Binding and Endocytosis of Glycoproteins by Isolated Chicken Hepatocytes†

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ABSTRACT: The binding and endocytosis of glycoproteins containing different terminal sugars by isolated chicken hepatocytes were studied. At 2 °C, where there is no endocytosis, the hepatocyte surface bound 30 800 $GlcNAc_{44}$-AI-BSA molecules [a bovine serum albumin (BSA) derivative which contains 44 residues of *N*-octylglucosamine (GlcNAc)] [Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963] and 32 900 asialoagalactoorosomucoid (AGOR) molecules per cell with estimated dissociation constants of 5×10^{-10} and 4×10^{-9} M, respectively. In the presence of digitonin or Triton X-100, each hepatocyte bound 7–18 times more ligand than in the absence of these detergents. Bound ^{125}I-AGOR could be dissociated from the cell surface by 5.5×10^{-5} M $GlcNAc_{44}$-AI-BSA with a $t_{1/2}$ of 30 min, while GlcNAc (10 mM) or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (4 mM) could dissociate over 98% of the surface-bound radioactivity within 10 min. Several neoglycoproteins inhibited the binding of ^{125}I-AGOR, requiring for 50% inhibition 2.1×10^{-9}, 4.0×10^{-7}, 1.6×10^{-6}, and 2×10^{-6} M for $GlcNAc_{44}$-, Glc_{37}-, Man_{43}-, and L-Fuc_{28}-AI-BSA, respectively. The bound AGOR and neoglycoproteins were internalized and degraded at 37 °C. [^{125}I]Iodide was the only labeled degradation product found. When the hepatocytes were exposed to 250 nM AGOR at 37 °C, ca. 100 000 molecules of AGOR were associated with the cell surface at the steady state of endocytosis. This is about a 3-fold increase over the corresponding value at 2 °C. Kinetic simulation of synchronous processing of surface-bound AGOR or $GlcNAc_{44}$-AI-BSA suggests that significant recycling of internalized ligand to the surface occurs during endocytosis at 37 °C.

In mammalian liver, there exists a receptor protein capable of mediating clearance of galactose- (Gal)[1,2] or GalNAc-terminated glycoproteins from the circulation and possibly other functions [for reviews, see Harford & Ashwell (1982) and Ashwell & Harford (1982)]. This Gal/GalNAc binding protein has been isolated and purified (Hudgin et al., 1974; Tanabe et al., 1979), and the surface binding properties and uptake processes have been characterized in both the perfused rat liver and isolated rat and rabbit hepatocytes by many laboratories [for example, see Wall et al. (1980), Weigel (1980), Wall & Hubbard (1981), Fiete et al. (1983), and Connolly et al. (1983)]. Whereas the Gal/GaNAc-specific receptor has been found in all mammalian liver examined to date, avian liver is apparently devoid of such a receptor (Lunney & Ashwell, 1976; Kawaskai & Ashwell, 1977). Instead, it contains a receptor capable of binding asialoagalactoorosomucoid (GlcNAc terminated) (Lunney & Ashwell, 1976). The receptor protein has been isolated and purified (Kawasaki & Ashwell, 1977) from whole chicken liver, and the GlcNAc-specific binding activity has been shown to be present in chicken hepatocytes (Schnaar et al., 1978; Kuhlenschmidt et al., 1982). The amino acid sequence of this receptor protein is also known (Drickamer, 1981).

Although one of the presumed roles for the mammalian Gal/GalNAc receptor is mediation of clearance of asialoglycoproteins from the serum (Ashwell & Morell, 1974), the lack of a similar activity in avian species suggests that the function of the respective receptors in chicken and mammals may be different. Therefore, detailed studies of receptor-mediated endocytosis of glycoconjugates by chicken hepatocytes were undertaken toward the goal of understanding the physiological role of carbohydrate receptor systems and of the process of receptor-mediated endocytosis. Some of the results obtained are presented in this report.

Experimental Procedures

Materials. The following animals or materials were obtained from the indicated sources: 21-day-old White Leghorn chickens from Truslow Farms, Chestertown, MD; carrier-free $Na^{125}I$ from Amersham Corp.; silicone oil (DC 550 fluid) from Accumetric Inc., Elizabethtown, KY; mineral oil, digitonin, and Triton X-100 from Sigma Chemical Co.; BSA (Pentex, fraction V) from Miles Laboratories, Inc.; HEPES from Research Organics, Inc. All other chemicals used in this work were of the highest purity available commercially and were used without further purification.

Preparation of Neoglycoproteins and AGOR. Neoglycoproteins of the amidino type (AI) containing 2–44 mol of thioglycosides per mol of BSA were prepared by the published method (Lee et al., 1976; Stowell & Lee, 1980). The neoglycoproteins were purified by gel filtration on Sephadex G-150 to obtain pure monomeric BSA derivatives prior to radioiodination. AGOR was prepared as previously described (Lunney & Ashwell, 1976).

Protein Determination. The protein concentrations of neoglycoprotein solutions were determined by the microbiuret method (Zamenoff, 1957). The protein concentration of orosomucoid derivatives was determined by using $E_{280nm} = 8.9$ (Schmid, 1975).

Iodination of Protein. Purified neoglycoproteins or orosomucoid derivatives (10–50 μg) were iodinated with 1 mCi of

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[1] All sugars are of the D configuration in pyranoside form unless otherwise indicated.

[2] Abbreviations: BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $GlcNAc_n$-AI-BSA, a bovine serum albumin derivative which contains *n* residues of *N*-acetylglucosamine; AGOR, asialoagalactoorosomucoid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Glc, glucose; Man, mannose; Fuc, fucose; Gal, galactose; EDTA, ethylenediaminetetraacetic acid; [I_{50}], concentration of inhibitor which produced 50% inhibition.

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$Na^{125}I$ by a modification (Connolly et al., 1981) of the procedure of Greenwood et al. (1963). The iodination mixture was chromatographed on a column (0.7 × 20 cm) of Sephadex G-25, and the radioactive material at the void volume was pooled. Recovery of protein after iodination and chromatography was estimated to be 60–70% as determined by a fluorescamine reaction (Stowell et al., 1978), and the specific radioactivity was 25–50 μCi/μg of protein. Radioiodinated ligand solutions were made 0.1% (w/v) BSA and were stored at 4 °C and used within 2 weeks. For most experiments, the preparation was diluted with the appropriate unlabeled protein for the desired specific activity unless the labeled ligand was less than 1 nM. To show that no harmful consequence resulted from radioiodination, the iodinated protein was diluted with the corresponding unlabeled protein, and the extent of binding was found to remain constant on the basis of the combined quantity of labeled and unlabeled ligands, either for AGOR or $GlcNA_{44}$-AI-BSA.

