Cell Surface Distribution and Intracellular Fate of Asialoglycoproteins: A Morphological and Biochemical Study of Isolated Rat Hepatocytes and Monolayer Cultures

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ABSTRACT A combination of biochemistry and morphology was used to demonstrate that >95% of the isolated rat hepatocytes prepared by collagenase dissociation of rat livers retained the pathway for receptor-mediated endocytosis of asialoglycoproteins (ASGPs). Maximal specific binding of ^{125}I-asialoorosomucoid (^{125}I-ASOR) to dissociated hepatocytes at 5°C (at which temperature no internalization occurred) averaged 100,000–400,000 molecules per cell. Binding, uptake, and degradation of ^{125}I-ASOR at 37°C occurred at a rate of 1×10^6 molecules per cell over 2 h. Light and electron microscopic autoradiography (LM- and EM-ARG) of ^{125}I-ASOR were used to visualize the surface binding sites at 5°C and the intracellular pathway at 37°C. In the EM-ARG experiments, ARG grains corresponding to ^{125}I-ASOR were distributed randomly over the cell surface at 5°C but over time at 37°C were concentrated in the lysosome region. Cytochemical detection of an ASOR-horseradish peroxidase conjugate (ASOR-HRP) at the ultrastructural level revealed that at 5°C this specific ASGP tracer was concentrated in pits at the cell surface as well as diffusely distributed along the rest of the plasma membrane. Such a result indicates that redistribution of ASGP surface receptors had occurred.

Because the number of surface binding sites of ^{125}I-ASOR varied among cell preparations, the effect of collagenase on ^{125}I-ASOR binding was examined. When collagenase-dissociated hepatocytes were re-exposed to collagenase at 37°C, 10–50% of control binding was observed. However, by measuring the extent of ^{125}I-ASOR binding at 5°C in the same cell population before and after collagenase dissociation, little reduction in the number of ASGP surface receptors was found. Therefore, the possibility that the time and temperature of the cell isolations allowed recovery of cell surface receptors following collagenase exposure was tested. Freshly isolated cells, dissociated cells that were re-exposed to collagenase, and perfused livers exposed to collagenase without a Ca^{++}-free pre-perfusion, were found to bind 110–240% more ^{125}I-ASOR after 1 h at 37°C that they did at 0 time. This recovery of surface ASGP binding activity occurred in the absence of significant protein synthesis (i.e., basal medium or 1 mM cycloheximide).

Suspensions of isolated, unpolarized hepatocytes were placed in monolayer culture for 24 h and confluent cells were demonstrated to reestablish morphologically distinct plasma membrane regions analogous to bile canalicular, lateral, and sinusoidal surfaces in vivo. >95% of these cells maintained the capacity to bind, internalize, and degrade ^{125}I-ASOR at levels comparable to those of the freshly isolated population. ASOR-HRP (at 5°C) was specifically

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bound to all plasma membrane surfaces of repolarized hepatocytes (cultured for 24 h) except those lining bile canalicular-like spaces.

Thus, both isolated, unpoliarized hepatocytes and cells cultured under conditions that promote morphological reestablishment of polarity maintain the pathway for receptor-mediated endocytosis of ASGPs.

The asialoglycoprotein (ASGP) receptor is localized exclusively to the parenchymal cells of mammalian liver and functions in the rapid removal of desialylated glycoproteins from the circulation by receptor-mediated endocytosis (1–5). The distribution of the ASGP receptor along the plasma membrane of hepatocytes *in situ* is restricted to the blood sinusoidal surface and is preferentially concentrated in coated pits present in this domain (6, 7). During the preparation of single hepatocytes by collagenase perfusion of the liver, cells lose the three morphologically identifiable plasma membrane domains (8, 9) (sinusoidal, bile canalicular, and lateral surfaces), yet retain the capacity to bind, internalize, and degrade ^{125}I-ASGPs (10–16). Previous studies of the location of the ASGP receptor in the isolated, unpolarized hepatocyte using fluorescence microscopy showed a diffuse distribution of ASGP binding over the entire cell (17), suggesting that redistribution of the ASGP receptor had occurred. However, the topographical distribution of this receptor at the ultrastructural level is not known. In addition, several laboratories have studied the pathway for receptor-mediated endocytosis of ASGPs in dissociated hepatocytes without knowing whether a representative population of the original surface receptors was still present or whether the enzymatic dissociation had depleted the surface pool. Finally, it is not yet known whether there is continued expression of the ASGP receptor on hepatocytes cultured under conditions that promote reestablishment of cell surface domains morphologically.

We have used a combined biochemical and morphological approach to address these issues. In the present report, we show that virtually all hepatocytes retain the pathway for receptor-mediated endocytosis of ASGPs after collagenase dissociation and that a representative number of the original surface population of ASGP receptors is preserved. In addition, we have localized the ASGP surface receptor on isolated cells at the ultrastructural level. Finally, we present evidence that the expression of this receptor and its activity are maintained in cultured hepatocytes.

MATERIALS AND METHODS

Reagents

Collagenase (Type 1, lot #108C-0062), 3-amino-1,2,4-triazole, 3,3′ diaminobenzidine (DAB) (grade II), insulin (bovine pancreas), hydrocortisone, glucose oxidase (type V), polyvinylpyrrolidone (PVP-40, pharmaceutical grade) and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). Lactoperoxidase (purified grade) was obtained from Calbiochem-Behring Corp. (La Jolla, CA), and glutaraldehyde, Ilford L4 and K5 emulsions from Electron Microscopy Sciences (Fort Washington, PA); carrier-free $Na^{125}I$ and {methyl-^{3}H}-thymidine (20 Ci/nmol) were obtained from New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, Hanks' balanced salt solution without Ca^{+2}, Mg^{+2}, and HCO_3^{-1}, penicillin/streptomycin (10,000 U/10,000 μg/ml) were obtained from Gibco (Grand Island Biological Co., Grand Island, NY). All other chemicals were reagent grade. Male Sprague-Dawley rats (120 gm) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Tissue culture dishes (35 mm) were obtained from Lux Scientific Corp. (Newbury Park, CA).

Solutions

(*1*) Ca^{+2}/Mg^{+2}-free Hanks' balanced salt solution (HBSS), pH 7.4

(*2*) 0.05% Collagenase in HBSS with 4 mM $CaCl_2$, pH 7.4

(*3*) Krebs-Henseleit (18) with 5 mM glucose, and with or without 2% PVP-40 (KH, KH-PVP-40), pH 7.4

Solutions 1–3 were continuously oxygenated with 95% O_2/5% CO_2 during use.

(*4*) Culture medium: Dulbecco's MEM with 17% heat-inactivated fetal bovine serum (FBS), 0.5 μg/ml insulin, 20 mM glucose, 1 μM hydrocortisone, penicillin/streptomycin (100 U/100 μg/ml) (DMEM)

Preparation of Dissociated Hepatocytes

Sprague-Dawley rats were starved overnight before use. The liver was first perfused *in situ* through the portal vein for three min with HBSS at 37°C and a flow rate of 20 ml/min and then removed to a recirculating liver perfusion system (19). Cells were prepared by a modification of the two-step procedure described by Seglen (20). HBSS was recirculated for 10 min at 40 ml/min and 37°C, followed by 0.05% collagenase in HBSS with 4 mM $CaCl_2$ for 15 min. The liver was minced and the suspension diluted with 20 vol of KH. The cell clumps were mechanically dissociated on a rotary evaporator with oxygenation for 15 min. The suspension was then filtered successively through 75-μm, 52-μm, and 30-μm screens (Tetko: Elmsford, NY) to select for single cells. Hepatocytes were purified by three cycles of sedimentation and resuspension in KH (50 *g* for 3 min or 1 *g* for 15 min). Cells prepared in this manner were routinely 99% parenchymal, 90% singles, and 85–97% intact by trypan blue exclusion. Yields averaged 1–2 × 10^8 cells per 5–7 g liver.

Hepatocyte Monolayer Culture

Dissociated hepatocytes were prepared as described above, except that sterile precautions were observed and 0.006% benzylpenicillin was included in the isolation media. Cells were suspended in DMEM, and 1-ml aliquots (1–1.3 × 10^6 cells/ml) were plated onto tissue culture dishes coated with denatured collagen (21), 40 μg/ml, 0.2 ml/dish. Cultures were maintained at 37°C in a humidified 5% CO_2/95% air incubator. The medium was replaced 4 h after plating and every 24 h thereafter.

The percent of cells plated that attached to the tissue culture dish after 4 h was equal to the percent that was viable immediately after isolation as indicated by trypan blue.

Determination of Cell Viability and Number

Cell viability was monitored in two ways—trypan blue exclusion and lactate dehydrogenase (LDH) release into the medium. Cell number was measured in three ways—direct counting with a hemocytometer, LDH activity, and DNA content.

TRYPAN BLUE: Cells were diluted 1:5 (vol/vol) with 0.4% trypan blue in 0.9% saline and examined by phase microscopy within 4 min.

LDH ACTIVITY: Cells were separated from the suspension medium by centrifugation (50 *g* for three min or 12,800 *g* for 10 s) and both the medium and cells were frozen at −20°C. After thawing, samples were solubilized in 0.5% Triton X-100 (TX-100, final concentration) and assayed for LDH activity as described by Schnaar et al. (22). Culture medium and monolayers (rinsed four times with cold KH-PVP-40) were frozen, thawed, and solubilized as described above for LDH assays. Activity present in the culture medium in the absence of cells was measured as a blank and accounted for only 3% of total cellular activity.

The percentage of cells which were stained with trypan blue (5–14%) was equivalent to the percentage of total LDH activity (cells and medium) in the medium (3–17%). Therefore, we used these two assays interchangeably as equally accurate indicators of cell viability.

DNA CONTENT: We compared LDH activity and DNA content both in the isolated hepatocyte preparations and in monolayer cultures, to be sure that these basic parameters were not changing with time in culture. Cell suspensions and monolayers were recovered as described above, frozen in one ml of KH-PVP-40, and the DNA content in thawed samples was determined by the method of Burton (23).

^{3}H-THYMIDINE INCORPORATION BY HEPATOCYTE MONOLAYERS: Although it is generally assumed that adult rat hepatocytes in primary culture are quiescent non-dividing cells (24), we examined cells in monolayer culture exposed to ^{3}H-thymidine by light microscopic autoradiography (LM-ARG) and confirmed that DNA synthesis and cell division were rare in these cultures. Dishes were

exposed to 5 μCi (3 μM) ^{3}H-thymidine between 4.5 and 20.5 h. Monolayers were then fixed, dried, and processed for LM-ARG as described below (Autoradiography). The dishes were exposed for 7 d at 4°C. The percentage of labeled nuclei was determined for 600 total nuclei per dish.

