturation: this could be sufficient to fill all coated pits participating in the endocytosis of ASG (65). Thus, if IgA and ASG were endocytosed into common vesicles, then saturation of SC by IgA could limit ASG-induced aggregation of its receptors or binding to receptors already clustered nearby; conversely, saturation of ASG receptors and the formation of ligand-receptor aggregates could limit binding of IgA to neighboring SC molecules or decrease the accessibility of SC to endocytic pits. Cross-interference of endocytosis would be measured as an increase in transport time to bile; inhibition of binding would be reflected both in transport time and in the rate of disappearance from blood.

Analysis of data from the inhibition studies revealed no evidence for cross-inhibition of either uptake or processing of IgA and ASG. Based on the arguments presented above, this suggests that the two ligands are endocytosed separately at the cell surface. The results are conceivably consistent with endocytosis of both ligands into common vesicles, but this is possible only providing the membrane receptor density or diffusion (not vesicle formation) is rate-limiting, and providing the fraction of IgA and ASG receptors clustered with each other on the membrane during endocytosis (including in coated pits) is small. In addition, the active steps in the intracellular processing of ASG have a lower capacity than the endocytic step (64). Since the biliary transport of radiola-

bel from ICI-IgA or BH-ASG is not kinetically cross-inhibitable, the handling of these two ligands after endocytosis must remain independent throughout intracellular processing.

In summary, this study demonstrates that proteins entering the bile transport and degradation pathways are recognized by separate receptors and sorted before reaching lysosomes; the data are most easily fitted assuming that the pathways share no intracellular compartments.

The Intact Transport of ASG from Blood to Bile Is Receptor-mediated

We have shown here that up to 4% of injected ASG is transported from blood to bile intact. Other workers (66–68) have described the non-receptor-mediated transfer or leakage of proteins including ASG into bile, with molecular weight-dependent and molecular weight-independent components. Nonspecific pinocytosis by hepatocytes accounts for at least part of this transfer, since injected horseradish peroxidase (for which the hepatocyte has no known receptor) can be detected in intracellular vesicles at about the rate expected from the known rate of fluid uptake (69, 70). For many of these proteins, clearance from the circulation is slow, and the ligand has up to several hours to reach bile via the nonspecific route. However, ASG has a circulating half-time of <1 min, and is transported to bile with a kinetic profile reflecting its rapid clearance. We have presented data in this paper demonstrating that intact ASG reaches bile through a receptor-mediated pathway specific for desialylated glycoprotein.

The key observation in reaching this conclusion is that persistence of glycoprotein in the circulation is not accompanied by an increase in the fraction transferred to bile. We achieved these conditions by injecting native fetuin, or by injecting desialylated protein in the presence of excess ASFet. If receptor recognition was not required for biliary transport of ASG, then the increased time that the glycoprotein was available in the circulation should have resulted in a concomitant increase in size of the fraction reaching bile. In fact, just the opposite occurred: the size of the transported fraction decreased significantly, indicating that biliary transport of intact ASG following injection of trace doses does indeed require receptor recognition. A precedent for receptor-mediated biliary transport of minor fractions of endocytosed protein has already been established: injected epidermal growth factor has been recovered in bile covalently attached to its receptor (71). We believe that a receptor-mediated minor pathway may account for the appearance in bile of trace amounts of other proteins primarily endocytosed for degradation (72–77).

In discussing the mechanism for biliary transport of intact ASG, it is again necessary to consider the possibility that intact protein in bile represents material that has been translocated through hepatocyte lysosomes but has survived degradation (Fig. 9, model 2). However, because of the absence of proteolytic intermediates of ASG in bile, because intact ASG appears in bile earlier than catabolites from BH-ASG, and because leupeptin does not markedly alter the proportion of injected ASG that pursues the intact transport pathway, we conclude that ASG in bile has bypassed lysosomes.

Two alternatives remain that do not invoke a special receptor or cell type for the biliary transport of intact ASG (Fig. 9, models 3 and 4): During the sorting of proteins that usually occurs before or just after endocytosis by hepatocytes, ASG

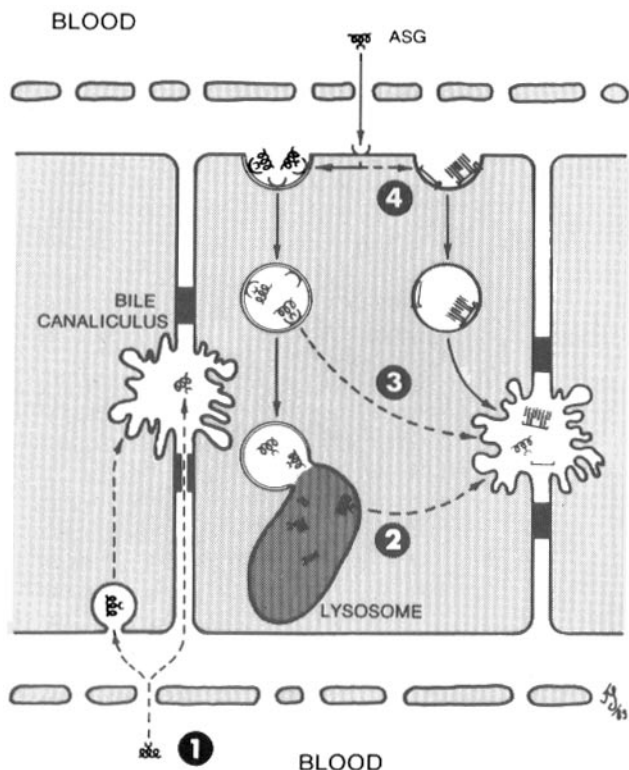


FIGURE 9 Models for transport of ASG intact to bile. Possible mechanisms for escape of ASG to bile (dotted arrows) are superimposed on a diagram of the principal specific transport pathways: (1) Nonspecific pinocytosis or leakage through tight junctions. (2) Specific transport through lysosomes, with a small proportion of endocytosed ASG surviving digestion. (3) Misdirection of ASG vesicles after IgA-ASG sorting is complete. (4) Receptor-dependent missorting of ligands: ASG is occasionally missorted into vesicles destined for the canaliculus; these vesicles may be (but are not necessarily) the same as those responsible for biliary transport of IgA. Models 3 and 4 are drawn assuming that ligand sorting occurs at the plasma membrane; analogous models can be drawn if ligand sorting occurs intracellularly, in which case the missorting of ASG could occur before or after release from its receptor. Of the models shown, only 3 and 4 are consistent with the data presented.

may occasionally be missorted into a different ligand-receptor pool, such as an IgA vesicle destined for the bile canaliculus. Alternatively, vesicles containing properly sorted ASG and normally destined for lysosomes may occasionally be misdirected and fuse with the bile canaliculus. Both of these models imply that the biliary transport of many proteins may have no essential physiological function, but occurs simply due to an inherent degree of error in the ligand sorting process. Occasional missorting may also result in the redirection of a fraction of internalized protein back to the surface from which it was originally endocytosed (78). Considering the complexity of metabolic pathways in the hepatocyte, it is perhaps not surprising that the fidelity of ligand partitioning is not complete.

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