Preparation of Chicken Hepatocytes and Receptor Protein. Chicken hepatocytes were prepared and characterized as described (Kuhlenschmidt et al., 1982). Cells were suspended in medium A, a modified Eagle's medium prepared as described in detail previously (Kuhlenschmidt et al., 1982). The cells were 85–95% viable as judged by trypan blue exclusion and by measurement of cellular lactate dehydrogenase activity and were 80–90% single cells. The cells were determined to be >95% hepatocytes as judged by morphological criteria in phase-contrast microscopy. The receptor protein from chicken liver was prepared by the published method (Kuhlenschmidt & Lee, 1984).

Assay for Ligand Binding and Uptake by Chicken Hepatocytes. Isolated chicken hepatocytes and ^{125}I-labeled ligand were incubated in medium A (Obrink et al., 1977) containing 0.6% (w/v) BSA. Some incubations were carried out in buffered isotonic salt solutions. Incubations were carried out in capped 12 × 75 mm or 17 × 100 mm polystyrene tubes (Falcon Catalog no. 2058 and 2001, respectively) which were rotated vertically (end to end) at 6 rpm. In some experiments, 1.5-mL polypropylene tubes (no. 690; Sarstedt, Princeton, NJ) were used for incubation. Assays were initiated by adding cells to an incubation mixture containing the ^{125}I-labeled ligand at the appropriate temperature. Nonspecific binding and uptake were determined by adding unlabeled $GlcNAc_{44}$-AI-BSA to a final concentration of 6 μM (ca. 10^4 times the estimated surface receptor number). To determine cell-associated ligands in assays carried out in 12 × 75 mm or 17 × 100 mm tubes, duplicate samples (400 μL) were taken from each assay tube, and the cells were centrifuged for 1 min through a mixture of (4:1) silicone oil–mineral oil in 1.5-mL polypropylene microfuge tubes by using an Eppendorf Model 5412 microcentrifuge. Cells incubated in 1.5-mL polypropylene tubes were processed by adding 0.5 mL of a (4:1) silicone oil–mineral oil mixture directly followed by centrifugation. In some cases, duplicate 100-μL samples were taken, and cells were centrifuged 20–30 s through 150 μL of (4:1) silicone oil–mineral oil in a 0.4-mL polypropylene microfuge tube (no. 710; Sarstedt, Princeton, NJ). After centrifugation, the polypropylene tube was usually cut at the middle of the oil layer, and the tips containing the cell pellet were counted for radioactivity.

Internalized ligand was determined by putting duplicate 200-μL samples into each of two tubes containing 0.5 mL of 8 mM EGTA, 100 mM HEPES, 7.8 mM KCl, and 120 mM NaCl, pH 7.45. After 10 min on ice, 600 μL of each cell suspension in EDTA was taken and centrifuged for 1 min through oil in 1.5-mL polypropylene tubes, and the pellet was assayed for radioactivity. This value represented the internalized ligand. The value of surface-bound ligand was computed by subtracting the value for internalized ligand (the value with EGTA treatment) from the total cell-associated ligand (without EGTA treatment).

Assay for Ligand Binding to Detergent-Treated Cells. Chicken hepatocytes were treated with Triton X-100 under conditions described in the figure legends. ^{125}I-labeled ligand was added and the incubation continued. At the end of the incubation period, bound ligand was precipitated with cold 50% saturated ammonium sulfate, a condition which does not precipitate free ^{125}I-AGOR or ^{125}I-GlcNAc-AI-BSA (Kawasaki & Ashwell, 1977; Kuhlenschmidt & Lee, 1984). Briefly, an equal volume (0.5 mL) of ice-cold saturated ammonium sulfate was added, and after 10 min on ice, 1 mL of a suspension of Celite (20 mg/mL rinse buffer) was added to aid filtration, and the entire suspension was filtered on Whatman GF-C filter disks and rinsed with ice-cold 50% saturated ammonium sulfate containing 10 nM $CaCl_2$ buffered at pH 7.8.

When chicken hepatocytes were treated with digitonin (Weigel & Oka, 1983a,b; Weigel et al., 1983), the cells were centrifuged through oil and the cell pellets counted for radioactivity. At high concentrations of digitonin, however, the cells were not completely pelleted, so the medium above the oil (300 μL) was mixed with 300 μL of saturated ammonium sulfate, and bound ligand was measured as described above. After correction for sample volume, the total cell-associated ligand was taken as the sum of the pellet and bound ligand which remained in the medium.

Assay of Ligand Degradation and Product Characterization. Degradation products were analyzed by chromatography as described by Hoppe & Lee (1983), in which undegraded ligand (at the void volume), KI, monoiodotyrosine, and diiodotyrosine were completely separated. Briefly, after centrifugation, the medium (200 μL) was made 25 mg/mL in KI and 50 μg/mL in mono- and diiodotyrosine before application to a Sephadex G-100 column (0.8 × 19 cm) equilibrated in 1 M acetic acid. The column was eluted with 1 M acetic acid.

Steady-State Analysis of Ligand Internalization and Hydrolysis. The rate constants for internalization and hydrolysis of ^{125}I-AGOR by chicken hepatocytes were determined by a modification of the method used by Wiley & Cunningham (1981, 1982). The internalization rate constant at 37 °C was determined by first allowing the hepatocytes to attain a steady level of surface binding of unlabeled ligand, and then carrier-free ^{125}I-labeled ligand was added in negligible volume to follow the uptake at steady state. At steady state, if there is no degradation of internalized ligand and if the rate of dissociation of the surface-bound ligand into the medium is negligible, the internalization process can be expressed as [internal ligand]/[surface ligand] = $k_{int}t$, where k_{int} is the rate constant of internalization and t is time.

The rate constant of hydrolysis was determined by incubating the cells with labeled ligand at 37 °C until steady state was reached. After the cells were washed in the cold to remove free labeled ligand, the cells were resuspended at 37 °C in unlabeled ligand at the same concentration. The decrease in cell-associated ligand was then followed and analyzed by a first-order kinetic plot to determine the steady-state hydrolysis rate constant.