Morphology

Cell suspensions and monolayer cultures were examined by light and electron microscopy. The primary fixative in each case was 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, for 30 min at 4°C. Cells fixed in suspension were rinsed with 0.1 M Na cacodylate, pH 7.4, and collected by centrifugation at 500 *g* for several sec. Fixed monolayers were rinsed *in situ* with the same buffer for 10 min on ice. The samples were then postfixed in 1% OsO_4 in 0.1 M Na cacodylate for 0.5–2 h on ice, rinsed in 0.9% saline, and stained with 0.5% uranyl acetate in acetate-veronal buffer, pH 6.0, (25) for 1–2 h at room temperature. The cell suspensions were rinsed with saline and then mixed with an equal volume of 3% agarose at 78°C and pelleted. The pellets were dehydrated with a graded series of ethanol, then propylene oxide, and embedded in Epon 812 (26). Cell monolayers were embedded by one of two procedures depending on whether or not an oriented specimen was required. (*a*) For randomly oriented cells, the monolayers were dehydrated through propylene oxide, at which step the sheets of cells lifted off the dish and were collected and pelleted (in propylene oxide). (*b*) For oriented specimens, monolayers were dehydrated to 100% ethanol, and embedded directly in Epon without a propylene oxide step. Small squares were then oriented for sectioning parallel or perpendicular to the plane of the dish.

Thick sections (0.5 μm) were collected on glass slides and stained with 1% toluidine blue in 1% Na borate. Thin sections (750–1000 Å) were collected on carbon- and formvar-coated copper grids, stained with saturated uranyl acetate for 10 min and lead citrate (27) for 2.5 min. The grids were viewed in a Siemens 101 or Zeiss 10A electron microscope operating at 80 kV.

^{125}I-ASOR Binding, Uptake, and Degradation in Isolated Hepatocytes and Monolayer Cultures

Biochemistry (^{125}I-ASOR binding, uptake, and degradation) and autoradiography were used to determine the number of ASGP binding sites and the fate of the ligand in hepatocytes in vitro.

IODINATION OF ASOR: ASOR was prepared by enzymatic hydrolysis of the terminal sialic acid residues from human orosomucoid (OR) and iodinated using insolubilized lactoperoxidase (LPO) and glucose oxidase (GO) as described (1) or chloramine T (28). Specific radioactivities ranged from 2 to 17 × 10^6 cpm/μg ASOR. The ^{125}I-ASOR was stored at −70°C in 0.9% saline containing bovine serum albumin BSA (200 μg/ml).

^{125}I-ASOR INCUBATIONS WITH ISOLATED HEPATOCYTES AND MONOLAYER CULTURES: The abilities of dissociated hepatocytes and cells in 24-h monolayer culture to bind, internalize, and degrade ^{125}I-ASOR were compared. Incubations were performed either at 37°C, where binding, internalization, and degradation of ^{125}I-ASOR occur, or at 5°C, where binding occurs but not internalization of the ligand. Cell-associated radioactivity was measured in two ways: (*a*) exposure of cells to ^{125}I-ASOR for timed intervals, followed by treatment with 7.5 mM EGTA (final concentration) to chelate Ca^{+2} and dissociate accessible ^{125}I-ASOR (surface binding); and (*b*) exposure of cells to ^{125}I-ASOR in the presence or absence of a 100-fold excess of unlabeled ASOR (specific binding).

The standard assay at either temperature was performed with 5 ml of cells (1 × 10^6 cells/ml) in KH-PVP-40 in each of two 25-ml flasks (A and B). The cells were preincubated at the appropriate temperature in a humidified, oxygenated atmosphere with gentle orbital shaking to prevent clumping. Unlabeled ASOR (500 μg) was added to flask B for 5 min. At time 0, 5 μg of ^{125}I-ASOR were added to both flasks. At timed intervals, two 0.25-ml aliquots were removed from flask A and one from flask B. One aliquot from A and the aliquot from B were each diluted to 1 ml with cold KH-PVP-40, and the other aliquot from A was diluted to 1 ml with 10 mM EGTA (7.5 mM final concentration) in KH and incubated for an additional 5 min on ice. The cells were then sedimented (50 *g*, 3 min, 4°C). The cell pellet was washed twice by resuspension and centrifugation in KH-PVP-40, after which the pellets and combined supernatants were counted in a gamma counter (Beckman 4000, Beckman Instruments, Inc., Fullerton, CA).

Metabolism of ^{125}I-ASOR was assessed at 5° and 37°C after precipitation of cells and medium with 5% TCA (final concentration). The acid-soluble radioactivity was analyzed by gel filtration under conditions where peptides, monoiodotyrosine (MIT), and I^- could be separated (29).

Analogous ^{125}I-ASOR incubations were performed on well-washed cell monolayers (four times with KH-PVP-40, 4°C). Incubations at 5° and 37°C were carried out in a 95% O_2/5% CO_2 and 95% air/5% CO_2 atmosphere, respectively. At various times after the addition of radioactivity, individual culture dishes were immersed in ice to stop the incubation. The monolayers were then washed with cold KH-PVP-40 (four times for 5 min each), and the cells were scraped from the dish for determination of radioactivity.

LIGHT AND ELECTRON MICROSCOPE AUTORADIOGRAPHY: Cells incubated with ^{125}I-ASOR were processed for LM-ARG and EM-ARG to determine the following: (*a*) whether all cells in the preparation bound ^{125}I-ASOR; (*b*) whether exposure to EGTA removed surface-bound ^{125}I-ASOR; and (*c*) whether the in vitro endocytic pathway resembled the in vivo pathway. Dissociated hepatocytes and cells in 24-h monolayer cultures grown on collagen-coated glass cover slips were incubated at 5° and 37°C with ^{125}I-ASOR for 0, 30 min, or 2 h with the appropriate controls in the manner described above. After rinsing, they were fixed in 2% glutaraldehyde in 0.1 M Na cacodylate with 2 mM $CaCl_2$, pH 7.4 for 30 min on ice. Dissociated hepatocytes were further processed for electron microscopy as described in "Morphology." The cells on cover slips were dehydrated through a graded series of ethanol and dried in air before coating with emulsion.

For LM-ARG, 0.5 μm sections of single cells or whole cells on cover slips were coated with Ilford K5 emulsion (4 g/12 ml ddH_2O) and exposed for 3–14 d at 4°C. They were then developed with D-19, fixed, and viewed in a Zeiss photomicroscope. For EM-ARG, the flat substrate technique of Salpeter and Bachman (30) was used with 750- to 1,000-Å thin sections of dissociated hepatocytes.

For EM-ARG analysis, randomly selected hepatocytes in the developed sections were divided into quadrants and photographed at 9,000 times' magnification. Quantitation was performed on prints at 23,400 times' magnification. Six cell compartments were chosen for analysis: (*1*) the plasma membrane which consisted of two half distances (1 H.D. = 1,000 Å) on either side of a band encompassing the microvillar projections; (*2*) the cytoplasm; (*3*) lysosomes, a heterogeneous population by size and content which were usually recognized as membrane-bounded vesicles often containing autophagic structures near the Golgi complex; (*4*) the Golgi complex which consisted of the region containing Golgi elements as well as cytoplasm 2 H.D. beyond the Golgi saccules; (*5*) the nucleus; and (*6*) mitochondria. A mitochondrial compartment was included in our analysis, because their large number contributed sufficient area to the cytoplasmic compartment such that the area contribution of small cytoplasmic vesicles in this latter compartment was diluted. ~100 grains were counted for each time-point and condition. The area was computed using a test grid (10 × 10 cm^2 or 3.74 intersection points/μm^2). Percent grains, percent area, and percent grains/percent area were calculated.

Analysis of the Effect of Collagenase on ASGP Binding Activity

Exposure of the hepatocyte cell surface to the mixture of proteases that constitutes crude collagenase could affect the apparent number of receptors detected by the ^{125}I-ASOR binding assays. Therefore, we first characterized the collagenase used, then determined its effect on the ligand, and finally tested its effect on the number of ASGP receptors per cell.

NONSPECIFIC PROTEASES: To assess the level of proteolytic activity, we compared the digestion by 0.01–0.05% collagenase of 1% agar gels containing a bovine casein substrate to a trypsin control (2.5–10 μg/ml), as directed in the Bio-Rad Protease Detection Kit (Bio-Rad Laboratories, Richmond, CA) (31). Proteases were detected with 50 mg of collagenase containing the equivalent of 1 mg of trypsin-like activity (data not shown). A variety of protease inhibitors—soybean trypsin inhibitor, ovomucoid, phenylmethylsulfonyl fluoride (PMSF)—were unable to inhibit the proteolysis.

NEURAMINIDASE (NANADASE): We screened several lots of collagenase for NANAdase activity, since enzymatic hydrolysis of terminal sialic acids from the ASGP receptor inhibits ligand binding (32). Collagenase (0.4 mg/ml) was incubated with bovine submaxillary mucin (4 mg/ml) in 0.1 M acetate, pH 5.0, 37°C for 30 min with shaking. The reaction was stopped with an equal volume of 5% phosphotungstic acid, and the supernatant was analysed for free sialic acid (33). Neuraminidase activity was not detectable in our lots of collagenase.

EFFECT OF COLLAGENASE ON ^{125}I-ASOR: If contaminating hydrolases in the collagenase preparation adsorbed to cells during the liver dissociation, then these enzymes could potentially degrade the ^{125}I-ASOR used in binding studies. Therefore, we incubated ^{125}I-ASOR (25 μg) with 25 to 450 μg collagenase in 0.4 ml of 0.1 M Tris-HCl, pH 7.4, 4 mM $CaCl_2$, for 15 min at 37°C with shaking. The reaction mixture was diluted to 1 ml and precipitated with 0.015% Na deoxycholate and 6% TCA, 4°C. The pellets and supernatants were solubilized, reduced, alkylated, and electrophoresed on a 8% polyacrylamide slab gel, according to Maizel (34). The gel was dried and autoradiographed at −70°C with a Cronex Lightening Plus intensifying screen. No detectable iodinated polypeptide fragments, no decrease in TCA-precipitable radioactivity, and no change in the intensity of the ^{125}I-ASOR bands by ARG were seen (data not shown).

ASGP BINDING ACTIVITY BEFORE AND AFTER COLLAGENASE DISSOCIATION: To examine the possibility that the population of functional

ASGP receptors at the surface was reduced during preparation of dissociated liver cells, we compared the number of cell surface receptors in the isolated perfused liver to the number on the same hepatocytes after enzymatic dissociation. Isolated livers were first perfused in the recirculating system with KH-PVP-40 for 30 min at 37°C and then with fresh medium at 6°C for 15 min. ^{125}I-ASOR was added (0.5 ml, 50-103.5 μg, 5.1×10^4–5.2×10^5 cpm/μg), and samples (0.5 ml) of perfusate were withdrawn periodically for determination of remaining radioactivity. After one h, ^{125}I-ASOR binding was saturated, and EGTA (final concentration, 5 mM) was added to the perfusate to dissociate accessible ^{125}I-ASOR from cell surfaces. The amount of EGTA-dissociable radioactivity was used to calculate the number of surface ASGP receptors. The perfusate was then replaced with HBSS at 37°C, and single cells were prepared as described earlier. Isolated cells were subsequently reexposed to ^{125}I-ASOR at 5°C (same concentration as above), and the specific and surface-bound ligand were measured as described earlier ("incubations").

