Characterization of the Asialoglycoprotein Receptor on Isolated Rat Hepatocytes*

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Rat hepatocytes, freshly isolated by a collagenase perfusion technique, bound $[^{3}H]$. Asialo-orosomucoid in a sugar-specific and calcium-dependent manner as expected for the hepatic asialoglycoprotein receptor. At least 90% of the total cell surface-bound $[^{3}H]$. Asialo-orosomucoid represented specific binding and was removed by washing with EDTA. Freshly isolated cells had about $7 \times 10^{9}$ surface receptors per cell. However, when cells were incubated at $37^\circ C$, the number of surface receptors per cell rapidly increased 2- to 3-fold to about $2.2 \times 10^{9}$. This increase in receptor number occurred in the absence of serum and began within minutes, depending on the particular conditions used to keep the cells in suspension. (The maximal rate of appearance of new receptors at $37^\circ C$ was about $2.2 \times 10^{9}$ per cell per s.) When cells were first exposed to a brief EDTA treatment at $4^\circ C$, before measuring the binding of $[^{3}H]$. Asialo-orosomucoid, the number of surface receptors per cell was found to increase by about 45%. Therefore, about 30% of the surface receptors on freshly isolated cells have already bound endogenous asialoglycoproteins or are present in the membrane in a cryptic form.

At $4^\circ C$ the binding of $[^{3}H]$. Asialo-orosomucoid was rapid ($k_{on} \geq 1.8 \times 10^{8} M^{-1} s^{-1}$), whereas the dissociation of bound $[^{3}H]$. Asialo-orosomucoid, measured in the presence of excess nonradioactive glycoprotein, was extremely slow ($k_{off} \leq 0.9 \times 10^{-3} s^{-1}$). The association constant calculated from these data ($K_{d} = 2.0 \times 10^{-9} M^{-1}$) agreed well with that obtained from equilibrium binding experiments ($K_{d} = 2.4 \times 10^{-9} M^{-1}$) using untreated cells or cells which had first been treated with EDTA or incubated at $37^\circ C$. In all cases, when the concentration of $[^{3}H]$. Asialo-orosomucoid was higher than about $600 \text{ ng/ml}$, the Scatchard plots were curvilinear. The data are, however, consistent with the conclusion that there is a single high affinity receptor on the hepatocyte surface. The additional receptors that appear on the surface when cells are incubated at $37^\circ C$ or exposed to EDTA are identical with those on untreated cells.

The hepatic asialoglycoprotein receptor was initially discovered and characterized by Morell et al. more than 12 years ago (1). This receptor functions in vivo to effect the rapid and efficient clearance of desialylated serum glycoproteins. It was the first mammalian protein identified as a lectin-like molecule and has since been studied extensively. The protein was first purified from rabbit liver (2) and well characterized by Kawasaki and Ashwell (3, 4). Pricer and Ashwell demonstrated (5) that the asialoglycoprotein is present in several subcellular fractions, including the Golgi complex, the lysosomes, and smooth microsomes as well as the plasma membrane (roughly 5% of the total hepatic pool of receptor was actually in the plasma membrane fraction). More recently, Tanabe et al. purified the receptor from rat liver and examined its topological distribution in subcellular membrane fractions (6). Surprisingly, they found viable receptor activity on the cytosolic surface of membranes in the lysosomal fraction. This result, together with in vivo experiments in which the half-life of the receptor was measured, led them to suggest that the receptor must be recycled during asialoglycoprotein catabolism (6).

Other workers have studied the clearance from plasma and the rapid degradation in vivo of various asialoglycoproteins including fetuin (7), $a_{1}$-antitrypsin (8), transcortin (9), transferrin (10), and orosomucoid (7). Regoeczi et al. also studied the turnover of the asialoglycoprotein receptor in vivo and concluded that it is not destroyed when it functions (7). Wall et al. recently reported (11) that surface-bound lactosyl ferritin, a derivative which is recognized by the receptor and rapidly taken up in vivo, is almost exclusively localized in coated pit regions of the sinusoidal plasma membrane. Tollehsaag et al. have studied the uptake and degradation (12) and the intracellular localization (13) of asialofetuin in isolated rat hepatocytes.

We have chosen to study the hepatic asialoglycoprotein receptor for several reasons. First, we are interested in the molecular details of receptor-mediated endocytosis. The asialoglycoprotein receptor appears to provide an excellent model system for such studies, since the receptor has already been purified and characterized (6) and is known to function in vivo in an endocytic capacity. In addition, there are many advantages of dealing with rat liver including the ability to do in vivo experiments, to obtain active plasma membrane fractions, to isolate and culture hepatocytes, and to obtain biochemically significant quantities of material. We recently reported a preliminary characterization of the endocytosis of asialo-orosomucoid by freshly isolated hepatocytes (14). Second, the asialoglycoprotein receptor appears to be involved in the specific adhesion of freshly isolated rat hepatocytes to synthetic carbohydrate surfaces (15). Rat hepatocytes will adhere in a sugar-specific and calcium-dependent manner to flat polycrystalline gels containing covalently linked galactosides (16). Cell adhesion will only occur, however, at or above a critical concentration of galactoside in the gel (17). This threshold binding response, which also occurs in the specific adhesion of chick hepatocytes to N-acetylglucosamine surfaces (17), may be a fundamental and important characteristic

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of cellular interactions with external surfaces. Preliminary experiments indicate that the distribution of asialoglycoprotein receptors on the rat hepatocyte surface changes when these cells adhere to galactoside surface (15). We are continuing to explore the role of the asialoglycoprotein receptor in this threshold binding response.

For both of the studies mentioned above, it was necessary to characterize the asialoglycoprotein receptors on freshly isolated rat hepatocytes, and we, therefore, undertook the present study. Steer and Ashwell subsequently reported on the receptor in isolated hepatocytes and provided additional evidence that the receptor is recycled during the catabolism of asialo-orosomucoid (18). A preliminary report of our results has appeared (19).