Data Analysis. The following computer programs were used to analyze the indicated types of data: SCAFIT, equilibrium binding of ligand to cell surfaces at low temperature; NEWKIN, kinetic data of ligand binding to cell surfaces at low temperature; KINSIM, kinetic simulation of synchronous processing

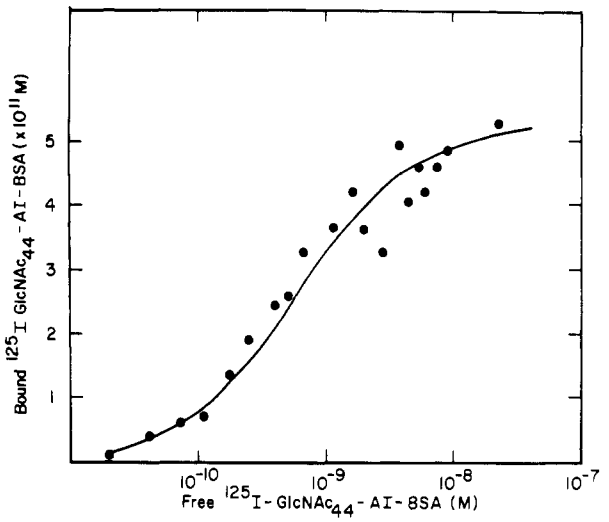


FIGURE 1: Equilibrium binding of ^{125}I-GlcNAc$_{44}$-AI-BSA to chicken hepatocytes at 2 °C. Chicken hepatocytes (1 × 10^6 cells/mL) were incubated with various concentrations of ^{125}I-GlcNAc$_{44}$-AI-BSA at 2 °C. After 2 h, samples were removed, and specifically bound ^{125}I-GlcNAc$_{44}$-AI-BSA was determined. The best fit of the data was obtained with a one-site model, using SCAFIT. Both the fitted curve (solid line) and the experimental points (●) are shown. The best-fit binding parameters (one-site model) are as follows: K_D = 5.5 × 10^{-10} M (±19%); R = 30800 (±13%) sites/cell.

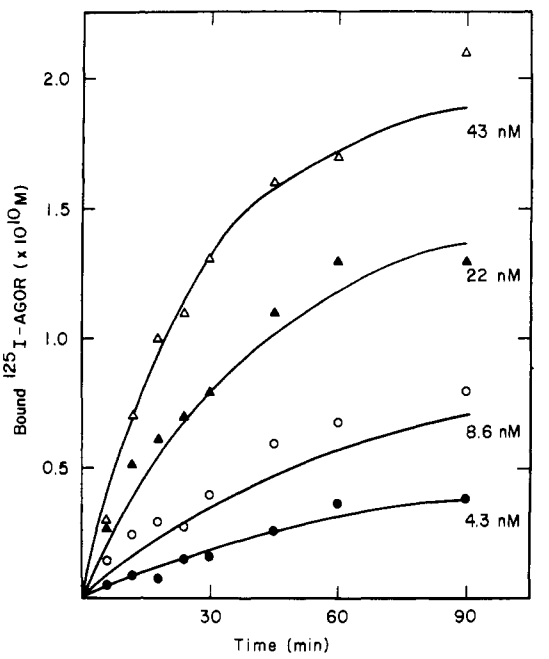


FIGURE 2: Time course of ^{125}I-AGOR binding to chicken hepatocytes at 2 °C. Chicken hepatocytes (5.14 × 10^6 cells/mL) were incubated for various lengths of time at 2 °C with ^{125}I-AGOR at 4.3 (●), 8.6 (○), 22 (▲), and 43 nM (△). At each time point, samples were removed, and specifically bound ^{122}I-AGOR was determined. The data were fitted to a reversible bimolecular reaction model for binding. Both the fitted curves (solid lines) and the experimental points are shown. The parameters obtained from this analysis were k_1 = 6.38 (±0.76) × 10^5 M^{-1} min^{-1}, K_D = 1.54 (±0.62) × 10^{-8} M, and k_{-1} = 0.99 (±0.42) × 10^{-2} min^{-1}. The number of ligands bound per cell was calculated by using the parameters obtained from curve fitting.

of surface-bound ligand at 37 °C.

The SCAFIT (Munson & Rodbard, 1980) and NEWKIN (DeLean & Rodbard, 1979) programs were obtained from the Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, TN. The KINSIM program (Barshop et al., 1983) was kindly provided by Dr. C. Frieden and his co-workers at Washington University, St. Louis, MO. All computer programs were implemented on a VAX 11/780 at the Engineering Computing Facility, The Johns Hopkins University.

Results

Binding of ^{125}I-GlcNAc$_{44}$-AI-BSA and ^{125}I-AGOR at 2 °C. Preliminary experiments showed that a 2-h incubation at 2 °C was sufficient time to establish equilibrium for ^{125}I-GlcNAc$_{44}$-AI-BSA binding at a dose of 220 pM or greater when the cell density was 4 × 10^6 cells/mL. This incubation time was used to obtain an isotherm of ^{125}I-GlcNAc$_{44}$-AI-BSA binding to isolated chicken hepatocytes (Figure 1).

Analysis of the data shown in Figure 1 by the nonlinear regression computer program SCAFIT (Munson & Rodbard, 1980) yielded a dissociation constant, K_D = 5.5 × 10^{10} M (±19%), and an apparent receptor site concentration, R = 30800 (±13%) sites per cell. These data fit a one-site model (shown by a solid line) better than two-site models. An analogous experiment with ^{125}I-AGOR and the data reduction yielded K_D = 4.0 (±1.5) × 10^{-9} M, with 32900 (±13%) sites/cell. In this case also, a one-site model fit the data better than two-site models.

Time Course of Binding of ^{125}I-AGOR to Isolated Chicken Hepatocytes at 2 °C. The time course of binding of ^{125}I-AGOR to isolated chicken hepatocytes at 2 °C was determined with ^{125}I-AGOR concentrations ranging from 4.3 to 43 nM and on incubation period up to 90 min (Figure 2). The data in Figure 2, when analyzed by the nonlinear regression program NEWKIN, yielded a binding rate constant of k_1 = 6.38 (±0.76) × 10^5 M^{-1} min^{-1} and an association constant K_A = 0.65 (±0.26) × 10^8 M^{-1} [or a dissociation constant K_D = 1.54 (±0.62) × 10^{-8} M]. Since $k_{-1} = k_1 K_D$, a rate constant for dissociation of ligand from the cell surface, k_{-1} = 0.99 (±0.42) × 10^{-2} min^{-1} ($t_{1/2}$ = 70 min), was obtained. The number of AGOR molecules bound to the cell surface was calculated to be 31900 (±13%) per cell.