To separate the effects of enzyme exposure from mechanical dissociation on subsequent ASGP binding, the following experiment was performed. The number of surface ASGP receptors was first determined on a liver perfused at 5°C as described above. The same liver was then rinsed free of radioactivity with 5 mM EGTA, warmed to 37°C, and exposed to 0.05% collagenase in KH-PVP-40 for 15 min. After removal of the enzyme solution the liver was rinsed with 500 ml of KH-PVP-40 and cooled to 5°C for determination of ASGP surface receptor number. To see whether the enzyme-related change in surface binding activity was reversible, the liver was warmed to 37°C for 1 h. It was then cooled to 5°C for a final measurement of ASGP surface receptors. Therefore, with a single liver, surface receptor number was determined under three conditions: (*a*) precollagenase; (*b*) postcollagenase; and (*c*) after a 1-h recovery period.

EFFECT OF COLLAGENASE ON DISSOCIATED HEPATOCYTES: The sensitivity of single hepatocytes to a second exposure of collagenase was determined by comparing the amount of ^{125}I-ASOR bound at 5°C by isolated cells that had been incubated in suspension with 0.05% collagenase to that bound by control cells (i.e., cells exposed to collagenase only during liver dissociation). Cells (3×10^6/ml) were incubated in KH-PVP-40 with or without 0.5 mg/ml collagenase at 37°C for 15 min in oxygenated flasks. The cells were quickly cooled to 4°C, sedimented by centrifugation (50 *g* for 3 min), resuspended in cold KH-PVP-40, and rinsed twice more. Cells were then exposed to ^{125}I-ASOR at 5°C, and the amounts of specific and surface-bound ligand were quantitated as described above.

The effect of incubation at 37°C for 1 h on the number of cell surface ASGP receptors was studied in isolated hepatocytes in the presence or absence of 1 mM cycloheximide to inhibit protein synthesis. The incubations were performed under the conditions described above.

ASOR-Horseradish Peroxidase (ASOR-HRP) Cytochemistry

In a series of experiments designed to visualize the ASOR binding sites on the surface of isolated hepatocytes at the ultrastructural level, we incubated cells with a cytochemical tracer, HRP conjugated to ASOR. ASOR-HRP was prepared and characterized as described by Wall et al. (35). Cells were incubated with ASOR-HRP at 5° or 37°C as described for ^{125}I-ASOR above. Cell pellets were washed twice and fixed with 2% glutaraldehyde/2 mM $CaCl_2$ in 0.1 M Na cacodylate as described above. The fixed cells were rinsed first in 0.1 M Na cacodylate and then in 0.1 M phosphate, pH 7.4, by resuspension and sedimentation. The pellets were resuspended in 0.1 mg/ml DAB, 0.02 M aminotriazole (to inhibit catalase), and 0.03% H_2O_2 in 0.1 M phosphate, pH 6.0 (36), and incubated for 2 h at 25°C. The cells were rinsed twice in 0.1 M phosphate, pH 7.4, once in 0.1 M Na cacodylate, and then postfixed in 1% OsO_4/0.1 M Na cacodylate, pH 7.4, for 1 h at 4°C. The cells were rinsed in saline and processed for electron microscopy as described above (Morphology).

RESULTS

Morphological Appearance of Dissociated Hepatocytes and Monolayer Cultures

Hepatocyte suspensions examined immediately after isolation were composed predominantly of single cells with 10%

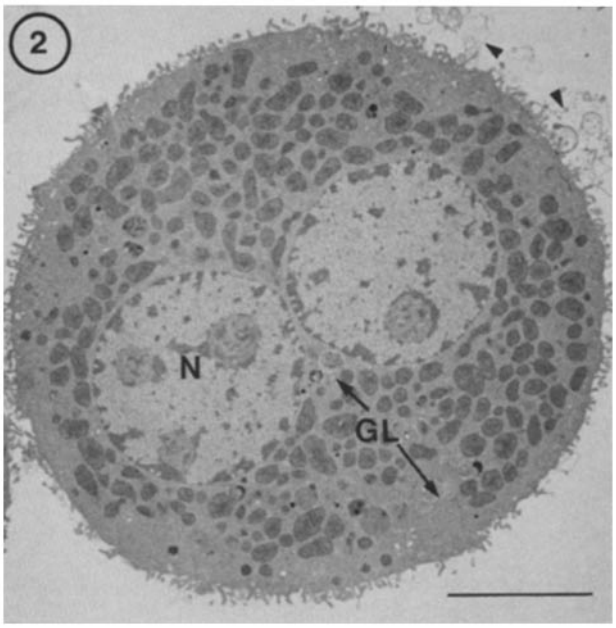

FIGURE 2 Electron microscopic appearance of a typical isolated binucleated hepatocyte. This cell preparation was maintained in suspension at 25°C for 2.5 h before fixation. The cell retains the intracellular organelles seen in vivo but has lost the cell surface specializations distinctive of sinusoidal, bile canalicular, and lateral surfaces. Debris generated during isolation is associated with a portion of the cell surface (arrowheads) and is scattered through most preparations. *GL*, Golgi-Lysosome areas. Bar, 5 μm. × 3915.

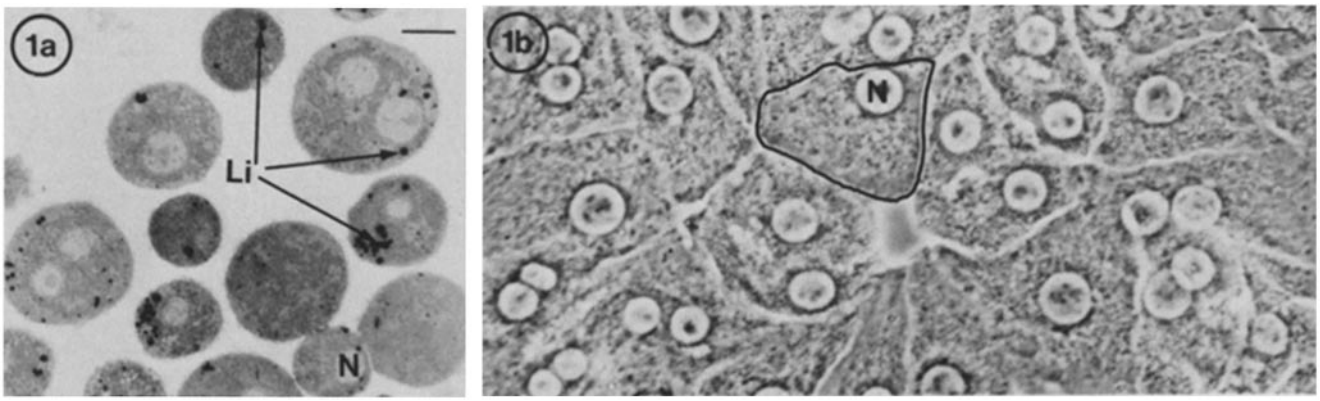

FIGURE 1 Morphological appearance of dissociated hepatocytes and monolayer cultures. (*a*) Freshly isolated hepatocytes were incubated in suspension for 1 h at 37°C, then fixed and embedded in Epon. *N*, nucleus. *Li*, lipid droplet. Bar, 10 μm. × 700. (*b*) Hepatocytes were maintained in monolayer culture for 18 h and then viewed by phase microscopy directly on the tissue culture dish. The cells have flattened and established a sheetlike architecture. The borders between adjacent cells are highly refractile (1 cell is outlined). *N*, nucleus. Bar, 10 μm. × 460.

doublet and triplet groups (Fig. 1 *a*). As can be seen in Fig. 2, the shape and surface ultrastructure of individual cells were drastically altered from those of cells *in situ*. That is, cells were spherical, uniformly covered with microvilli, and lacking in junctional complexes. Occasionally, an entire tight junction plus a portion of the cytoplasm from a neighboring cell remained. Golgi complexes and lysosomes were concentrated near the nucleus in isolated cells in contrast to their biliary location *in situ*. Given the time and temperature of the dissociation procedure (2 h, 25° and 37°C), the loss of distinctive cell surface morphology and cell shape could have been well underway by the time of fixation for electron microscopy.

Thirty min after plating on a collagen-coated substrate, the cells were attached, spherical, and distinct from their neighbors. At 4 h, the cells had begun to flatten and to reassociate with neighboring cells. By 24 h, they had flattened further to form a continuous monolayer (Figs. 1 *b* and 3 *b*). Cell surface specializations were detected between 24 and 48 h (Fig. 3 *a* and *c*): that is, desmosomes, tight junctions and intercellular pockets resembling bile canaliculi could be seen. This time-course of reestablishment of cell surface polarity agrees with the finding of others (8, 37, 38).

Cell Viability and Survival in Suspension and Monolayer Culture

Trypan blue exclusion, LDH activity, and DNA content were compared in isolated cells and cells after 24 h in monolayer culture to establish a reliable, sensitive, and rapid method for measuring cell number in monolayers and to determine cell survival after isolation and in culture. First, the percentage of cell-associated LDH activity in isolated cells was equal to the percentage of cells that excluded trypan blue (89%), indicating that LDH could be used to assess cell viability (Table I). In addition, using either assay, we established that isolated cells could be maintained in suspension for up to 5 h with no more than a 10–15% decrease in viability. Cells in culture exhibited similar levels of viability (88% viable, Table I). The DNA content of cells after 24 h was 85–95% that of the freshly isolated cell suspension from which they were derived (data not shown). Since DNA synthesis as measured by [^{3}H]thymidine incorporation was insignificant during the first 24 h of culture (only 1% of the nuclei were labeled as detected by LM-ARG), we have concluded that 85–95% of the cells that were initially plated had survived. Finally, the ratio of LDH activity