EXPERIMENTAL PROCEDURES

Materials

Human orosomucoid (a, acid glycoprotein) was the generous gift of Dr. M. Wickerhauser of the American National Red Cross. Asialo-
orosomucoid and asialo-agalacto-orosomucoid were prepared as described by Schachter et al. (20). Triton X-100, collagenase (type I or type IV), BSA (Fraction V), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were from Sigma Chemical Co. Sprague-Dawley rats (125 to 150 g) were obtained from Timco Breeding Laboratories, Houston, Texas. Scintillation fluid (3a70B) and high specific activity NaB'H, (20 Ci/mmol), obtained in 25-mCi ampules, were from Research Products International Corp.

Freshly isolated hepatocytes were suspended in Medium 1 which contained a modified Eagle's medium (Grand Island Biological Co. catalogue 420-1400) supplemented with 2.4 g/liter of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 0.22 g/liter of NaHCO3. All other chemicals were reagent grade.

Cell Preparation

Hepatocytes were prepared from male rats (female animals were used occasionally; no sex-related differences were observed) by the collagenase perfusion procedure of Seglen (21) with several minor modifications (17). Final cell pellets were suspended in Medium 1, and were routinely 60 to 85% single cells and 85 to 95% viable as judged by either trypan blue exclusion (in the absence of serum or BSA) or the release of lactate dehydrogenase (22). All experiments were performed in the absence of serum. In some cases 0.1% BSA was present.

Preparation of Radioactive Asialoglycoprotein

The reductive methylation procedure of Means and Feeney (23) was used to make [3H]asialo-orosomucoid. Typically, 2 mg of protein in 1 ml of 0.2 M sodium borate, pH 9, was mixed, on ice, with 1 ml of 2% aqueous formaldehyde and added to a freshly opened ampule containing 25 ml of NaB'H,. At 5-min intervals, 1-ml additions of formaldehyde were made and the contents thoroughly mixed. After 30 min, the reaction mixture was dialyzed exhaustively, with repeated changes, against phosphate-buffered saline until the dialysate had less than 106 cpm/ml. [3H]Asialo-orosomucoid could be stored at 0°C for months with no loss of activity. Reproducibly, the specific activity was in the range 2.0 to 2.5 X 106 dpm/µg of protein.

Binding Assays

The binding of [3H]asialo-orosomucoid to suspensions of freshly isolated hepatocytes was performed at 4°C and assessed using a centrifugation procedure. In some experiments a sample was removed from a large volume of cell suspension at various times after adding the radioactive glycoprotein, while in others, smaller volumes (typically 1 ml) were used, each at a different time or protein concentration. Cells were kept in suspension either by gently swirling the tubes by hand every 10 to 15 min or with the use of a multipurpose rotator (Scientific Products, isolated hepatocyte). Rotating at 6 rpm, to which the tube was attached. In either case, binding of [3H]asialo-orosomucoid was determined by washing cells and then measuring radioactivity in a lysed cell pellet. Samples were first diluted with a 7- to 10-fold excess of the same ice-cold medium, in which the cells were suspended in disposable borosilicate tubes (15 X 100 mm). Diluted suspensions were well mixed by gentle inversion of the tubes (sealed with parafilm; American Can Co.), and then pelleted at 10000 g for 3 min. After removing the supernatant fluid by aspiration, the pellet was gently resuspended in the same volume (usually 6 to 8 ml) of ice-cold wash medium and recentrifuged. The final cell pellet was dissolved in 1.2 ml of 0.2% Triton X-100. One milliliter of the lysate was mixed with 9 ml of scintillation fluid to determine [3H]radioactivity. Nonspecific binding was assessed by including in the binding medium an excess of nonradioactive asialo-orosomucoid (at least 50-fold).

Various Treatments of Cell Suspensions

EDTA Treatment—To a cell suspension at 4 x 105 cells/ml in Medium 1 on ice was added a stock solution of 0.5 mM sodium-EDTA. EDTA was used to give a final concentration of 10 mM. The cell suspension was immediately swirled and gently pipetted up and down several times to achieve mixing and then incubated for 5 min with gentle swirling every 30 to 40 s. The cells were then centrifuged (800 rpm for 2 min), the medium removed by aspiration, and the pellet resuspended in Medium 1 (with or without 0.1% BSA) usually to a concentration of 4 x 105/ml. This EDTA treatment did not affect cell viability and did not increase the percentage of single cells. Note also that Medium 1 contains 1.8 mM CaCl2.

Treatment at 37°C—Cell suspensions (4 ml) at 2 x 105 cells/ml in Medium 1 plus 0.1% BSA were incubated in 16-ml polystyrene tubes (Falcon, catalogue 2001) for 30 to 60 min in a 37°C incubator. The tubes were inverted by rotating them at 6 rpm on a multipurpose rotator. Under these incubation conditions cell-cell adhesion does not occur (16), and cell viability and the pH of the medium are not affected. The cell suspensions were chilled on ice and then centrifuged for 2 min at 1500 rpm. The supernatant fluid was removed by aspiration and the cell pellets resuspended to the desired cell concentration in Medium 1 (with or without 0.1% BSA, depending on the experiment).

General

Protein was determined by the method of Lowry (24). Centrifugations of cell suspensions were done with a G-1 table top centrifuge (Sorvall Instrument Co.). Radioactivity was determined on 10% aqueous samples in a Triton/toluene scintillation fluid (3a70B from RPI Corp.) using a Packard Tri-Carb model 2002 liquid scintillation spectrometer.