This program also enables examination of changes in binding affinity as a function of site saturation by considering an interaction factor, δ, which is defined as K_e/K_f, where K_e and K_f are the association equilibrium constants of the ligand–receptor complex when the receptors are "empty" (no ligand bound) and "full" (receptor saturated), respectively. When the δ value is greater than unity, the presence of "negative cooperativity" is indicated. Conversely, a δ value smaller than unity suggests positive cooperativity. When the AGOR binding data of Figure 2 were analyzed, the δ value was 1.09, suggesting that, by this criterion, cooperativity is virtually absent in the binding of AGOR by these cells.

Increased Binding of ^{125}I-GlcNAc$_{44}$-BSA upon Treatment of Isolated Chicken Hepatocytes with Detergent at 2 °C. The binding by the Triton-treated cells shows a striking increase over that by the untreated cells (Figure 3). When the binding data were analyzed with the SCAFIT program as described previously, the Triton-treated cells had a K_D = 4.5 (±2.0) × 10^{-10} M for the GlcNAc$_{44}$-AI-BSA–receptor complex and a receptor concentration (R) of 4.3 × 10^5 (±25%) sites/cells. The untreated cells had a K_D = 3.1 (±1.2) × 10^{-10} M and a receptor concentration corresponding to 24000 (±25%) sites/cell in the same experiment.

Treatment of the cells with digitonin (Weigel et al., 1983; Weigel & Oka, 1983b) also showed increased ^{125}I-GlcNAc$_{44}$-AI-BSA binding capacity. The binding of ^{125}I-GlcNAc$_{44}$-AI-BSA to digitonin-treated cells was measured as a function of digitonin concentration, and the release of cellular lactate dehydrogenase activity was followed (Figure 4). With no detergent present , there were 36900 molecules of ^{125}I-GlcNAc$_{44}$-AI-BSA associated per cell, and after treatment

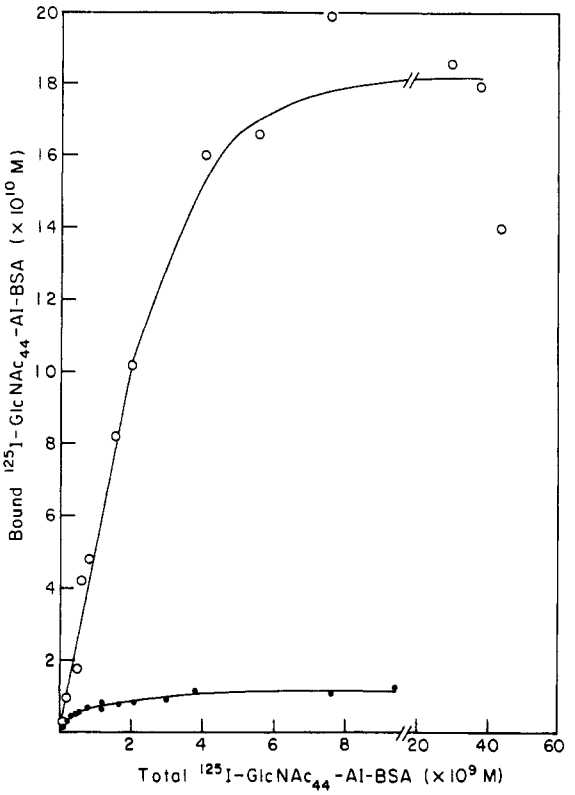


FIGURE 3: Effect of Triton X-100 on the binding of ^{125}I-GlcNAc$_{44}$-AI-BSA to chicken hepatocytes at 2 °C. Hepatocytes (2 × 10^6 cells/mL) were incubated at 2 °C with various concentrations of ^{125}I-GlcNAc$_{44}$-AI-BSA in the absence of detergent (●). After 2 h, the cells incubated without detergent were analyzed for specifically bound ^{125}I-GlcNAc$_{44}$-AI-BSA, as described under Experimental Procedures. For the detergent treatment experiment, the same stock of cells (1 × 10^7 cells/mL) was mixed with 0.1 volume of 10% Triton X-100, homogenized by using a few strokes in a Dounce homogenizer, and allowed to sit for 30 min on ice. Portions of the detergent-treated cells, a final concentration equivalent to 2 × 10^6 cells/mL, were then incubated with various levels of ^{125}I-GlcNAc$_{44}$-AI-BSA overnight in an ice bath (○). ^{125}I-GlcNAc$_{44}$-AI-BSA bound specifically to detergent-treated cells was analyzed as described under Experimental Procedures.

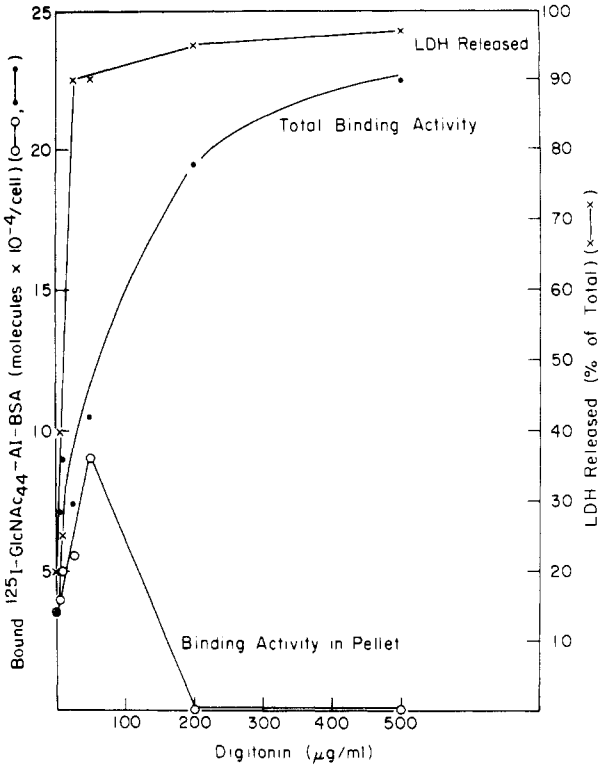


FIGURE 4: Effects of digitonin on the binding of ^{125}I-GlcNAc$_{44}$-AI-BSA to chicken hepatocytes. Hepatocytes (1 × 10^7 cells/mL) were incubated with ^{125}I-GlcNAc$_{44}$-AI-BSA (8.5 × 10^{-8} M) for 2 h at 2 °C in the presence or absence of digitonin. Because digitonin caused difficulties in sedimenting "cellular material", in addition to measuring the ^{125}I-GlcNAc$_{44}$-AI-BSA bound specifically to the cells by direct centrifugation through the oil layer, a portion (0.3 mL) of the supernatant was removed and made 50% saturated in ammonium sulfate to measure ligand specifically bound to the receptor in the supernatant. The data are expressed as the total ^{125}I-GlcNAc$_{44}$-AI-BSA molecules bound in the supernatant and the pellet.