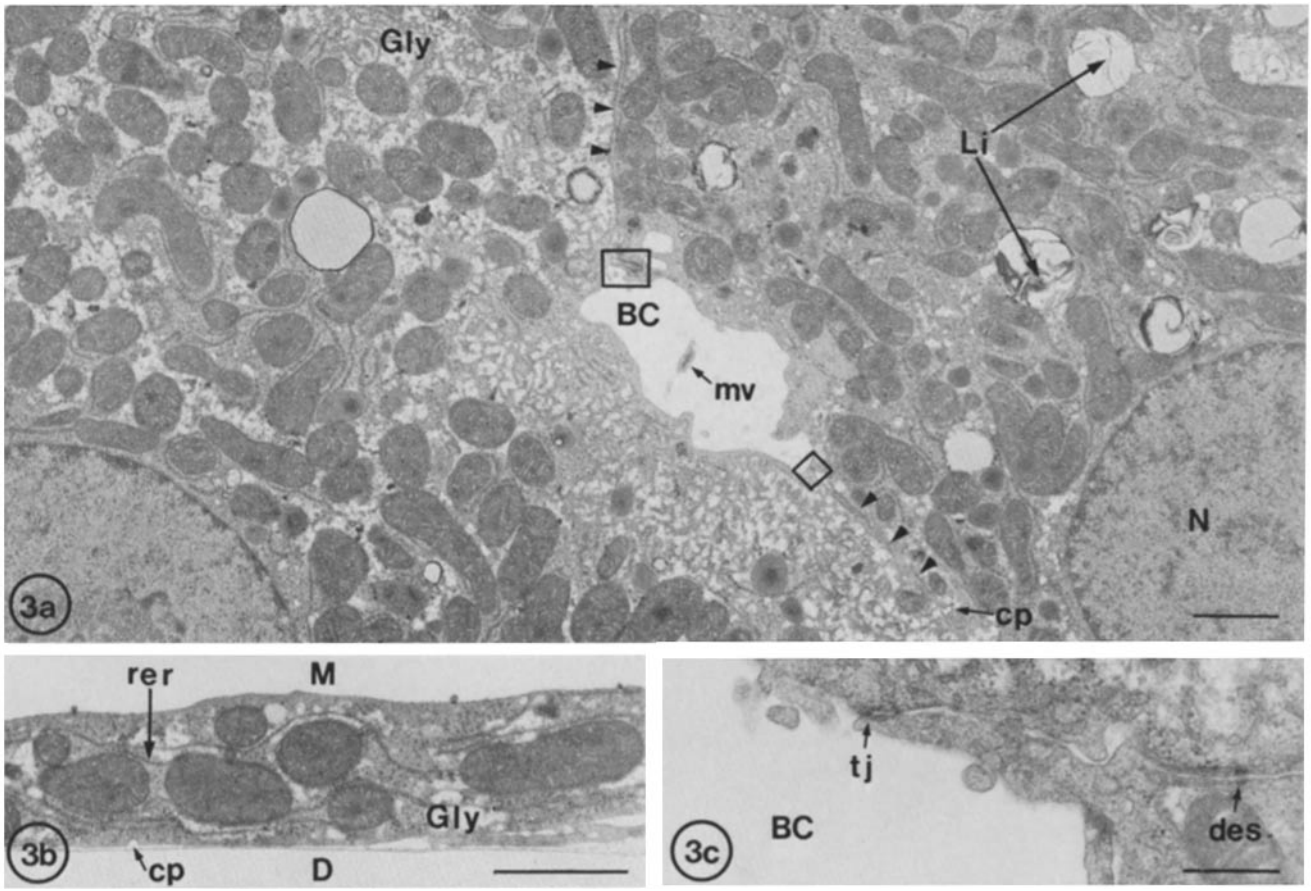

FIGURE 3 Ultrastructural appearance of hepatocytes after 24 h in monolayer culture. (*a*) Electron micrograph prepared from a section cut parallel to the plane of the tissue culture dish. The two cells shown are associated in a manner similar to that observed in vivo. That is, a bile canalicular-like region (*BC*) is formed between lateral surfaces (arrowheads) with junctional complexes (rectangles) composed of tight junctions and a variable number of adhering junctions and desmosomes. The bile canalicular-like region is somewhat dilated and has fewer microvilli than in vivo. The cells are packed with glycogen (*Gly*) and lipid droplets (*Li*). *N*, nucleus. *Cp*, coated pit. Bar, 2 μm. × 5750. (*b*) Electron micrograph prepared from a section cut perpendicular to the plane of the tissue culture dish. The extreme flattening of these cells is illustrated here. *D*, surface in contact with the dish. *M*, surface in contact with the medium (note the absence of microvilli). *RER*, rough endoplasmic reticulum. *Gly*, glycogen. *Cp*, coated pit. Bar, 2 μm. × 8250. (*c*) Typical appearance of junctional complexes after 24 h in culture. *tj*, tight junction. *des*, desmosome. *BC*, bile canalicular-like space. Bar, 0.25 μm. × 52,000.

to DNA content was very similar in freshly isolated and cultured cells (Table I, 235 vs. 252 U LDH/μg DNA), establishing that the cell-associated LDH activity could be used reliably to determine cell number in monolayers.

^{125}I-ASOR Binding in Dissociated Hepatocytes

Having established conditions for the successful isolation and culture of dissociated hepatocytes, we used biochemical and morphological methods to determine whether ASGP binding activity was expressed by single cells.

THE ^{125}I-ASOR BINDING ASSAY FOR FRESHLY DISSOCIATED HEPATOCYTES AT 5°C: ^{125}I-ASOR binding activity present on the cell surface of freshly isolated hepatocytes and on cells cultured for 24 h was determined between 4° and 10°C, temperatures reported to inhibit internalization of the ligand (39, 40). To measure the surface population of ASGP receptors, cells were first exposed to ^{125}I-ASOR at 4°–10°C and the amount of ligand accessible to (i.e., released by) subsequent addition of EGTA was used to calculate the number of surface-bound ASOR molecules. To confirm that the EGTA-accessible radioactivity at 4°C was located at the cell surface, we visualized the ^{125}I-ASOR associated with cells before and after EGTA treatment by LM-ARG. Fig. 4*a* and *b* illustrate the results obtained. ARG grains representing the total cell-associated ^{125}I-ASOR at 4°C were predominantly located at the cell surface (Fig. 4*a*). After EGTA treatment of cells previously exposed to ^{125}I-ASOR at 5°C, virtually no ARG grains were observed (Fig. 4*b*).

Specific binding was determined as follows: cells at 5°C were exposed to ^{125}I-ASOR in the presence of a 100-fold excess of unlabeled ASOR, and the amount of radioactivity associated with these cells was subtracted from that associated with cells exposed to only ^{125}I-ASOR, the difference representing specific binding. LM-ARG of cells exposed to an excess of unlabeled ASOR were devoid of ARG grains (Fig. 4*c*). Nonspecific binding usually averaged 5–23% of the total radioactivity bound and tended to increase with the age of the iodinated probe. EGTA-inaccessible binding at 5°C was generally equal to nonspecific binding. From these controls we conclude that the ^{125}I-ASOR binding measured at 4°–10°C accurately reports the number of specific ASGP receptors on the cell surface, assuming that one molecule of ASOR bound is equivalent to one ASGP receptor.

The concentration of ^{125}I-ASOR necessary to saturate the cell surface was next investigated with isolated hepatocytes at

TABLE I

Biochemical and Morphological Parameters of Viability in Isolated and Cultured Cells *

	Isolated hepatocytes	24-h Monolayer cultures
mU LDH/cell	4.5 ± 0.16‡ (6)§	—
pg DNA/cell	18.8 ± 1.10 (8)	—
U LDH/μg DNA	235 ± 15.1 (6)	252 ± 29 (4)
% Trypan blue exclusion	89 ± 0.86 (7)	N.D.
% LDH in cells	89 ± 1.46 (6)	88 ± 0.94 (4)

* Trypan blue exclusion was measured for each cell preparation directly after isolation. LDH activity and DNA content were measured in dissociated cells and cells in monolayer culture as described in Materials and Methods. Cell number was determined by hemocytometer counting. 1 U of LDH activity = {(ΔOD_{340}/min) × (dilution)}/0.0015

‡ mean ± standard deviation

§ (number of experiments)

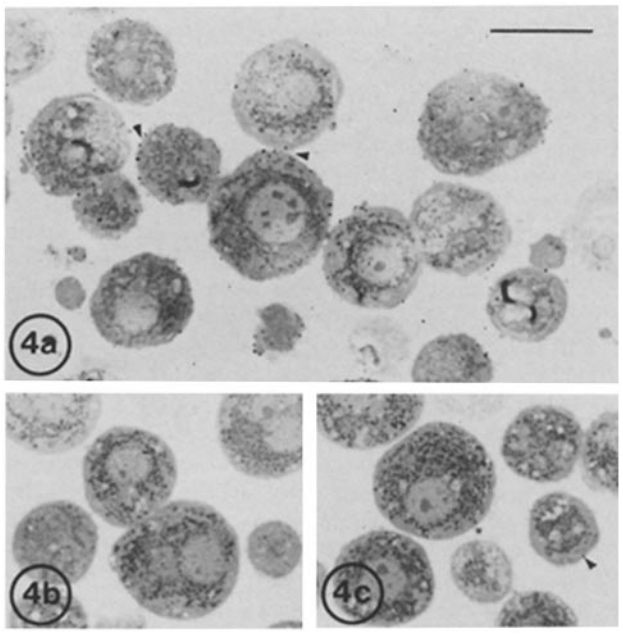

FIGURE 4 LM-ARG of dissociated hepatocytes incubated with ^{125}I-ASOR at 4°C. Cells were incubated with 0.18 μg/ml ^{125}I-ASOR (8.2 × 10^6 cpm/μg) for 2 h at 4°C in the presence or absence of 50 μg/ml unlabeled ASOR. 14-d exposure, Bar, 20 μm. × 675. (*a*) Incubation with ^{125}I-ASOR alone. The ARG grains are localized predominantly at the cell surface (arrowheads). (*b*) Incubation with ^{125}I-ASOR alone, then treatment with 7.5 mM EGTA. Cells are free of ARG grains. (*c*) Incubation with ^{125}I-ASOR in the presence of excess ASOR. ARG grains are virtually absent. Arrowhead, ARG grain.

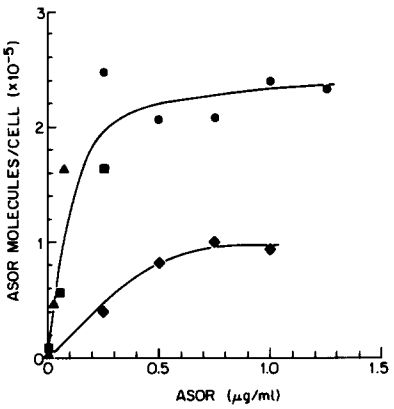

FIGURE 5. ^{125}I-ASOR binding to isolated hepatocytes at 5°C: the effect of increasing concentrations of ^{125}I-ASOR. Dissociated hepatocytes (four different cell preparations) were maintained at 22–25°C for 30–60 min and then cooled to 5°C for 30 min. Total and nonspecific binding were determined as described in Materials and Methods. ^{125}I-ASOR was diluted with unlabeled ASOR and added in the concentrations indicated. For each concentration, a 100-fold excess of unlabeled ASOR was included to measure nonspecific binding. All data points were taken at 4 h, at which time binding had reached a maximum. Some variation in the level of saturation binding was observed among the cell preparations as demonstrated by the two curves drawn. The symbols (●, ▲, ■, ◆) represent data points from four different cell preparations.

5°C and ^{125}I-ASOR concentrations from 0 to 1.5 μg/ml. The results are presented in Fig. 5. The data were collected from four separate cell preparations with viabilities >86% and yields between 1.5 × 10^8 and 2.3 × 10^8/5–7 g liver. Nonspecific binding of ^{125}I-ASOR ranged between 4% and 23%. All data points represent the binding measured at 4 h because maximum binding was observed by this time at all concentrations tested. Binding became saturated at >0.5 μg/ml ASOR but the

amounts bound at saturation varied between cell preparations. This is illustrated by the two curves drawn in Fig. 5. In general, ~65% of our isolations have yielded cells with between 200,000 and 400,000 surface receptors/cell and 35% with ~40,000 to 200,000 surface receptors/cell. The variability was not correlated with viability, yield, or level of nonspecific binding. Therefore, we considered whether one or more of the steps in the cell preparation (e.g., collagenase exposure, mechanical cell dissociation, the time and temperature of hepatocyte purification) was generating the variable levels of ASGP binding activity.

THE EFFECT OF COLLAGENASE ON ASGP BINDING ACTIVITY: The first step in liver dissociation is exposure of cells *in situ* to collagenase. Therefore, we asked whether the level of ASGP binding activity on the surface of isolated hepatocytes might be decreased by the enzyme preparation. First, we established the level of contamination of collagenase by hydrolases: (*a*) neuraminidase was not detectable; and (*b*) 50 mg of collagenase contained the equivalent of 1 mg of trypsin as detected by casein digestion. Secondly, we measured the number of cell surface receptors in the perfused liver and compared it with the number after collagenase dissociation to single cells. Thirdly, we studied the effect of collagenase on surface ASGP binding after a second exposure to collagenase. And, finally, we examined the effect on surface binding of incubating collagenase-treated livers or isolated cells at 37°C for 1 h (i.e., allowing a "recovery" period).