RESULTS

Characterization of the Binding Assay—The specific binding of [3H]asialo-orosomucoid to isolated rat hepatocytes in suspension was measured using a centrifugation assay. This assay was linear with increasing cell number and gave a constant recovery of cell protein from about 100 X 106 cells/ml (Fig. 1). The specificity of binding, determined by the ability of excess nonradioactive asialo-orosomucoid to prevent the binding of [3H]asialo-orosomucoid was usually 90% (e.g. see Fig. 4A). The purified asialo-glycoprotein receptor (6) as well as the functioning molecules in vivo (1) and in isolated plasma membranes (25) have been shown to bind specifically to terminal galactosyl side chains of various glycoproteins. This specificity is also clearly expressed by the isolated hepatocytes (Fig. 2). Neither orosomucoid (in which galactose is the penultimate sugar in the peripheral oligosaccharide chains) nor asialo-agalacto-orosomucoid (from which both the terminal sialic acid and the penultimate galactose residues have been removed) competed for the binding of [3H]asialo-orosomucoid by hepatocytes, whereas unlabeled asialo-orosomucoid competed effectively as expected (Fig. 2).

Calcium ions are required for the successful binding of asialoglycoproteins by both the purified (6) and membrane-bound asialoglycoprotein receptor (25). In hepatocytes also required Ca2+ to express fully the activity of this receptor. For example, at 37°C the binding and uptake of [3H]asialo-orosomucoid by hepatocytes in suspension can be inhibited 98% by 20 mM EDTA (not shown). At 4°C, the binding of [3H]asialo-
orosomucoid was decreased by about 80% by first washing

1 The abbreviation used is: BSA, bovine serum albumin.
Asialo-orosomucoid was added to all tubes to a final concentration of asialo-orosomucoid and on cell recovery. Freshly isolated cells were incubated in ice-cold Medium 1 to a density of 12 × 10⁶ cells/ml, and different volumes of the suspension were added to glass tubes (13 × 10⁶ mm) on ice. 

The cell pellet was resuspended in ice-cold Medium 1 and centrifuged again. The supernatant fluid was removed by aspiration, and the cell pellets were resuspended in 7 ml of ice-cold Medium 1 and centrifuged again. The final cell pellet was dissolved in 1.2 ml of 0.1 N NaOH and incubated at 55°C for 20 min. One milliliter of this lysate was removed for the determination of radioactivity (●—●). The ordinate is a logarithmic scale (protein concentration). The recovery of cell protein was measured on washed cell pellets as described under "Experimental Procedures." The data shown on the ordinate. (If the data were extrapolated just to zero specific binding, one would predict that the Buffer 1 plus 0.1% BSA contained about 15 μM Ca²⁺. This is equivalent to 1 mol of Ca²⁺ per mol of albumin or a 0.03% (w/w) contamination by calcium ions, a very likely possibility. Furthermore, when EDTA is added to this buffer, the residual, apparently Ca²⁺-independent specific binding of [³H]asialo-orosomucoid is completely abolished.)

and then resuspending the cells in a buffer with no added Ca²⁺ (see legend, Fig. 3). Moreover, when cells were washed briefly with EDTA prior to measuring [³H]asialo-orosomucoid binding, the apparent Kₘ (i.e. half-saturation value) for Ca²⁺ shifted from about 20 μM to 40 μM (Fig. 3). This indicates, as one would expect, that a significant pool of Ca²⁺ ions was associated with the hepatocytes. However, the binding of [³H]asialo-orosomucoid could be reversed or prevented by EDTA (Fig. 4D). In the other experiment, cells (●—●, 25 ml, 90% viable, 77% singles, 4 × 10⁶ cells/ml) were treated with EDTA as described under "Experimental Procedures" and then washed once with 25 ml of Buffer 1 plus 0.1% BSA. Cells (1.25 ml) were incubated for 124 min at a final concentration of 3.2 × 10⁶ cells/ml, and bound [³H]asialo-orosomucoid was determined as described above. The amount of nonspecific binding was independent of the calcium ion concentration.

The ordinate is a logarithmic scale (protein concentrations ranging up to about 2700 ng/ml).

**Fig. 1.** Effect of cell number on the specific binding of [³H]asialo-orosomucoid and on cell recovery. Freshly isolated cells (89% viable, 60% singles, 4 × 10⁷/ml) were treated with 10 mm EDTA on ice for 5 min and centrifuged at 800 rpm for 2 min (see under "Experimental Procedures"). The cell pellet was resuspended in ice-cold Medium 1 to a density of 12 × 10⁶ cells/ml, and different volumes of the suspension were added to glass tubes (13 × 10⁶ mm) on ice. [³H]Asialo-orosomucoid was added to all tubes to a final concentration of 1.0 μg/ml, and the final volume was brought to 1.25 ml with Medium 1. Duplicate samples were used with and without 71 μg/ml of nonradioactive asialo-orosomucoid to assess specific binding. After incubation on ice for 120 min (with gentle shaking every 10 to 15 min, to keep the cells suspended), 7 ml of ice-cold Medium 1 was added to each sample. The cell suspensions were mixed by gently inverting the tubes several times and then centrifuged at 1000 rpm for 15 min. The supernatant fluid was removed by aspiration, and the cell pellets were resuspended in 7 ml of cold Medium 1 and centrifuged again. The final cell pellet was dissolved in 1.2 ml of 0.1 N NaOH and incubated at 55°C for 20 min. One milliliter of this lysate was removed for the determination of radioactivity (●—●), and the remainder was used to determine protein concentration. The recovery of cell protein (□—□) is expressed as a percentage relative to the initial protein concentration of the EDTA-treated cell suspension.

**Fig. 2.** Effect of orosomucoid derivatives on the binding of [³H]asialo-orosomucoid by rat hepatocytes. Cells (1 ml, 81% viable, 90% singles) at a final concentration of 2.4 × 10⁷/ml were incubated in glass tubes (13 × 10⁶ mm) on ice for 2½ h with 600 ng of [³H]asialo-orosomucoid/ml and the indicated concentrations of unlabeled orosomucoid (□—□), asialo-orosomucoid (●—●), or asialo-agalacto-orosomucoid (○—○). Bound [³H]asialo-orosomucoid was measured on washed cell pellets as described under "Experimental Procedures." The ordinate is a logarithmic scale (protein concentrations ranging up to about 2700 ng/ml).