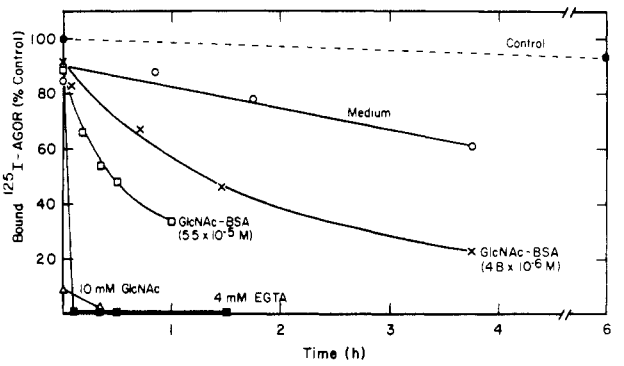


FIGURE 5: Dissociation of bound ^{125}I-AGOR from chicken hepatocytes. Hepatocytes (7.7 × 10^6 cells/mL) and ^{125}I-AGOR (5.3 × 10^{-8} M) were incubated for 2 h at 2 °C. Cells were centrifuged in the incubation tube at 100g in the cold for 2 min, and the medium was removed. The cell pellet was washed once in ice-cold medium to remove unbound ligand. Cells were then resuspended to the original volume with ice-cold medium (○), medium containing 4.8 × 10^{-6} (×) or 5 × 10^{-5} M (□) GlcNAc$_{44}$-AI-BSA, medium containing HEPES–EGTA (4 mM) (■), or medium containing 10 mM GlcNAc (△). One set of samples was not centrifuged but was allowed to continue incubation at 2 °C (●). After various times at 2 °C, samples were removed from the incubation mixtures, and specifically bound ^{125}I-AGOR was measured. All values are expressed as a percentage of the initial amount bound.

with 200 μg/mL digitonin, 2.24 × 10^5 molecules of ligands were associated with the cellular material.

Dissociation of the Surface-Bound Ligands. Figure 5 shows the time course of dissociation of surface-bound ^{125}I-AGOR upon treatment with GlcNAc$_{44}$-AI-BSA (unlabeled), GlcNAc, or EGTA, at 2 °C. The $t_{1/2}$ for displacement of bound AGOR with 5.5 × 10^{-5} and 4.8 × 10^{-6} M GlcNAc$_{44}$-AI-BSA was found to be 0.5 and 1.4 h, respectively. Some slow loss of bound ^{125}I-AGOR from the cell surface was seen in the absence of ligand or inhibitor ($t_{1/2}$ = ca. 5 h), while with the cells that were kept undisturbed in the presence of the same amount of ^{125}I-AGOR the initial amount of bound ^{125}I-AGOR was virtually unchanged (94% of the starting level) after 6 h at 2 °C.

By contrast, displacement of bound AGOR from chicken hepatocytes with 4 mM EGTA was extremely fast. This result was fully anticipated on the basis of the requirement of Ca^{2+} for activity by the isolated chicken hepatic lectin (Kawasaki & Ashwell, 1977). Greater than 98% of the bound ligand was released from the cell after treatment with 4 mM EGTA for 5 min. Likewise, dissociation with 10 mM GlcNAc was complete within 20 min.

Sugar Specificity for Binding at 2 °C. The isolated chicken liver binding protein was able to recognize derivatives of BSA to which GlcNAc, Man, Glc, or L-Fuc was attached, indicating a broad sugar specificity for binding (Kuhlenschmidt & Lee, 1984). Among the neoglycoproteins, however, GlcNAc derivatives were bound the tightest, 100–1000 times the affinity of Glc, Man, or L-Fuc.

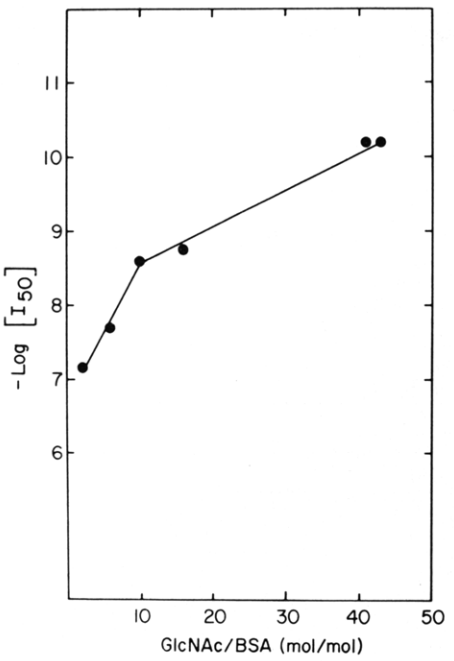


FIGURE 6: Dependence of the inhibitory potency of GlcNAc density on BSA for chicken hepatocytes. Hepatocytes (1.3×10^6 cells/mL) and ^{125}I-GlcNAc$_{44}$-AI-BSA (5×10^{-11} M) were incubated for 2 h at 2 °C in the presence of various concentrations of BSA to which different levels of GlcNAc were attached. Specifically cell-associated radioactivity was determined (●).