When ^{125}I-ASOR binding to perfused liver at 5°C was compared with binding to the same cells after collagenase dissociation, no significant difference was seen. A typical experiment is shown in Fig. 6. A perfused liver was exposed to 1.2 nmol of ^{125}I-ASOR at 5°C, and after 90 min EGTA was added. The amount of EGTA-dissociated radioactivity was used to calculate the number of surface receptors with the assumption that 1 g of liver contains 1×10^8 cells (21). The EGTA-releasable radioactivity was equivalent to 214,000 molecules of ASOR bound/cell. ^{125}I-ASOR binding at 5°C to the same cells after dissociation (2 h later) is presented in Fig. 6*b*, where it can be seen that 280,000 molecules of ASOR were bound per cell. The average number of ASOR receptors in the isolated, perfused liver from three experiments was 230,000 (SD 44,000), and after isolation was 211,000 (SD 100,000), suggesting that under our isolation conditions no substantial reduction in binding activity was detectable. It should be noted that the collagenase-treated cells were maintained at room temperature (22°–25°C) for ~1 h before the binding assay was performed.

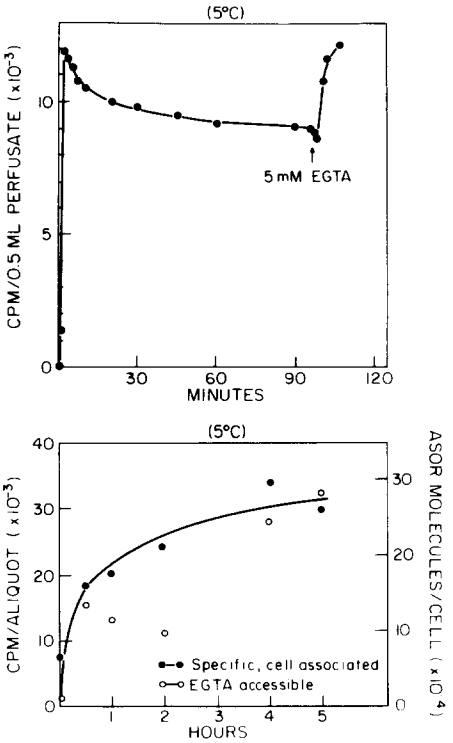

FIGURE 6. ^{125}I-ASOR binding at 5°C before and after collagenase dissociation. (*A*) ^{125}I-ASOR binding in the perfused liver. A 9-g liver (wet weight minus 25% as volume of the perfusate) was cooled to 6°C in the recirculating system for 15 min. (Bile was collected as a measure of metabolic activity during temperature shifts.) ^{125}I-ASOR (103.5 μg, 5.1×10^4 cpm/μg) was added, and 0.5-ml aliquots of perfusate were sampled until binding was maximal (90 min). EGTA (final concentration 5 mM) was added, and the perfusate was again sampled for 10 min. The difference between peak radioactivity in the perfusate and the level where binding was maximal was used to calculate the number of receptors per cell. The perfusate was then removed, and the liver was rinsed by single-pass perfusion. The liver was warmed to 37°C, the perfusate was replaced with HBSS and dissociated hepatocytes were prepared as described in Materials and Methods. (*B*) ^{125}I-ASOR binding to dissociated hepatocytes. Total, nonspecific, and EGTA-inaccessible binding were measured using 0.7 μg/ml ^{125}I-AOR (1.5×10^6 cpm/μg) and 40 μg/ml unlabeled ASOR. Nonspecific binding averaged 17% with a 40-fold excess of unlabeled ASOR. The scatter in the EGTA-accessible data points reflects brief warming of aliquots during the rinse procedure.

In contrast to the apparent lack of effect of collagenase on surface ASGP receptors measured after mechanical dissociation, we found that collagenase did reduce the number of

TABLE II

*Effect of a Second Exposure to Collagenase on ^{125}I-ASOR Binding to Isolated Hepatocytes at 5°C**

Experiment	Viability		Specific binding (Receptors per cell × 10^{-5})		Surface binding (EGTA-released ASOR per cell × 10^{-5})		Nonspecific binding		Ratio of specific binding
	Con. %	*Exp.* %	*Con.*	*Exp.*	*Con.*	*Exp.*	*Con.* %	*Exp.* %‡	$\frac{Exp.}{Con.} \times 100$
1	87	76	8.86	2.92	8.70	2.97	3	8	33
2	92	93	2.40	1.30	1.21	1.87	10	26	54
3	91	91	5.42	1.57	4.58	1.18	21	48	29
4	90	88	3.24	0.321	3.03	0.339	5	43	10

* Isolated hepatocytes ($2–3 \times 10^6$ cells/ml) were incubated in suspension in the absence (control) or presence (experimental) of 0.05% collagenase in KH-PVP-40 for 15 min at 37°C. The cells were cooled to 4°C, washed by three cycles of centrifugation and resuspension, and then assayed for specific and EGTA-accessible binding over 4 h at 5°C. ^{125}I-ASOR (1.6—7.2×10^6 cpm/μg) at 1 μg/ml and 100 μg/ml unlabeled ASOR were used. Viability was determined by trypan blue exclusion before the binding assay.

‡ Collagenase treatment was associated with an increase in nonspecific binding as compared to control cells.

TABLE III

Collagenase-associated Reduction in Surface ASGP Binding and Recovery at 37°C in the Absence of Significant Protein Synthesis *

Condition	Specific ASGP Binding‡	Initial Binding§
	^{125}*ASOR/cell*	%
1. Perfused liver	170,000‖	100
Perfused liver, post-collagenase	68,000‖	40
Perfused liver, 1 h (37°C) post-collagenase	190,000‖	110
2. Isolated cells, 15 min, 37°C (*a*)	110,000	100
Isolated cells, 15 min, 37°C plus collagenase (*b*)	56,000	51
(*a*) Post-recovery, 1 h, 37°C	270,000	240
(*b*) Post-recovery, 1 h, 37°C	240,000	220
3. Isolated cells (*a*)	220,000	100
(*a*) Post-recovery, 1 h, 37°C	380,000	170
(*a*) Post-recovery, 1 h, 37°C plus 1 mM cycloheximide	460,000	210

* Isolated hepatocytes were incubated in suspension in the presence or absence of 0.05% collagenase as described in Table II.

‡ Specific binding was assessed at 5°C in the presence and absence of a 100-fold excess of unlabeled ASOR as described in Materials and Methods. Nonspecific binding ranged between 10 and 19%.

§ Initial binding (100%) was defined independently in each of the four experiments shown above.

‖ Specific binding in the perfused liver was measured at 5°C by quantitating the disappearance of ^{125}I-ASOR from the perfusate and the subsequent fraction that was released from the liver by 5 mM EGTA. Since input was subsaturating (6.7 μg ASOR/g liver), numbers were multiplied by 2 (see Fig. 4 in reference 53).

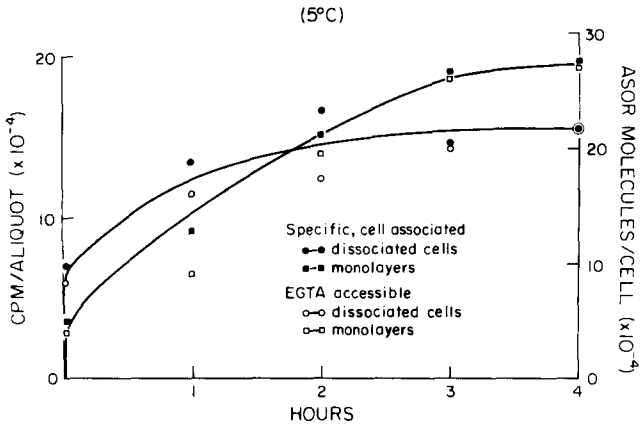

FIGURE 7 ^{125}I-ASOR binding at 5°C to dissociated cells and monolayer cultures. Dissociated hepatocytes and cells from the same preparation after 24 h in culture were preincubated at 5°C for 1 h, and total, nonspecific, and EGTA-inaccessible radioactivities were assayed as described in Materials and Methods. ^{125}I-ASOR (9.7×10^6 cpm/μg) at 1 μg/ml and ASOR at 100 μg/ml were used. Cell number was determined by LDH assays on duplicate tissue culture plates as described in Materials and Methods.

receptors on isolated hepatocytes exposed twice to collagenase. Cells were incubated for 15 min at 37°C in the presence or absence of 0.05% collagenase, washed well, and assayed at 5°C for ^{125}I-ASOR binding. The results of four experiments (Table II) showed that cells exposed twice to collagenase retained 10–50% of the binding activity of those cells exposed only once. The average number of ASGP receptors after one exposure to collagenase and incubation at 37°C was 500,000/cell and after two exposures at 37°C was 150,000/cell.

The experiments described above raised two questions: (*1*) Why was the collagenase-associated reduction in surface ASGP receptors detected on isolated cells treated twice with the enzyme and not on dissociated cells treated once? and (*2*) Why did control cells (one exposure to enzyme) subsequently incubated at 37°C have relatively high levels of binding compared to freshly isolated cells? A number of processes may be occurring simultaneously to give these results: (*a*) inactivation by enzymes in the collagenase preparation of the receptor and/or some other component of the plasma membrane involved in ASGP binding; (*b*) loss of surface receptor by some mechanism other than inactivation such as internalization of portions of plasma membrane; (*c*) recovery of functional receptor at 37°C by insertion of receptor from some intracellular compartment or by new synthesis and insertion. To separate the effects of enzyme exposure from subsequent events, ^{125}I-ASOR binding at 5°C was compared in a perfused liver before and after collagenase treatment at 37°C (no Ca^{+2}-free pre-perfusion). A 60% decrease in cell surface binding was observed immediately after collagenase exposure (Table III, Experiment 1). This decrease was not observed after collagenase treatment at 5°C (not shown). The liver was then warmed to 37°C for 1 h to simulate a recovery period and surface binding activity increased to 110% of the initial value. This experiment verifies that collagenase exposure alone reduces ASGP binding activity and illustrates the ability of perfused liver to recover that binding activity at 37°C upon removal of the enzyme.

The previous experiment was repeated for isolated cells and the same result was obtained (Table III, Experiment 2). That is, collagenase reduced cell surface ^{125}I-ASOR binding by 49%, but after 1 h at 37°C, control cells and collagenase-treated cells exhibited increased binding activity (240% and 220%, respectively).

Although the buffered salt solution used for these experiments would not be expected to sustain significant protein synthesis (41, 42), we studied the increase in cell surface ^{125}I-ASOR binding in the presence of 1 mM cycloheximide, an inhibitor of protein synthesis. The incorporation of [^{3}H]leucine into TCA-insoluble material in KH-PVP-40 with or without 1 mM cycloheximide was 7–15% of the incorporation seen in DMEM (unpublished observations). We found that 0.5–1 mM cycloheximide had no effect on the recovery phenomenon.