**Fig. 3.** Effect of the concentration of CaCl₂ on the binding of [³H]asialo-orosomucoid. Two separate experiments are shown. In one, cells (○—○; 7 ml, 96% viable, 60% singles, 4.7 × 10⁷/ml) were washed twice by centrifugation at 4°C with 40 ml of Buffer 1 plus 0.1% BSA. (Buffer 1 contains, per liter, 8.3 g of NaCl, 0.5 g of KCl, and 2.4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid adjusted to pH 7.4 with NaOH.) The final cell pellet was resuspended in the same medium to a final concentration of 2.3 × 10⁶ cells/ml, and 1.25-ml portions in glass tubes (13 × 10⁶ mm) were incubated on ice for 100 min with 960 ng of [³H]asialo-orosomucoid/ml and the indicated concentrations of CaCl₂. The tubes were then centrifuged for 2 min at 1000 rpm, the supernatant fluid removed by aspiration, and the cell pellets resuspended in 7 ml of Medium 1 plus 0.1% BSA and centrifuged again. The final cell pellets were dissolved in 1.2 ml of 0.2% Triton X-100, and [³H]radioactivity was determined as described under "Experimental Procedures." In the other experiment, cells (●—●, 25 ml, 90% viable, 77% singles, 4 × 10⁶ cells/ml) were treated with EDTA as described under "Experimental Procedures" and then washed once with 25 ml of Buffer 1 plus 0.1% BSA. Cells (1.25 ml) were incubated for 124 min at a final concentration of 3.2 × 10⁶ cells/ml, and bound [³H]asialo-orosomucoid was determined as described above. The amount of nonspecific binding was independent of the CaCl₂ concentration. In the absence of added CaCl₂, the specific binding of [³H]asialo-orosomucoid by hepatocytes was 19% of the total specific binding at 200 μM CaCl₂. This value has been subtracted from the data shown on the ordinate. (If the data were extrapolated back to zero specific binding, one would predict that the Buffer 1 plus 0.1% BSA contained about 15 μM Ca²⁺. This is equivalent to 1 mol of Ca²⁺ per mol of albumin or a 0.03% (w/w) contamination by calcium ions, a very likely possibility. Furthermore, when EDTA is added to this buffer, the residual, apparently Ca²⁺-independent specific binding of [³H]asialo-orosomucoid is completely abolished.)

**Fig. 4.** Effect of the concentration of CaCl₂ on the binding of [³H]asialo-orosomucoid by Hepatocytes—The on rate (kₒ) for the binding of [³H]asialo-orosomucoid is rapid, even at 4°C (Fig. 4A). Total binding of the glycoprotein was 50% complete within about 6 min. The extent of nonspecific binding (measured in the presence of excess nonradioactive asialo-orosomucoid) increased rapidly for the first 5 min, then leveled off and increased gradually with time. The specific binding increased in a hyperbolic manner for about 60 min and, thereafter, increased at a rate slightly greater than that for the nonspecific binding. Nonspecific binding after 1 h was about 10% in this experiment (Fig. 4A). Neither the specific nor the nonspecific binding of [³H]asialo-orosomucoid appeared to saturate within 2 to 3 h.

As discussed below, hepatocytes treated in several different

² P. H. Weigel, unpublished results.
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Fig. 4. The kinetics of binding and release of $^{3}H$asialo-orosomucoid at 4°C. A, kinetics of $^{3}H$asialo-orosomucoid binding to hepatocytes. Cells (91% viable, 76% singles, 2.3 x 10^7 ml final concentration) were incubated on ice with 960 ng of $^{3}H$asialo-orosomucoid/ml in the presence (O—O) or absence (C—C) of 101 ng/ml of nonradioactive asialo-orosomucoid. At the indicated times, 0.8-ml samples were diluted into 8 ml of ice-cold Medium 1, and bound $^{3}H$asialo-orosomucoid was determined as described under "Experimental Procedures." B, kinetics of $^{3}H$asialo-orosomucoid binding to hepatocytes at 4°C after various treatments. Kinetic experiments of the type shown in A were performed with two different cell suspensions that were (O—O) or were not (C—C) treated with 10 mM EDTA at 0°C for 5 min as described under "Experimental Procedures." In one case (C) 0.1% BSA was added to the final cell suspension. The binding data for the first 5 min are not included (see text). The correlation coefficient for the line is 0.95. C, kinetics of release of bound $^{3}H$asialo-orosomucoid in the presence of excess nonradioactive asialo-orosomucoid. The correlation coefficient for the line is essentially constant. In the experiment shown in Fig. 4C, when an excess of nonradioactive asialo-orosomucoid was added to cells which had already bound $^{3}H$asialo-orosomucoid, $k_{on}$ measured over a 4-h period, was 1.1 x 10^8 s^{-1}. In a different experiment (not shown), when cells were first treated with EDTA (see under "Experimental Procedures"), allowed to bind $^{3}H$asialo-orosomucoid at 4°C, and washed and incubated with nonradioactive asialo-orosomucoid, the measured $k_{on}$ was 0.8 x 10^8 s^{-1}. In the absence of excess nonradioactive asialo-orosomucoid the amount of cell-bound $^{3}H$asialo-orosomucoid did not decrease. In these experiments, the final extent of dissociation (about 15%) could only be assessed over a period of about 4 to 5 h, since cell viability decreased considerably after that. It is, therefore, assumed (but not demonstrated) that all of the receptor-ligand complexes can be dissociated at an average rate (± S.D.), $k_{off}$ = 0.90 ± 0.17 x 10^7 s^{-1} (N = 3).