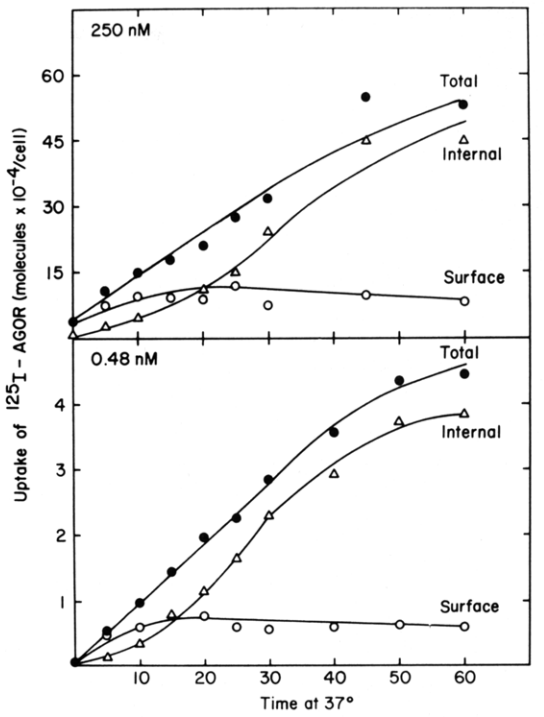


FIGURE 7: Uptake of ^{125}I-AGOR at 37 °C by chicken hepatocytes. Hepatocytes (3.5×10^6 cells/mL) and 250 nM ^{125}I-AGOR, or 0.48 nM ^{125}I-AGOR, were incubated at 37 °C. At the indicated times, samples were removed, and specifically bound cell-associated radioactivity (●) and internal radioactivity (△) were measured by the EGTA release technique as described under Experimental Procedures. Surface-bound material (○) was calculated as the difference between total cell-associated ligand and internal ligand.

The hepatocytes exhibited the same affinity hierarchy for sugars as the isolated binding protein (GlcNAc >> Glc, Man, L-Fuc >> Gal) (Kuhlenschmidt & Lee, 1984). The [I_{50}] values for GlcNAc$_{44}$-AI-BSA, Glc$_{37}$-AI-BSA, Man$_{43}$-AI-BSA, and L-Fuc$_{28}$-AI-BSA were 2.1×10^{-9}, 4.0×10^{-7}, 1.6×10^{-6}, and 2.0×10^{-6} M, respectively.

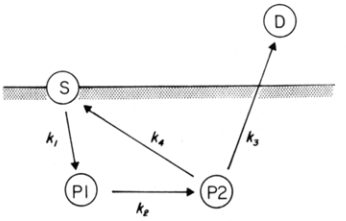
Although monosaccharides were drastically inferior to the macromolecular ligands containing clusters of sugars, the most potent inhibition of ^{125}I-GlcNAc-AI-BSA binding was by GlcNAc. This is also in agreement with the results of Sikder et al. (1983).

Effect of Sugar Density on the Binding of ^{125}I-GlcNAc-AI-BSA to Isolated Chicken Hepatocytes. Figure 6 shows the relationship of inhibitory potency vs. number of GlcNAc molecules per BSA derivative used as inhibitors of ^{125}I-Glc*NAc$_{44}$-AI-BSA* binding to hepatocytes. Generally speaking, the [I_{50}] value decreased exponentially while the GlcNAc density on BSA increased arithmetically.

Steady-State Analysis of Internalization and Hydrolysis of ^{125}I-AGOR by Chicken Hepatocytes. The amount of surface-bound ^{125}I-AGOR reached a steady state after 10–20 min at 37 °C for a dose of either 250 or 0.48 nM ^{125}I-AGOR (Figure 7). To estimate the internalization rate constant, hepatocytes and unlabeled AGOR (3×10^{-7} M) were incubated until the surface-bound ligand reached a steady level (30 min) as shown in Figure 7. A small amount of ^{125}I-AGOR was then added so that the internalization activity could be followed. At various times, samples were removed, and surface and internal radioactivities were measured. The rate constant of internalization was obtained from these data by the method of Wiley & Cunningham (1981, 1982).

Since more than 98% of the cell-surface-bound ^{125}I-AGOR at 2 °C could be dissociated with 4 mM EGTA (Figure 5), the amount of internal radioactivity after various periods of time at 37 °C could be determined by treatment of cells with 4 mM EGTA for 10 min. By subtraction of the value for the internal ligand from the total cell-associated radioactivity, the value for surface-bound ligand during endocytosis can be obtained. Thus, the rate constant for internalization can be determined (Kuhlenschmidt, 1983) from the slope of the plot of the internal radioactivity/surface radioactivity vs. time. An internalization rate constant, K_{int} = 0.10, 0.12, and 0.18 min^{-1}, from three separate experiments was obtained. A similar steady-state approach was used to determine the rate constant for hydrolysis (Kuhlenschmidt, 1983) to be 0.009 min^{-1} ($t_{1/2}$ = 75 min).

Scheme I



Synchronous Processing of Surface-Bound Ligand. In the cold, GlcNAc-containing ligands can be bound to the cell surface without becoming internalized. The hepatocytes were loaded with labeled ligand (AGOR or GlcNAc$_{44}$-AI-BSA) in the cold and washed to remove unbound ligand. Processing of the surface-bound ligand was initiated by raising the temperature to 37 °C (Figure 8). For the first 10–15 min, the ligand very rapidly disappeared from the surface, but the rate of disappearance was subsequently much reduced. The level of internal ligand reflected these changes. The degradation product (radioactive iodide) was not observed until 30 min after the temperature was raised.

These events could be simulated by a kinetic model (Scheme I) which features recycling of the internalized ligand to the cell surface. In this model, S, P1, P2, and D represents ligand