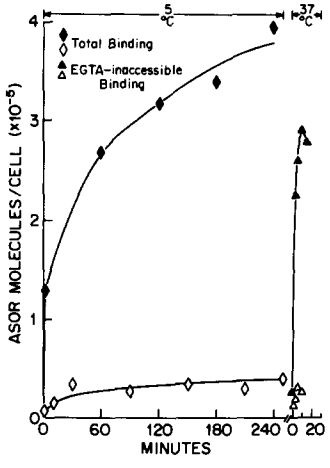

FIGURE 8 ^{125}I-ASOR binding to isolated hepatocytes at 5–7°C followed by warming to 37°C. Total and EGTA-inaccessible binding were measured as described in the Materials and Methods. Isolated hepatocytes were incubated at 5–7°C with 0.18 μg/ml (4.7×10^6 cpm/μg, ◇——◇) or 0.95 μg/ml (1.4×10^7 cpm/μg, ◆——◆) ^{125}I-ASOR for 4 h. Cells were washed three times at 4°C, resuspended, and then warmed to 37°C. Aliquots were removed and treated with EGTA. EGTA-inaccessible radioactivity represented internalized ^{125}I-ASOR (▲, △).

That is, an increase in ASGP binding at the cell surface was still observed after a 1-h incubation in 1 mM cycloheximide at 37°C (Table III, Experiment 3).

The series of experiments described above illustrates that although collagenase contains proteolytic activity and reduces the number of cell surface ASGP receptors in perfused liver and single cells, the dissociation process allows a significant, but variable, degree of recovery.

^{125}I-ASOR BINDING TO CELLS IN MONOLAYER CULTURE AT 5°C: Having determined the effect of collagenase on the surface ASGP receptor in dissociated hepatocytes, we next compared the binding activity at 5°C in freshly isolated cells to that in the same preparation of cells after 24 h in culture. The time-course of ^{125}I-ASOR binding in both cell systems at 5°C is presented in Fig. 7. Although the kinetics of binding were somewhat different, the numbers of surface receptors at saturation were similar in each system—215,000 receptors/cell in freshly isolated suspensions, and 295,000 receptors/cell after 24 h in culture.

Later Steps in the ASGP Pathway

It was important to follow the intracellular fate of ^{125}I-ASOR at 37°C in both freshly isolated and cultured hepatocytes to establish that the pathways were similar to the route in vivo and that all cells in the two populations manifested the pathway.

^{125}I-ASOR BOUND TO THE CELL SURFACE AT 4–10°C WAS INTERNALIZED AFTER WARMING TO 37°C: After saturation of cell surface receptors with ^{125}I-ASOR at 4–10°C, the bound ligand was rapidly internalized by isolated cells that were warmed to 37°C. Similar results were obtained with two cell preparations, one with relatively low and the second with relatively high numbers of cell surface ASGP receptors (Fig. 8). In both cases, 70% of the total radioactivity bound at 5°C was subsequently internalized by 10 min at 37°C. This result demonstrated that the ASGP internalization pathway was functional in cells with low (38,000) and high (400,000) numbers of surface ASGP receptors.

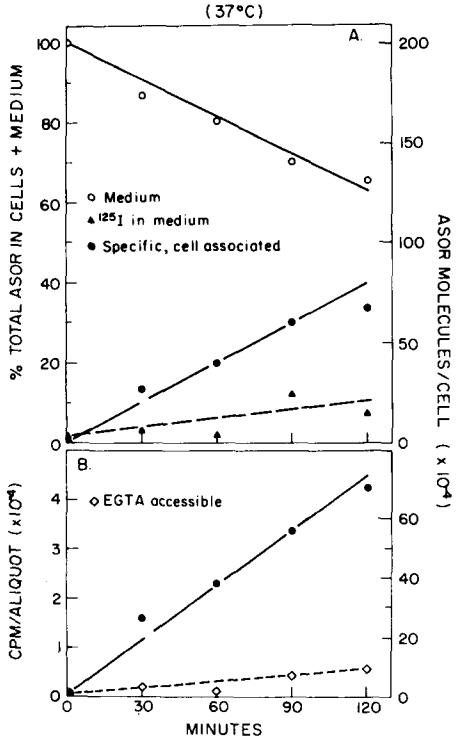

FIGURE 9 ^{125}I-ASOR binding, uptake, and degradation in isolated hepatocytes at 37°C. Binding, uptake, and degradation of ^{125}I-ASOR were measured with 0.7 μg/ml ^{125}I-ASOR (3.3 × 10^6 cpm/μg) and 80 μg/ml ASOR for nonspecific binding (3%). (*A*) Disappearance of radioactivity from the medium with the simultaneous increase in cell-associated radioactivity and $^{125}I^-$ in the medium. (*B*) Increase in EGTA-inaccessible radioactivity (the difference between the two curves) indicates internalization of ^{125}I-ASOR.

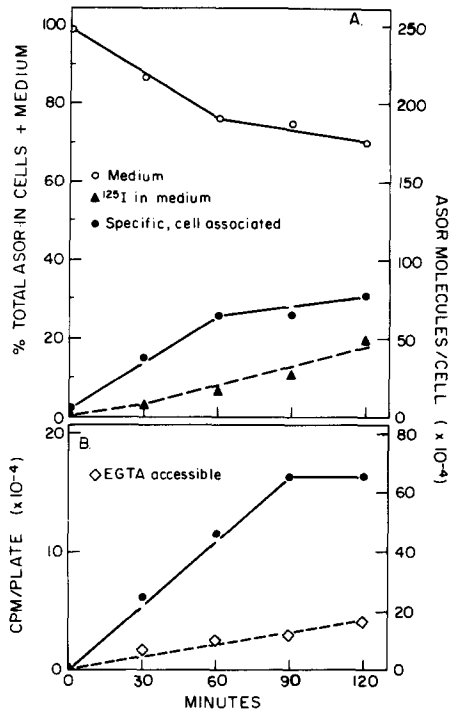

FIGURE 10 ^{125}I-ASOR binding, uptake, and degradation in cells at 37°C after 24 h in culture. Cells in culture were rinsed four times with warm KH-PVP-40, returned to the incubator for 15 min, and then assayed for ^{125}I-ASOR binding, uptake, and degradation. Individual dishes were exposed to 0.16 μg/ml ^{125}I-ASOR (3.5 × 10^6 cpm/μg) in the absence or presence of 50 μg/ml unlabeled ASOR (10% nonspecific binding). At timed intervals, dishes were cooled on ice and washed as described in Materials and Methods. The medium and the rinsed monolayers (rinses discarded) were precipitated with 10% TCA (final concentration). Aliquots of the TCA-soluble supernatant were analyzed by Sephadex G-25 chromatography as described in Materials and Methods. Cell number was estimated by LDH activity. (*A*) The disappearance of radioactivity from the medium occurs with a simultaneous increase in cell-associated radioactivity and $^{125}I^-$ in the medium. (*B*) Distribution of the cell-associated radioactivity. An increase in EGTA-inaccessible radioactivity (the difference between the two curves in *B*) indicates uptake of ^{125}I-ASOR.

^{125}I-ASOR BINDING, UPTAKE, AND DEGRADATION AT 37°C: Next, the capacities of both dissociated hepatocytes and reassociated cells in monolayer culture to bind, internalize, and degrade ^{125}I-ASOR at 37°C were compared (Figs. 9 and 10). By 2 h, single cells had internalized 40% of the added label and the monolayers 50%. These values represent 1 × 10^6 molecules and 1.25 × 10^6 molecules per cell, respectively. Degradation of the endocytosed ligand occurred by cells in both preparations as evidenced by the appearance of $^{125}I^-$ in the medium, beginning at ~30 min and increasing linearly with time.

The preceding results suggested that the ASGP pathway was maintained in both in vitro systems, making them valid models for studying the dynamics of the ASGP pathway. We next used LM-ARG to determine whether all of the cells in each system were actively taking up ^{125}I-ASOR. Dissociated cells (not shown) and cells in culture (Fig. 11) were incubated with ^{125}I-ASOR at 37°C. Virtually all viable cells (>95%) in both cases contained grains representing the ligand, whereas control cells (excess ASOR) were free of grains. Dead cells and debris did not accumulate significant numbers of grains in either system. In the monolayer cultures, cells on the edge of clusters

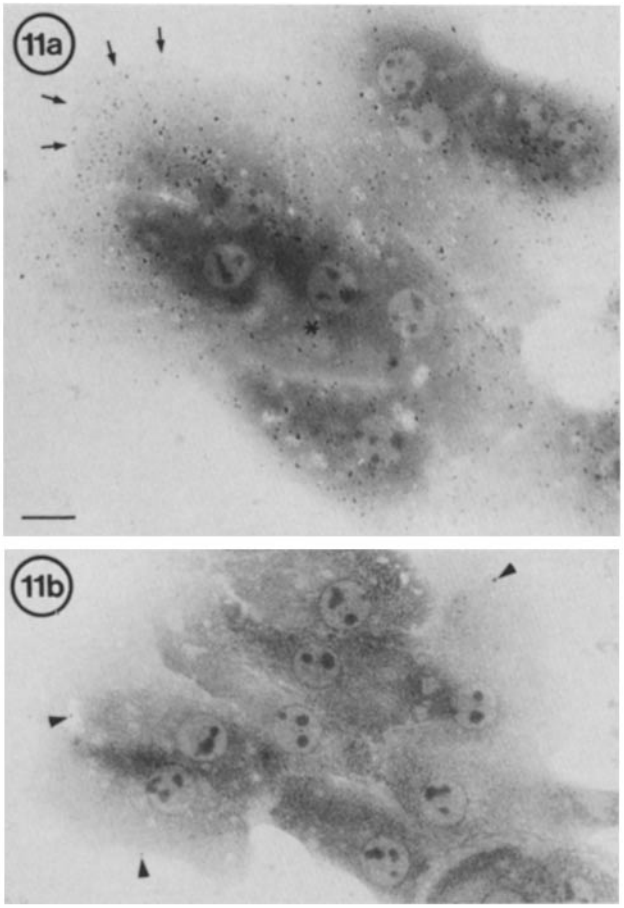

FIGURE 11 LM-ARG of cells in culture incubated with ^{125}I-ASOR at 37°C for 30 min. Cells after 24 h in culture were rinsed four times with KH-PVP-40 and preincubated at 37°C for 30 min. Individual dishes were then exposed to 0.5 μg/ml ^{125}I-ASOR (4.25 × 10^6 cpm/μg) in the absence or presence of 100 μg/ml ASOR. 2-d exposure, Bar, 20 μm. × 350. (*a*) Incubation with ^{125}I-ASOR at 37°C for 30 min. ARG grains are deposited over the entire cell cytoplasm but are out of focus at cell edges (arrows). Cells in the middle of clusters appear to have fewer ARG grains (*). (*b*) Incubation with ^{125}I-ASOR at 37°C for 30 min in the presence of excess ASOR. Very few ARG grains (arrowhead) are visible.

appeared to have accumulated more ligand than did cells toward the center (Fig. 11 *a*).