Since the asialoglycoprotein receptor requires Ca^{2+} in order to bind asialo-orosomucoid (Fig. 3), a divalent cation chelator such as EDTA could be used to remove $^{3}H$asialo-orosomucoid bound to the cell surface if the Ca^{2+} complex were reversible. This is apparently true since EDTA causes the
Effect of EDTA pretreatment on the binding of [\(^{3}H\)]asialo-orosomucoid by hepatocytes. A, effect of EDTA concentration. Cell suspensions (1 ml, 4 \times 10^9/ml of Medium 1, 89% viable, 60% single) were treated in glass tubes (13 \times 100) on ice for 5 min with the indicated final concentrations of EDTA and then centrifuged at 1500 rpm for 1 min. Cell pellets were resuspended in 1 ml of Medium 1 containing 1.25 \mu g of [\(^{3}H\)]asialo-orosomucoid/ml and incubated on ice for 120 min with gentle shaking every 10 to 15 min. Bound [\(^{3}H\)]asialo-orosomucoid was determined as described under "Experimental Procedures." The 100% value at time zero was 130 fmol of [\(^{3}H\)]asialo-orosomucoid/10\(^6\) cells. B, effect of the duration of exposure of hepatocytes to EDTA on the binding of [\(^{3}H\)]asialo-orosomucoid. Cell suspensions (1 ml, 90% viable, 77% singles, 4 \times 10^9/ml of Medium 1. 895 viable, 77% singles) were incubated in 1.25 \mu g of [\(^{3}H\)]asialo-orosomucoid per ml. The suspensions were incubated for 10 min on ice, and bound [\(^{3}H\)]asialo-orosomucoid was determined as described under "Experimental Procedures."
hepatocytes kept at 37°C expressed on their surfaces a different number of receptors per cell compared to cells at 4°C. When hepatocytes that had been kept on ice after isolation were incubated in suspension at 37°C (in the absence of serum) for increasing periods of time and then chilled on ice, the number of specific surface asialoglycoprotein receptors measured per cell increased 2- to 3-fold (depending on the experiment) within 30 to 45 min (Fig. 6). This increase in cell surface receptor number at 37°C was very reproducible (regardless of the cell preparation) and usually led to a maximum of between 1.8 × 10^5 to 3.0 × 10^5 receptors per cell. This effect was independent of the exact incubation conditions employed. Cells were kept in suspension either by inversion of capped tubes (Fig. 6A), by stirring in spinner flasks (Fig. 6B), or by shaking in a gyratory water bath. When samples of the same cell preparation were incubated at 37°C, in these three different ways, qualitatively and quantitatively identical results were obtained (Fig. 5C). Differences in the rate of receptor number increase paralleled the rate of temperature equilibration. The rate of temperature equilibration was faster for cells under the gyratory conditions than in the rotating tubes, which was, in turn, faster than the spinner culture. The increase in receptor number per cell occurred (for all three different geometries) under conditions in which the percent-age of viability, the percentage of single cells, and the pH of the medium did not change. This result suggests that freshly isolated cells rapidly insert (or expose) more receptors in the plasma membrane when they are warmed to 37°C. The rate of appearance of new receptors ranged from about 25 to 70 receptors per cell per s (Fig. 6C).

Additional specific asialoglycoprotein receptors appear on the surface of hepatocytes exposed to EDTA or incubated at 37°C. These two receptor populations are not, however, overlapping; that is, those sites exposed by EDTA do not seem to be the ones that appear at 37°C. Thus, when cells are first treated at 4°C with EDTA (as under “Experimental Procedures”) and subsequently incubated at 37°C as in Fig. 6, the same increase in the number of surface receptors per cell occurs (not shown). Also, preliminary experiments indicate that additional receptors can still be exposed by treatment with EDTA even on cells that have been at 37°C for up to 90 min.

Saturation of ([H]Asialo-orosomucoid Binding by Hepatocytes—The specific binding of [H]asialo-orosomucoid by hepatocytes, which were first treated with EDTA as described under “Experimental Procedures,” did not fully saturate even at relatively high ligand concentrations (Fig. 7). At the lowest [H]asialo-orosomucoid concentrations tested (from 1 to 20 ng/ml), the binding was linear (see inset, Fig. 7A), and no discontinuities or breaks in the binding curve were observed, whereas at higher glycoprotein concentrations the curve appeared nonhyperbolic. The same data are also presented on a logarithmic scale (Fig. 7B). Although complete saturation was not observed, half-saturation of receptor binding occurred at approximately 60 ng/ml of [H]asialo-orosomucoid (1.5 nm). The same results were obtained whether or not the cells were first treated with EDTA or exposed to a 37°C incubation prior to measuring the binding of [H]asialo-orosomucoid.

Since the binding of [H]asialo-orosomucoid by hepatocytes is apparently a reversible and an equilibrium process (Fig. 4, A to D), a series of equilibrium binding experiments was performed at 4°C in order to determine the total number and identity of asialoglycoprotein receptors on cells, which had been subjected to the various treatments discussed above. In a typical experiment (shown in Fig. 8), cells which had been treated at 37°C for 35 min (see under “Experimental Proce-