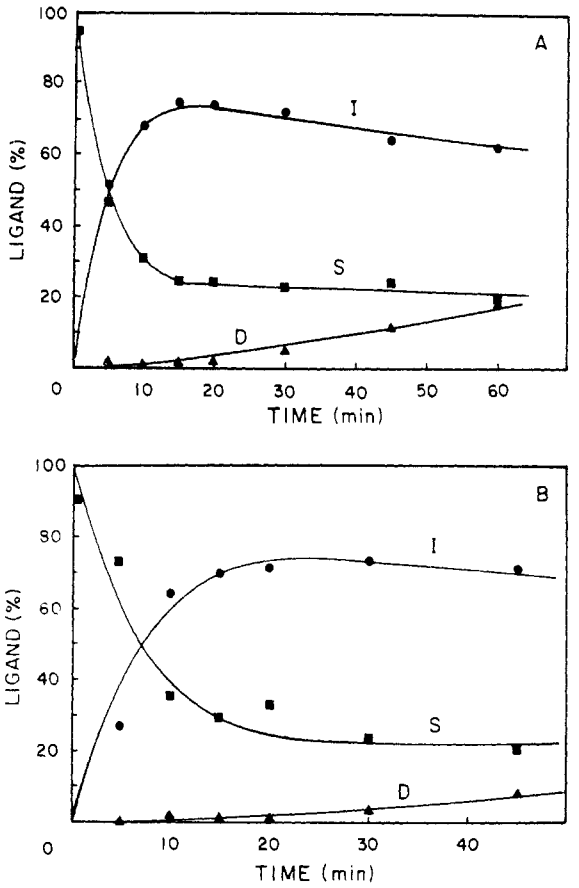


FIGURE 8: Synchronous processing of surface-bound ^{125}I-AGOR and ^{125}I-GlcNAc$_{44}$-AI-BSA by chicken hepatocytes. (A) Chicken hepatocytes (7.3×10^6 cells/mL) were incubated at 2 °C for 2 h with ^{125}I-AGOR (5.1×10^{-8} M). At this time, 158 fmol of ^{125}I-AGOR/10^6 cells was bound to the cell surface. The cells were washed with cold medium and then suspended to 7.3×10^6 cells/mL in warm (37 °C) medium. At various times, samples were taken to determine EGTA-resistant (●) and EGTA-releasable (■) ligand associated with the cells and degraded (▲) ligand to the medium. (B) Hepatocytes (4.5×10^6 cells/mL) were incubated at 2 °C for 2 h with ^{125}I-GlcNAc$_{44}$-AI-BSA (4.5×10^{-9} M), at which time 38 fmul of ^{125}I-GlcNAc$_{44}$-AI-BSA/10^6 cells was surface bound. The cells were washed in cold medium and suspended to 4.5×10^6 cells/mL in 37 °C medium. The remainder of the operation was the same as in (A). The lines are those obtained by simulation using the kinetic constants shown in Table I.

Table I: Kinetic Simulation Parameters

ligand	kinetic constants[a] k_1	k_2	k_3	k_4[b]	RMS[c]
AGOR	0.14	0.08	0.012	0.12	6.0
GlcNAc$_{44}$-AI-BSA	0.10	0.065	0.007	0.07	106.1

[a] All kinetic constants have units of reciprocal minutes. The kinetic constants that resulted in the best fit (smallest RMS) are listed. [b] Kinetic constant for the reaction leading to relocalization of ligand on the surface. [c] Residual mean square, defined as (sum of residual squares)/(number of data points − number of parameters − 1).

compartments corresponding to cell surface, two intracellular pools, and the extracellular medium. Experimentally, however, P1 and P2 could not be measured separately, and the sum of P1 and P2 was expressed as total intracellular ligands (I). The parameters used for the best simulation (Figure 8) are listed in Table I. The values of kinetic constants obtained by the steady-state approach (Wiley & Cunningham, 1981, 1982) are in agreement with these values.

Analysis of Degradation Products from ^{125}I-AGOR and ^{125}I-GlcNAc$_{44}$-AI-BSA Processing by Chicken Hepatocytes.

Table II: Comparison of Some Carbohydrate Binding Receptors on the Surfaces of Different Cells

cells	K_d (nM)	no. of receptors (1000 ligands/cell)
chicken hepatocytes		
GlcNAc$_{44}$-AI-BSA	0.55	31
AGOR	4.0	33
rabbit alveolar macrophages[a]		
Man$_{43}$-AI-BSA	2.2	43
rabbit hepatocytes[b]		
Gal$_{39}$-AI-BSA	1.1, 40	302
ASOR[c]	3.0, 67	410

[a] Hoppe & Lee (1983). [b] Connolly et al. (1983). [c] Asialoorosomucoid.

Table III: Comparison of Kinetic Parameters for Ligand Binding by Different Cells

parameters	rabbit hepatocytes[a] (ASOR)	rabbit macrophages[b] (Man$_{43}$-BSA)	chicken hepatocytes[c] (AGOR)
binding (2–4 °C)			
k_1 ($\times 10^6$ M^{-1} min^{-1})	1.1	1.2	0.64
k_{-1} ($\times 10^3$ min^{-1})	6.6	5.9	9.9
endocytosis (37 °C)			
k_{int} (min^{-1})	0.098	1.23	0.18
k_{hydr} (min^{-1})		0.074	0.009

[a] Connolly et al. (1983). [b] Hoppe & Lee (1983). [c] This study.

The ^{125}I-labeled degradation products externalized by the hepatocytes into the medium were analyzed by Sephadex G-10 chromatography (Hoppe & Lee, 1984). Only two radioactive peaks, one corresponding to undegraded material and another corresponding to KI, were seen. No radioactivity was eluted in the area corresponding to mono- or diiodotyrosines.

Discussion

Comparison of Physical Constants of Binding. The apparent affinity for binding comparable macromolecular ligands by rabbit hepatocytes, rabbit alveolar macrophages, and chicken hepatocytes falls in the same range having $K_D = \sim 1$ nM (Table II).

The affinity of AGOR binding by the isolated hepatocytes ($K_D = 4 \times 10^{/9}$ M) is comparable to the corresponding values for the isolated protein [$K_D = (1.4–1.8) \times 10^{-9}$ M] previously reported (Kawasaki & Ashwell, 1977; Kuhlenschmidt, 1983). In contrast to the mammalian hepatocytes, which consistently showed tighter binding of all classes of ligands than the isolated Gal/GalNAc binding protein (Lee et al., 1984), the chicken hepatocytes showed somewhat weaker binding than the isolated GlcNAc binding protein.

The number of receptors is usually measured by the number of ligand molecules bound. This is valid when there is a one to one correspondence between the receptor and the ligand. With multivalent ligands such as those used in our studies, the stoichiometry of the binding is more difficult to define and determine (Hoppe & Lee, 1983). Nevertheless, we assume a one to one correspondence when the term "binding sites" is referred to in this report.

At first sight, the values of 31 000 and 33 000 molecules of GlcNAc$_{44}$-AA-BSA or AGOR bound to each chicken hepatocyte at 2 °C (Table III) appears to be much lower than those observed with the binding of mammalian hepatocytes to Gal-terminated glycoconjugates (Weigel, 1980; Connolly et al., 1983). However, the average diameters of chicken and rat hepatocytes are ca. 13 and 23 μm (Obrink et al., 1977),

respectively, and if isolated hepatocytes are approximately spherical, then the chicken and rat hepatocytes would have 56–66 and 66–451 sites/μm^2, respectively. Thus, by this comparison, the number of receptors on the chicken hepatocytes is not especially low. The rabbit lung macrophages, being the same size as the chicken hepatocytes, possess about the same number of receptors on the cell surface (Table II).