We then turned to EM-ARG to visualize the intracellular pathway taken by ^{125}I-ASOR in isolated hepatocytes continuously exposed to the ligand at 37°C. Table IV contains a summary of the autoradiographic quantitation. At zero time, 61% of the grains were associated with the plama membrane compartment, and 39% were intracellular. This relatively large intracellular amount at the initial time-point probably reflects both the rapid internalization and the finite time it took to sample and dilute the aliquot on ice. The grain density (% grains/% area) was 3.3 for the plasma membrane compartment and only 0.91 in the cytoplasmic compartment. By 30 and 60 min, the grain concentration shifted to the lysosomal compartment. The decrease within this latter compartment at 60 min from 3.9 to 2.0 was consistent with the loss of degraded ^{125}I-ASOR in the form of $^{125}I^-$ as predicted from the biochemical results (Fig. 9). Cells incubated with ligand for 30 and 60 min and then briefly (5 min) exposed to EGTA had substantially reduced numbers of grains in the plasma membrane compartment (5.9% and 0%, respectively), confirming at the ultrastructural level that EGTA could be used to discriminate between surface and intracellular ligand.

The ARG grain localization seen in cells incubated with ^{125}I-ASOR for 30 min is illustrated in Fig. 12. A substantial concentration of grains was apparent in structures identified as lysosomes as well as around the cell periphery. With respect to the grains associated with (or near) the cell surface, there was no obvious concentration to particular regions of the cell.

ASOR-HRP Cytochemistry in Dissociated Hepatocytes

The experiments described above demonstrated that single hepatocytes and cells in monolayer cultures resembled hepatocytes in vivo, both quantitatively and qualitatively, in terms of binding activity, internalization, and fate of ASGPs. We next investigated the distribution of ASGP surface receptors on freshly isolated cells, because these cells have no recognizable blood front domain, the predominant location of the ASGP receptor *in situ* (6, 7). Qualitatively, the EM-ARG results indicated a random distribution of binding sites over the entire surface. However, the resolution of this technique was not adequate for precise ultrastructural localization of ASGP surface binding sites. Therefore, preliminary experiments were carried out with ASOR-HRP, a cytochemical tracer. In cells exposed to saturating concentrations of ASOR-HRP at 5°C for 2 h, reaction product was concentrated in pits at the cell surface (Fig. 13 *a*). The cell surface between pits and along microvilli was also stained, but less intensely. Examination of cross sections of >50 cells revealed that the entire cell circumference was stained, with clusters of darkly stained pits seen in some areas and occasional empty pits found alongside those containing the ligand. Cells that were treated with 7.5 mM EGTA after 2 h of continuous exposure to ASOR-HRP at 5°C were devoid of cell surface reaction product (Fig. 13 *b*), indicating that both the light and heavy staining required Ca^{+2}. Finally,

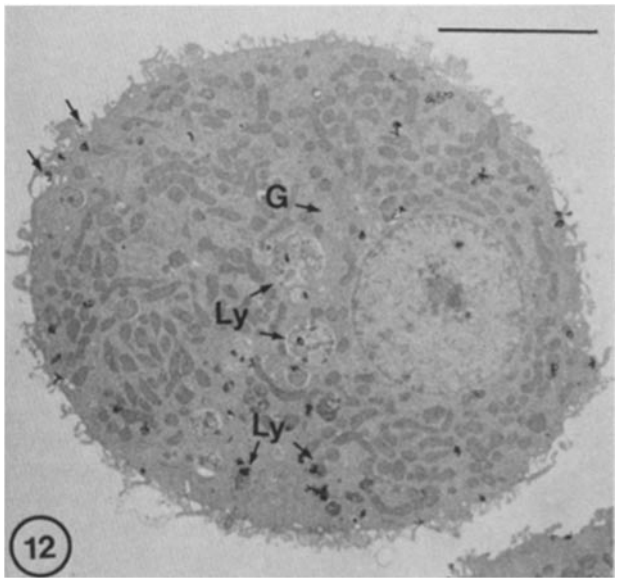

FIGURE 12 EM-ARG of an isolated hepatocyte incubated with ^{125}I-ASOR at 37°C for 30 min. Typical EM autoradiograph after 30-min continuous exposure to ^{125}I-ASOR under the conditions described in Table IV. ARG grains are concentrated on the plasma membrane (arrowheads) and in the Golgi-lysosome areas (*G*, Golgi complex. *Ly*, lysosome). Bar, 5 μm. × 4300. Five-month exposure.

cells that were exposed to ASOR-HRP in the presence of 100-fold excess ASOR (not shown) or that were never exposed to the conjugate were also free of staining. Therefore, ASOR-HRP reaction product deposited on the cell surface at 5°C represents specific binding of ASOR-HRP.

We next examined the endocytic pathway of ASOR-HRP in isolated hepatocytes. When cells were continuously exposed to ASOR-HRP at 37°C for 30 min, reaction product was found in vesicles in the Golgi-lysosome region. Furthermore, pits, vesicle profiles, and irregularly shaped tubules near the surface also contained reaction product (Fig. 14*a* and *b*) as well as regions of the cell surface between pits. This latter staining was variable in extent on the single cell, probably reflecting the rapid rate of internalization at 37°C. Treatment of these cells with EGTA removed all cell surface staining (Fig. 14*b*), but irregularly shaped tubules and vesicle profiles near the surface remained stained, demonstrating the proximity of this intermediate compartment to the cell surface in isolated cells.

Preliminary observations with ASOR-HRP at 5°C in hepatocytes cultured in monolayers for 4 and 24 h indicated that specific reaction product was located in pits along surfaces facing the medium, the collagen substratum, and on lateral membranes (Fig. 15*a* and *b*). In addition, staining was seen between pits and along microvilli (Fig. 15*a* and *b*). At 24 h, two preliminary observations were made: (*a*) The reaction product in pits at the cell surface appeared less concentrated than in pits on isolated hepatocytes or cells cultured for 4 h in a monolayer (Fig. 15 *b*); and (*b*) ASOR-HRP was absent from

TABLE IV

*Distribution of ARG Grains Associated with Isolated Hepatocytes Exposed to ^{125}I-ASOR at 37°C**

	Cellular Compartment					
	Plasma Membrane	Cytoplasm	Lysosomes	Golgi	Nuclei	Mitochondria
0 min						
% total grains (28)	61	36	0	0	0	3
% total area (928)	18	39	2.5	0.84	8.0	31
% grains/% area	3.3	0.91	0	0	0	0.12
30 Min						
% total grains (109)	19	60	16	3.7	0	4.6
% total area (1345)	14	42	4.2	2.6	12	25
% grains/% area	1.4	1.4	3.9	1.4	0	0.18
30 Min + EGTA						
% total grains (136)	5.9	61	27	1.5	0.74	3.7
% total area (1191)	9.2	39	4.7	1.6	15	30
% grains/% area	0.64	1.6	5.8	0.94	0.048	0.12
60 Min						
% total grains (103)	25	61	6.8	0.97	0.97	4.8
% total area (1189)	16	46	3.4	1.5	9.9	24
% grains/% area	1.6	1.3	2.0	0.66	0.10	0.20
60 Min + EGTA						
% total grains (75)	0	49	36	2.7	0	12
% total area (622)	13	44	5.1	1.6	1.9	35
% grains/% area	0	1.1	7.0	1.7	0	0.34

* Cells were incubated with 0.17 μg/ml ^{125}I-ASOR (1 × 10^7 cpm/μg) at 37°C, and aliquots were removed and either treated with EGTA or washed directly as described in Materials and Methods. The cell pellets were then fixed and processed for EM-ARG (Materials and Methods). Quantitation was performed on micrographs of sections exposed for 7 wk. (Numbers in parentheses refer to the total number of grains or intersection points counted in a given category.)

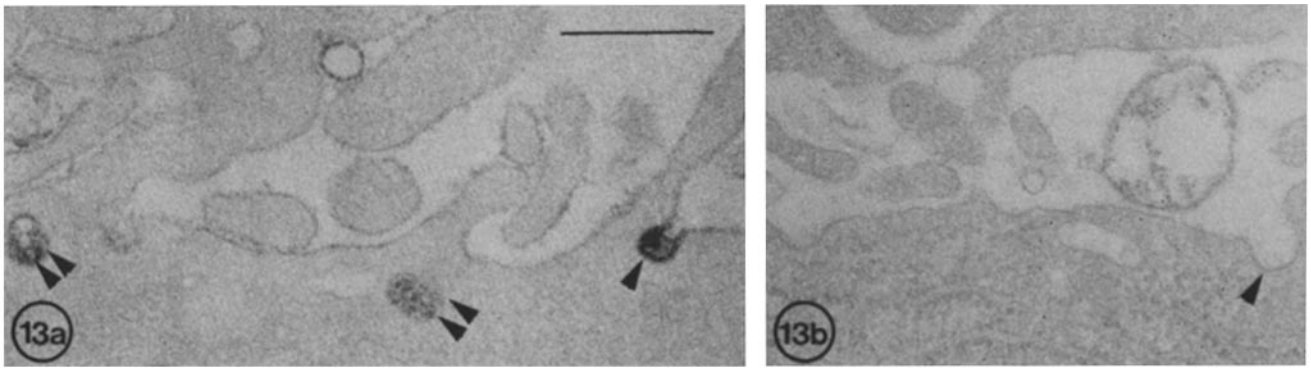

FIGURE 13 ASOR-HRP binding to the surface of dissociated hepatocytes at 5°C. (*a*) Isolated hepatocytes were incubated with 4 μg/ml ASOR-HRP for 2 h at 5°C, and then washed, fixed, and incubated with DAB-H_2O_2 as described in Materials and Methods. Reaction product is deposited diffusely over the cell surface and is heavily concentrated in pits (single arrowhead) and small vesicles presumed to be in contact with the medium (double arrowhead). (*b*) Cells from (*a*) that were incubated with ASOR-HRP for 2 h at 5°C, were treated with EGTA as described in Materials and Methods before processing. Reaction product is absent from the cell surface, pits (single arrowhead) and vesicles near the surface. Unstained sections, Bar, 0.2 μm. × 50,400.

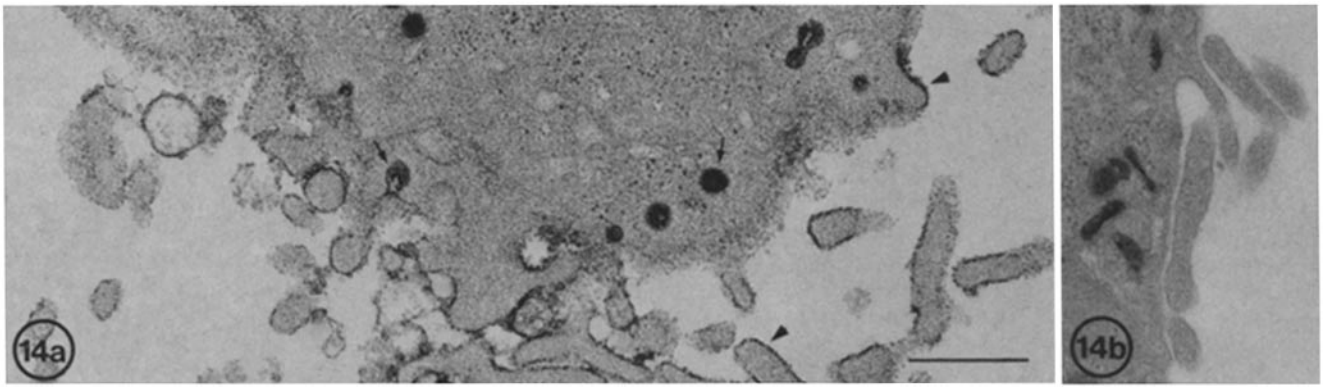

FIGURE 14 ASOR-HRP binding and uptake by dissociated hepatocytes at 37°C. (*a*) Isolated hepatocytes were incubated with 2 μg/ml ASOR-HRP for 30 min at 37°C, washed, fixed, and then incubated with DAB-H_2O_2 as described in Materials and Methods. Reaction product is concentrated in pits and vesicle profiles (arrows) near the surface, surface membrane outside pits and including microvilli (arrowheads), irregularly shaped tubules, and vesicles near the surface (intermediate compartment [5]), and in vesicles in the Golgi-lysosome region (not shown). (*b*) Cells from (*a*) that were incubated with 2 μg/ml ASOR-HRP for 30 min at 37°C, were treated with 7.5 mM EGTA before processing. Reaction product is absent from the cell surface but remains in small irregularly-shaped tubules near the surface (intermediate compartment). Bar, 0.5 μm. × 32,000.

bile canalicular-like spaces. Work is in progress to permeabilize the tight junctions in cultured cells under conditions that will prevent receptor redistribution to establish the presence or absence of the sinusoidal marker from the "new" bile canalicular domain.