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**FIG. 6. Effect of prior incubation at 37°C on the binding of [H]asialo-orosomucoid by hepatocytes.** A, suspensions in inverting tubes; effect of incubation time. Cell suspensions (90% viable, 50% singles, 2 × 10^7/ml) in Medium 1 plus 0.1% BSA were incubated at 37°C in rotating tubes for the indicated times as described under “Experimental Procedures.” The tubes were then chilled on ice, and 0.95-ml portions of cell suspension were transferred to glass tubes (13 × 100 mm) and centrifuged at 800 rpm for 2 min. The supernatant fluids were discarded, and the cell pellets were suspended in 0.6 ml of Medium 1 plus 0.1% BSA containing 1.5 μg of [H]asialo-orosomucoid/ml with (●, ○) or without (□, △) a 50-fold excess of nonradioactive asialo-orosomucoid. After incubation on ice for 90 min, bound [H]asialo-orosomucoid was determined as described under “Experimental Procedures.” B, suspensions in spinner flasks; effect of incubation time. A cell suspension at 4°C (92% viable, 85% singles, 2 × 10^7/ml, 80 ml) was transferred to a 100-ml spinner flask (Bellco Glass, Inc., cat. 1960-00100) in a 37°C incubator. At the indicated times 1.8-mI samples were removed into tubes (13 × 100 mm) on ice, and the binding of [H]asialo-orosomucoid was determined as in A. C, comparison of different conditions for keeping cells in suspension; effect of incubation time. Portions of cell suspensions (87% viable, 84% singles, 2 × 10^7/ml) were incubated at 37°C in polystyrene tubes (4 ml/tube, ●) as described under “Experimental Procedures,” or in a spinner flask (60 ml; □) as in B, or in 50-ml Erlenmeyer flasks (6 ml/flask; ○) in a gyratory shaking water bath (New Brunswick Scientific Co.) at 100 rpm. At the indicated times, samples (2 ml) of cell suspensions were diluted into 6 ml of ice-cold Medium 1 plus 0.1% BSA, mixed, and kept on ice. One set of samples (△) was on ice throughout the experiment. All samples were then centrifuged at 800 rpm for 2 min, the supernatant fluid aspirated off, and the pellets suspended in 1 ml of Medium 1 plus 0.1% BSA containing 1.3 μg of [H]asialo-orosomucoid/ml. The suspensions were incubated at 4°C for 85 min, and bound [H]asialo-orosomucoid was determined as described under “Experimental Procedures.”
The fraction of total receptors that are high affinity is calculated to a final cell density of $2.4 \times 10^9$/ml. One milliliter of ice-cold cell suspension was then added to glass tubes (13 x 100 mm) containing different concentrations of $[^3H]$asialo-orosomucoid (from 5 to 2500 ng/ml). Duplicate samples with 60 ng/ml of nonradioactive asialo-orosomucoid were used to determine specific binding. The final volume in all cases was 1.25 ml. After incubating the samples at 4°C for 100 min (with gentle shaking every 10 to 15 min to keep cells suspended), the tubes were centrifuged for 2 min at 2000 rpm. A portion (0.5 ml) of each supernatant fluid was removed to determine free $[^3H]$asialo-orosomucoid, and the remainder was aspirated off and discarded. The cell pellets were resuspended in 6 ml of Medium 1 containing 0.1% BSA, recentrifuged, and the amount of bound $[^3H]$asialo-orosomucoid in the final cell pellets was determined as described under "Experimental Procedures." Binding data for the total sample is presented. Specific binding was at least 95% of the total binding at each concentration of $[^3H]$asialo-orosomucoid. The correlation coefficients for the lines estimating the uncorrected binding parameters were $-0.99 (- - -)$ and $-0.94 (- - -)$.

### Table I

**Summary of binding data**

<table>
<thead>
<tr>
<th>Cell surface asialoglycoprotein receptors</th>
<th>High affinity</th>
<th>Apparent low affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell treatment</td>
<td>$K_a$</td>
<td>No. of sites/cell</td>
</tr>
<tr>
<td>None</td>
<td>$1.5 \times 10^8$</td>
<td>$7.5 \times 10^8$</td>
</tr>
<tr>
<td>EDTA</td>
<td>$2.2 \times 10^8$</td>
<td>$9.2 \times 10^8$</td>
</tr>
<tr>
<td>EDTA, BSA</td>
<td>$1.9 \times 10^8$</td>
<td>$9.9 \times 10^8$</td>
</tr>
<tr>
<td>37°C, BSA</td>
<td>$2.7 \times 10^8$</td>
<td>$9.5 \times 10^8$</td>
</tr>
<tr>
<td>37°C, CBA</td>
<td>$2.4 \times 10^8$</td>
<td>$21 \times 10^8$</td>
</tr>
<tr>
<td>37°C, CBA</td>
<td>$3.7 \times 10^8$</td>
<td>$24 \times 10^8$</td>
</tr>
<tr>
<td>Mean values</td>
<td>$K_a = 2.4 \pm 0.8 \times 10^6 M^{-1}$</td>
<td>$K_a = 0.37 \pm 0.10 \times 10^6 M^{-1}$</td>
</tr>
</tbody>
</table>

* n.d., not determined. In these experiments the concentration of $[^3H]$asialo-orosomucoid was not high enough to assess accurately the apparent low affinity receptors.

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**Surface Asialoglycoprotein Receptors on Rat Hepatocytes**

Fig. 7 (left and center). Saturation of the binding of $[^3H]$asialo-orosomucoid by hepatocytes. A, B, effect of increasing $[^3H]$asialo-orosomucoid concentration on the specific binding of $[^3H]$asialo-orosomucoid. Cell suspensions (92% viable, 70% singles, 4 x 10^9/ml) were treated with 10 mM EDTA (see under "Experimental Procedures") and incubated in glass tubes (13 x 100 mm) on ice for 120 min with the indicated final concentrations of $[^3H]$asialo-orosomucoid (volume differences were made up with Medium 1). Duplicate samples were used with and without nonradioactive asialo-orosomucoid (60 ng/ml final concentration) to assess specific binding. The inset shows that $[^3H]$asialo-orosomucoid binding was linear at the lowest protein concentrations used. Note that in A the abscissa is a linear scale, whereas in B the abscissa is logarithmic.

Fig. 8 (right). Scatchard plot for the binding of $[^3H]$asialo-orosomucoid to cells first incubated at 37°C. Cell suspensions (92% viable, 63% singles, 2 x 10^9/ml) were incubated at 37°C for 35 min as described under "Experimental Procedures," then chilled on ice, centrifuged, and resuspended in Medium 1 containing 0.1% BSA (92% viable, 63% singles, 2 x 10^9/ml) were treated with 10 mM EDTA, with or without 0.1% BSA as described under "Experimental Procedures." Binding data were treated according to the procedure of Scatchard (27) as presented in Fig. 8. The numbers below were calculated for each portion of the binding curve (like that in Fig. 8) from linear regression lines with the best correlation coefficients.
Hepatocytes in Culture—The experiments presented above were all performed with nonattached, freshly isolated cells over relatively short periods of time (1 to 2 h). Several preliminary experiments were also performed with cells cultured on 32-mm tissue culture dishes for 24 or 48 h (details will be presented elsewhere). After 24 h in culture, hepatocytes bound about 380 fmol of [H]asialo-orosomucoid per 10^4 cells, which corresponds to about 2.3 X 10^4 receptors per cell. This value is comparable to those shown in Table I and Fig. 6 for cells incubated at 37°C without serum for ≤1 h. Attached cells, cultured for 24 h, also showed an increase in the number of surface asialoglycoprotein receptors measured per cell when they were first treated with EDTA at 4°C, as discussed above for freshly isolated cells.