It is known that rat hepatocytes previously exposed to a 37 °C environment show an increased capacity for binding ligands (Weigel & Oka, 1980; Fiete et al., 1983). The chicken hepatocytes incubated with 250 nM AGOR at 37 °C maintained a steady level of ca. 100000 molecules of surface-bound AGOR, which is nearly 3-fold the value determined in the cold. Preincubation of the chicken hepatocytes for 1 h at 37 °C also increased the rate of ligand uptake more than 2-fold (Kuhlenschmidt, 1983).

It is not clear whether the temperature-induced increase in the ligand bound to the surface is due to an increase in the number of receptor molecules on the surface, perhaps by recruiting the receptor molecules from the internal pool, or due to the changes in the distribution, orientation, or conformation of the receptors on the surface. Unfortunately, rapid internalization of the surface-bound ligands makes it difficult to obtain binding parameters at higher temperatures directly.

Chicken hepatocytes are different from mammalian hepatocytes and alveolar macrophages in that the bound macromolecular ligand can be displaced with a similar macromolecular ligand at concentrations equivalent to 100–1000-fold K_D with perceptible rates (Figure 5). It should be noted also that, in contrast to mammalian hepatocytes (Connolly et al., 1983), chicken hepatocytes bind the analogous ligands less tightly than isolated receptors (Kuhlenschmidt, 1983).

Just as Gal or GalNAc was effective in releasing macromolecular ligands bound to mammalian hepatocytes (Bridges et al., 1982), the monosaccharide GlcNAc was more effective in releasing bound macromolecular ligands from chicken hepatocytes. GlcNAc at 10 mM, ($[I_{50}]$ = 1 mM) (Kuhlenschmidt, 1983) completely dissociated bound AGOR in less than 20 min. However, GlcNAc did not show any stimulative effect on ligand binding by the chicken hepatocytes such as that seen with the monosaccharide mannose in rabbit alveolar macrophages (Hoppe & Lee, 1982).

Effect of Detergent on Receptor Activity. As in the case of mammalian hepatocytes (Tanabe et al., 1979; Weigel & Oka, 1983a), addition of detergent to isolated chicken hepatocytes had a large effect on the extent of ligand binding (Figures 3 and 4). Apparent 7-fold and 18-fold increases in the total activity were observed for digitonin and Triton treatment, respectively. Because there were no appreciable changes in the binding affinity before and after Triton treatment, it is most likely that the enhanced binding activity was due to exposure of new sites and not to a change in affinity. This is analogous to rat hepatocytes (Weigel & Oka, 1983b).

Interestingly, the maximal release of lactate dehydrogenase occurs at digitonin concentrations lower than the concentration required to expose the maximal number of sites, indicating that although enough plasma membrane disruption of the cell had occurred to release cytoplasmic enzyme, and presumably to allow ^{125}I-$GlcNAc_{44}$-AI-BSA to enter the cell, intracellular sites were not fully exposed.

Sugar Specificity of Binding and Uptake by the Isolated Hepatocytes. The chicken hepatocytes displayed the same sugar specificity hierarchy for binding as the isolated protein, that is, GlcNAc > Man, Glc, Fuc > Gal (Kuhlenschmidt, 1983). For the monosaccharides tested, only GlcNAc was potent enough to inhibit binding, having an $[I_{50}]$ value of 1 mM. Since the usual medium contained 5 mM Glc, the inhibition by monosaccharides was tested in medium without Glc (the $[I_{50}]$ value for Glc was later determined to be in excess of 20 mM under such conditions).

That the chicken hepatocyte surface possesses the same sugar binding specificity as the GlcNAc binding protein isolated from the whole liver strongly points to their identity. This is supported by the fact that antibody raised against the isolated GlcNAc binding protein from chicken liver (Kuhlenschmidt et al., 1982) was effective in abolishing binding of $GlcNAc_{44}$-AI-BSA and Man_{43}-AI-BSA by the chicken hepatocytes (Kuhlenschmidt, 1983).

There was a dramatic effect of sugar density on the neoglycoproteins on the relative affinity of the hepatocyte surface to bind ligand. The $[I_{50}]$ value for $GlcNAc_{44}$-AI-BSA was 10^6–10^7-fold lower than that of GlcNAc (monosaccharide), representing a far greater increase in affinity than can be attributed to the only 44-fold increase in sugar concentration [$[I_{50}](GlcNAc_{44}$-AI-BSA) = 10^{-10}–10^{-9} M vs. $[I_{50}]$(GlcNAc) = 10^{-3} M].

Kinetic Analysis. Kinetic parameters of binding at 2 °C are summarized for hepatocytes and lung macrophages from rabbit and hepatocytes from chicken (Table III). Analysis of the association kinetic data of binding AGOR at 2 °C yielded an "on rate", $k_1 = 6.38 \times 10^5$ M^{-1} min^{-1}, and a ligand–receptor dissociation constant, $K_D = 1.5 \times 10^{-8}$ M (Figure 3). The kinetically determined K_D was within 2–3-fold of that determined by equilibrium binding. From the k_1 (on rate) and K_D (dissociation constant), a k_{-1} (off rate) value of 0.99×10^{-2} min^{-1} can be estimated.

The values of kinetic constants obtained from the method of Wiley & Cunningham (1981, 1982) were useful guides for the simulation parameters. In simulation of the data shown in Figure 8, only the simplest models that can accommodate the experimental data were considered. Linear transport models such as proposed for rat hepatocytes (Bridges et al., 1982) could not accommodate the experimental data. The models which fit the experimental data (Scheme I) consider return of the internalized ligand to the cell surface. A model featuring direct return of the internalized ligand from the first compartment (P1 to S) was not satisfactory.

Justification for incorporating a step of ligand recycling is provided by the fact that, in mammalian hepatocytes, internalized ligands often (Connolly et al., 1982; Townsend et al., 1984; Weigel & Oka, 1984) exocytose with or without the presence of a dissociating agent (EDTA, GalNAc). The depicted model also predicts that in the presence of excess EDTA or GlcNAc, exocytosis of internalized GlcNAc-BSA can be demonstrated. This is indeed the case, and the details of data related to exocytosis will be published elsewhere. Whether the ligand recycling is accompanied by parallel receptor recycling has not been determined. It is interesting that a hypothesis of two intracellular compartments of unequal functions has been proposed for rat hepatocytes recently (Weigel & Oka, 1983c, 1984).

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