DISCUSSION

We have demonstrated that both freshly isolated hepatocytes and cells cultured for 24 h in a monolayer express the receptor-mediated pathway for ASGP uptake and catabolism. A combined biochemical and morphological approach was used to quantitate and visualize the ligand pathway.

Collagenase Causes a Reduction in Cell Surface ASGP Receptors that is Reversible with Incubation at 37°C

Because we were specifically interested in the localization of cell surface ASGP binding sites on hepatocytes after dissociation, we were concerned that the proteases known to be present in collagenase preparations (43, 44) might inactivate a significant number of receptors. By manipulating the number of exposures to collagenase and the time and temperature of subsequent incubations, we were able to distinguish between a collagenase-associated reduction in cell surface ASGP binding activity and a time- and temperature-dependent recovery of that activity in both perfused liver and isolated cells. The ability of isolated hepatocytes to increase the number of surface ASGP receptors during incubation at 37°C has been reported by others (14, 45, 46). We found that dissociated cells exposed twice to collagenase were able to increase their surface receptor population even in the presence of 1 mM cycloheximide.

Our finding that collagenase-treated hepatocytes "recover" a variable fraction of surface ASGP receptors in the absence of new protein synthesis suggests that a previously inaccessible pool of receptors appears at the cell surface with time at 37°C. Recent reports indicate that the cell surface population in the isolated hepatocyte may represent as little as 5% or as much as 50% of total cellular receptor (14, 47, 48). However, the exact size and site(s) of the internal receptor pool remain controversial (48, 49). The results of several studies, including our own, demonstrate clearly that the surface population of receptor is insufficient to account for all of the ligand internalized at 37°C. Thus, receptor reutilization has been postulated. However, the role of the internal pool in this reutilization is unclear at present. Nevertheless, our results suggest very strongly that in isolated hepatocytes the internal receptor pool can contribute to the surface population. We are continuing these relocation studies.

Although the mechanisms of collagenase-associated reduction and subsequent recovery of ASGP binding activity remain unclear, it appears that the variability seen among our cell preparations could be explained by the variable degrees of loss and recovery of surface receptors. Thus, we urge caution in the interpretation of receptor number in isolated hepatocytes.

Surface Binding and Intracellular Fate of ASGP Receptors in Isolated Hepatocytes and Monolayer Cultures Resembled Events in Perfused Liver

As stated above, we observed substantial variability in the number of surface ASGP receptors at 5°C among freshly isolated cell preparations, with most of our preparations yielding cells having 100,000—400,000 surface receptors after isolation. The extremes we have found are in agreement with both the low values (i.e., <100,000 receptors/cell [14, 45, 46]) and high values (i.e., 100,000–500,000/cell [15, 50–52]) reported by others.

When isolated cells were maintained in monolayer culture for 24 h, we found that the numbers of surface ASGP receptors they retained were comparable to those found in cells in suspension and in perfused livers (53). Furthermore, we verified by LM-ARG that >95% of the cells in the culture actively internalized ^{125}I-ASOR. The preliminary finding that hepatocytes surrounded by neighboring cells contained fewer ARG grains than cells with large surface areas available to the medium could reflect either a greater number of cell surface receptors or greater accessibility to ligand in the medium. Experiments are in progress to address this question.

In this study we have found that $\sim 1 \times 10^6$ molecules of ASOR were processed over a 2-h period by both dissociated

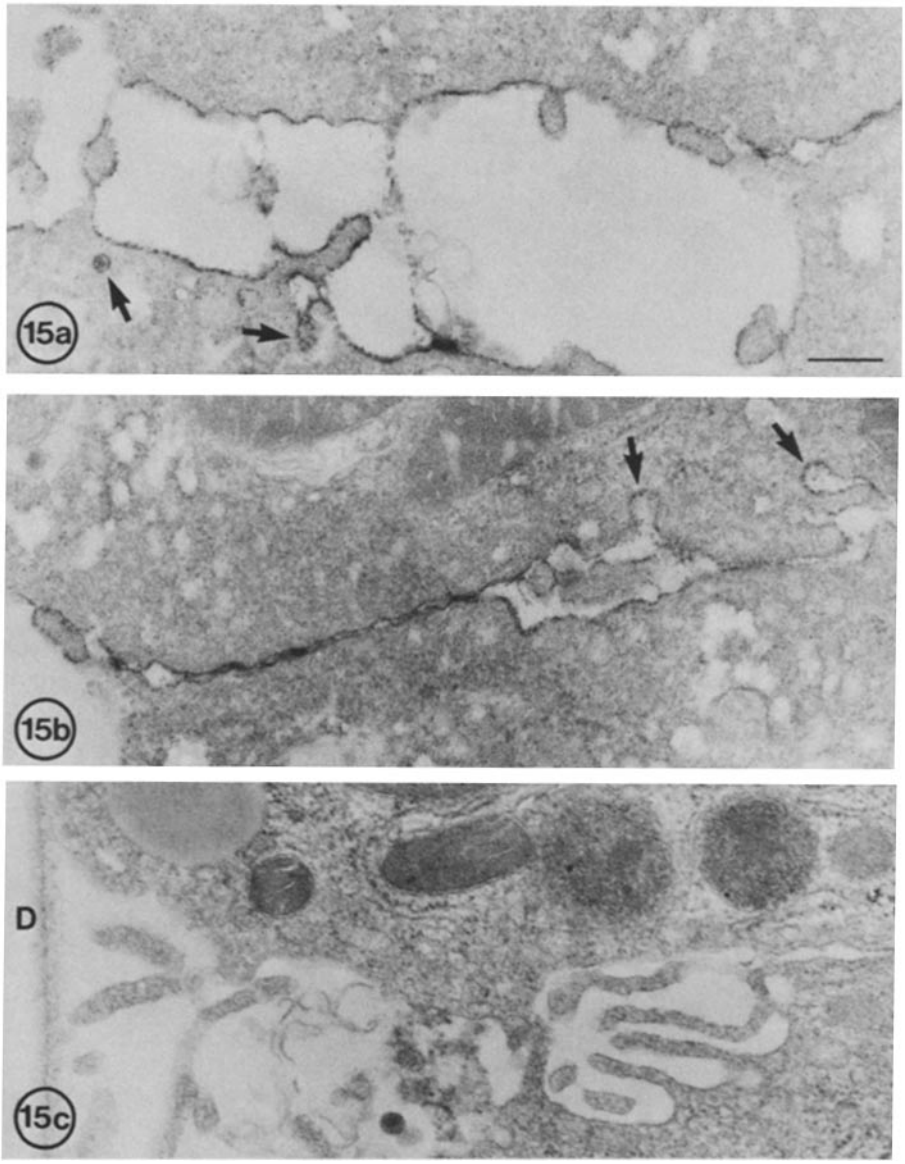

FIGURE 15 ASOR-HRP binding at 5°C to the surface of hepatocytes cultured for 4 and 24 h in a monolayer. (*a*) Cells were cultured in a monolayer for 4 h and then rinsed and incubated with ASOR-HRP (2 μg/ml) at 5°C for 4 h as described in Materials and Methods. The monolayers were processed for electron microscopy as pellets as described in Materials and Methods. The reaction product is uniformly distributed along membrane surfaces. (arrows indicate pits and vesicles near the cell surface) Bar, 0.33 μm. × 30,400. (*b*) Cells from the same plating as (*a*) were cultured for 24 h and then incubated with ASOR-HRP under the same conditions. Reaction product is seen evenly deposited along lateral membrane surfaces and pits (arrows) opening onto these surfaces. × 30,400. (*c*) Cells from (*a*) were incubated with ASOR-HRP for 4 h at 5°C in the presence of 100 μg of unlabeled ASOR. Reaction product is absent from all membrane surfaces. (D, surface in contact with the dish) × 30,400.

cells and cells cultured for 24 h. This rate represents 10–30% of the rates reported by others (10, 14, 16, 51). At present, we do not have an explanation for the slower rate observed; however, the reduction in rate of uptake could possibly be exploited to detect ligand or receptor in compartments that would be depleted too rapidly for detection in vivo. Nonetheless, we are confident from our autoradiography results that the intracellular fate of internalized ASOR remains qualitatively equivalent to the in vivo pathway.

ASGP Binding Sites Were Not Uniformly Distributed on the Surface of an Isolated Hepatocyte

Our cytochemical results using ASOR-HRP support the hypothesis that, with isolation and loss of cell surface specializations, integral membrane proteins are no longer segregated to one domain. That is, the ASGP receptor, a marker of the sinusoidal domain in hepatocytes *in situ*, was distributed around the entire periphery of the single cell. However, we did observe a concentration of ASOR-HRP reaction product in coated pits as well as a lighter staining on the rest of the cell surface, a pattern analogous to that seen in the perfused liver (53). It is interesting that the preferential concentration in coated pits is maintained in the unpolarized cell because previous studies of membrane topography in other epithelial systems have not detected such a distribution for membrane proteins after disruption of cell junctions. For example, Sang et al. (54) dissociated MDCK cells with EDTA and noted the simultaneous loss of tight junctional elements and the disap-

pearance of differential intramembrane particle densities by freeze-fracture. Pisam and Ripoche (55) studied dissociated frog urinary bladder epithelium by a variety of ultrastructural and cytochemical techniques and found that, as tight junctions were opened, apical glycoprotein labels "invaded" the basolateral domain. Ziomek et al. (56) observed slow and uniform diffusion of alkaline phosphatase and leucine aminopeptidase (two integral membrane enzymes normally segregated to the brush border of intestinal epithelium) over the entire surface of the single cell. We and others have yet to determine whether redistribution of membrane proteins is accompanied by insertion from an intracellular pool.

Finally, we have found that during reestablishment of ultrastructural differentiation in cells cultured as a monolayer for 24 h, the pathway for uptake of desialylated glycoproteins was maintained. The sites of ASGP uptake in this system remain to be investigated, to establish whether a specific domain marker, the ASGP receptor, can be resegregated into a single domain of the cultured hepatocyte surface.

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