**DISCUSSION**

This paper describes some properties and characteristics of asialoglycoprotein receptors on freshly isolated rat hepatocytes. We view this as a necessary first step in an effort to understand the overall molecular details of how this receptor functions during the endocytosis of soluble asialglycoproteins and during the adhesion of hepatocytes to synthetic surfaces containing β-galactosides (18).

Hepatocytes showed a calcium-dependent (Fig. 3) and sugar-specific (Fig. 2) binding of asialo-orosomucoid consistent with the properties of the membrane-bound and solubilized asialoglycoprotein receptor. At 37°C, both the binding and uptake of [H]asialo-orosomucoid occur very rapidly and cannot easily be studied independently. At 4°C, only binding to the cell surface occurs, and the kinetics of this process was, therefore, studied in detail. The rate of binding, k on, (assuming a biomolecular reaction), was at least 1.8 X 10^4 M^-1 s^-1 and was not affected by 0.1% BSA or the cell treatment procedures discussed above. The time course of [H]asialo-orosomucoid binding (Fig. 4A) shows that at later times (i.e., higher percentage of receptor occupancy) the rate of specific binding decreases markedly, although it still continues at a low level. This indicates that complete saturation of receptor, with respect to time, is difficult to achieve (possibly because there is a lower affinity class of receptors or negative cooperative interactions between receptors occur under conditions of high receptor occupancy). The rate of dissociation of the receptor-[H]asialo-orosomucoid complexes, although slow, was measurable (Fig. 4C); k off = 0.9 ± 0.17 X 10^-1 s^-1 (± S.D., N = 3). It could not be shown directly that all receptor complexes dissociate at this rate, since cell integrity could not be maintained long enough (t d, for dissociation was about 16 h). However, in the presence of the divalent cation chelator EDTA, more than 90% of the specifically bound [H]asialo-orosomucoid was rapidly released (Fig. 4D), indicating that virtually all the specific receptor complexes are, in fact, reversible.

The questions, how many of each type of receptor are there? and how many of each type of receptor per cell? turned out to be more complicated than expected as a consequence of two new observations. First, treatment of hepatocytes with EDTA, under defined and relatively mild conditions, exposed about 45% more specific surface receptors. Second, when the temperature was raised to 37°C the number of surface receptors per hepatocyte increased markedly (2- to 3-fold).

We first considered whether the same type of receptor was present, in different numbers per cell, in each of these three cases (i.e., untreated, EDTA treated, and 37°C incubated cells). Equilibrium binding experiments (as in Fig. 8) were performed to answer this question. In all cases the cells possessed a high affinity receptor, which had an association constant ranging from 1.5 to 3.7 X 10^4 M^-1. The value for the apparent association constant calculated from the kinetic data K = k on/k off = 2 X 10^4 M^-1 is in good agreement with that obtained from equilibrium binding experiments (Table I), which gave an average value (± S.D.) of 2.4 ± 0.8 X 10^4 M^-1 (N = 7). The apparent dissociation constant, K d = k off/K a, is, therefore, about 42 nm. Using [125I]asialo-orosomucoid, Steer and Ashwell recently reported a K d value of 34 nm for this receptor on isolated hepatocytes (18). In their study the rate of binding of [125I]asialo-orosomucoid to hepatocytes was slower than reported here, and half-saturation of binding at 4°C occurred at approximately 1000 ng/ml of ligand (compared to about 60 ng/ml in this study). The reason(s) for these differences are unknown. However, significantly different procedures were used; for example, in the preparation of radioactive asialo-orosomucoid, the preparation of hepatocytes and the medium used to culture or maintain cells (e.g., no serum was used in the present study, whereas these workers used 17.5% heat-inactivated horse serum (18)). It was also found, in all cases in which the concentration of [H]asialo-orosomucoid was greater than about 600 ng/ml, that the Scatchard plots were curvilinear. This was consistent with the inability to saturate completely the binding of [H]asialo-orosomucoid by hepatocytes (Fig. 7). Although this could suggest that the properties of the membrane-bound and soluble asialo-orosomucoid receptor complexes at other affinity sites, we believe it is more likely that there is really one type of receptor (designated the high affinity receptor in Table I) which exhibits negative cooperativity characteristics in its binding at high receptor occupancy. This conclusion is supported by the following observations. (i) The ratio of high affinity to apparent total receptors was remarkably constant (0.66 to 0.68; Table I) regardless of any cellular treatment. (ii) Both the rat asialoglycoprotein receptor and the asialo-orosomucoid used here are multivalent. The stoichiometry of the in vivo binding reaction between the cell surface receptor and ligand is not known (it was assumed in the experiments presented here to be one for one). Since multivalent interactions should be more likely to occur as the amount of bound ligand increases, this could explain the apparent negative cooperativity of binding when the percentage of occupancy of receptors is high (either because of decreased accessibility or actual modulation of unoccupied receptor sites). (iii) The relative difference in affinities (if there were two distinct types of receptors) would only be about 6-fold. In most other cases that have been described the difference between high and low affinity receptors is usually several orders of magnitude. (iv) It is also known that the isolated receptor oligomerizes readily (2, 3). If receptor-receptor interactions also occur on the intact cell surface and such interactions are altered when a receptor binds an asialoglycoprotein then this could also contribute to the appearance of negative cooperative binding. Therefore, although it is possible that there are other types of asialglycoprotein receptors, we conclude at this point that the data in toto suggest there is only one type of receptor which is represented (in both affinity and number of sites per cell) by the high affinity receptor shown in Table I. The apparent lower affinity receptors are presumed to reflect multivalent interactions between receptor (complexes) and ligand. Freshly isolated cells had about 7 to 8 X 10^4 surface receptors per cell. Cells which had been treated with EDTA had close to 10^5 surface receptors per cell, whereas cells which were incubated at 37°C had about 2.3 X 10^5 surface receptors per cell. Steer and Ashwell found that hepatocytes cultured for 30 and 300 min at 37°C had, respectively, about 5 X 10^4 and 7 X 10^5 surface receptors per cell (18).

Two possible explanations for the exposure of new receptor sites by EDTA are that (i) some of the receptors on the surface of isolated cells are occupied by endogenous asialogly-
coproteins (bound in vivo or during the isolation procedure), or (ii) there is a population of unoccupied receptors present in the membrane in a cryptic, nonfunctional state, and these receptors can be activated by exposure to EDTA. If the first possibility is correct then asialoglycoproteins should be released into the medium during the EDTA treatment, and this cell-free supernatant fluid should have components that compete for the binding of [3H]asialo-orosomucoid by fresh hepatocytes. Although this explanation seems more likely, initial experiments have not yet detected such endogenous asialoglycoproteins. The second possibility should be seriously considered for several reasons. First, when freshly isolated cells are placed at 37°C (under which conditions rapid endocytosis of asialoglycoproteins occurs (14)), EDTA-exposable sites can still be detected. In addition, hepatocytes in culture for 24 to 48 h also have a portion (30 to 50%) of their total surface receptors which can be exposed by EDTA treatment. If these EDTA-sensitive sites represent occupied receptors then they are evidently not endocytosed rapidly like the bulk of the receptor complexes (14). Alternatively, if these sites are internalized, then new ones must be continually appearing at 37°C. Second, preliminary experiments indicate that when fresh cells are treated with EDTA at 4°C and then incubated in suspension at 37°C (in the absence of serum), new EDTA- strippable receptor sites appear as a function of time. This also showed that after subcellular fractionation the receptor is occupied by endogenous asialoglycoproteins. Third, the exposure of additional receptors by EDTA occurs slightly faster and at lower concentrations than does the removal of bound [3H]asialo-orosomucoid from the cell surface (not shown). This could be due to differences in affinity between the receptor and endogenous asialoglycoproteins compared to asialo-orosomucoid, which has the highest known affinity for mammalian asialoglycoprotein receptors. Alternatively the different EDTA sensitivities might indicate that EDTA does two different things.

The observation that a portion of the asialoglycoprotein receptors on isolated hepatocytes may be in a cryptic form, which can be exposed and made functional by EDTA, may be important in light of recent evidence that this receptor is recycled and conserved during endocytosis. In vivo experiments led Rogoeczi et al. (7) and Tanabe et al. (6) to conclude that the receptor is not degraded along with the internalized glycoprotein, but rather is reused and mediates the catabolism of many glycoprotein molecules per receptor. Tanabe et al. also showed that after subcellular fractionation the receptor appeared to be localized on the cytosolic side of membranes in a lysosomal fraction, whereas it was also present on the intravesicular side in several other membrane fractions (6). In addition, Steer and Ashwell have shown (18) that the receptor is also recycled in hepatocytes isolated by a collagenase perfusion technique. Whatever the mechanism by which the receptor is spared from degradation (e.g. by a change in orientation or sidedness within the membrane or by interaction with other protecting components, etc.), it is reasonable to assume that the progression from an initial receptor-glycoprotein complex to the regenerated receptor will be complicated and involve numerous steps. It is possible that in one of these steps the receptor is transiently cryptic within the membrane, and can be converted into a functional state by exposure to EDTA. Experiments are in progress to test this hypothesis.

The observation that hepatocytes expose additional receptors in the plasma membrane at 37°C may also be explicable in terms of receptor recycling. Preliminary experiments suggest that occupied and unoccupied receptors may be endocytosed at the same rate; that is, there may not be a preferential uptake of occupied receptors (14). If this is true and receptors continually recycle into and out of the cell, then under steady state conditions the total cellular pool of functioning receptors would be present in particular amounts at each stage of the recycling process. If this steady state is perturbed during the isolation procedure, then when the cells are rewarmed to 37°C they might be increasing the concentration of surface receptors in an attempt to reestablish the status quo of the cycle.

The additional surface receptors generated at 37°C or by exposure to EDTA appear to function like the receptors on untreated cells. The binding characteristics for [3H]asialo- orosomucoid are virtually identical (as discussed above), and preliminary experiments indicate that the rate and extent of endocytosis of the asialoglycoprotein by the receptors in each case are the same.

Lastly, the increase in the number of surface receptors per cell at 37°C may explain an earlier observation about the adhesion of rat hepatocytes to synthetic culture surfaces. When rat hepatocytes adhere to flat polyacrylamide surfaces containing covalently attached galactosides, there is a characteristic lag period at 37°C. This lag could be entirely eliminated by prior incubation of the cells at 37°C for 45 min under conditions (i.e. the rotating tubes) identical with those used in this study (16). Preliminary experiments indicate that the asialoglycoprotein receptor is the cell surface molecule that mediates this cell-gel binding (15). The observation that additional receptors are inserted into the membrane at 37°C for about 40 min (Fig. 6) suggests that freshly isolated cells do not yet have enough receptors to allow cell adhesion to the galactoside surfaces and, therefore, provides an explanation for the lag period. In addition, experiments with fluorescein asialo-orosomucoid indicate that clusters of asialoglycoprotein receptors form when hepatocytes bind to these galactoside surfaces (15). The role of asialoglycoprotein receptors in this binding interaction, in particular the nature of the threshold binding response (17), is being examined.

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REFERENCES
Surface Asialoglycoprotein Receptors on Rat Hepatocytes