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Binding and endocytosis of glycoproteins and neoglycoproteins by isolated rabbit hepatocytes

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Rabbit hepatocytes were isolated by a collagenase perfusion technique, and used to study the binding and endocytosis of the glycoprotein, asialo-orosomucoid, and the neoglycoprotein, Gal_{39}-bovine serum albumin. Both of these proteins contain exposed galactosyl residues, and were avidly bound by the lectin on the hepatic parenchymal cell surface. Steady state and kinetic experiments performed at 2°C and at 37°C revealed the presence of two apparent classes of binding sites totalling 4.7×10^5 sites/cell at 2°C, and 6.3×10^5 sites/cell at 37°C. At 37°C, both classes of sites participated in internalization of bound ligand. The cells were capable of internalizing about 60000 molecules/min per cell. The process appeared to be first-order, with a rate constant $k = 0.098\,min^{-1}$ and $t_{\frac{1}{2}} = 7.1 \pm 0.6$ min. Binding could be inhibited by galactose-containing compounds, EGTA, and by anti-(hepatic lectin) immunoglobulin G. The inhibition by antibody appeared to be reversible upon removal of antibody-containing medium.

The hepatic lectin or asialoglycoprotein receptor has proved to be a useful model in studies of the morphological (Wall *et al.*, 1980) and biochemical (for review, see Harford & Ashwell, 1982) events of receptor-mediated endocytosis. These processes have been investigated both *in vivo* and *in vitro* using the perfused liver (Dunn *et al.*, 1979) or isolated rat hepatocytes (Weigel & Oka, 1982). This receptor binds and internalizes ligands with terminal galactosyl and *N*-acetylgalactosaminyl residues (Harford & Ashwell, 1982). This leads to lysosomal degradation of the ligand within hepatic parenchymal cells (LaBadie *et al.*, 1975). Receptor dynamics are not as well described; however, evidence suggests that the receptor is recycled (Tanabe *et al.*, 1979) and dissociates from the ligand soon after internalization (Bridges *et al.*, 1982). Recently, it has been demonstrated in this (Connolly *et al.*, 1982) and another (Regoeczi *et al.*, 1982) laboratory that certain ligands can bypass lysosomal degradation after internalization, with subsequent exit from hepatic parenchymal cells. To provide a basis for quantifying the initial cellular processes involved in endocytosis as well as exocytosis, we have now conducted studies on the binding and internalization by isolated rabbit hepatocytes of two ligands, asialo-orosomucoid and a neoglycoprotein, Gal_{39}-AI-BSA (Lee *et al.*, 1976).

The binding of both ^{125}I-ASOR and ^{125}I-Gal_{39}-AI-BSA at 2°C was analysed according to a reversible equilibrium model for binding by non-linear regression analysis and by Scatchard plot analysis. Binding analysis was also conducted at 37°C using methods of data analysis adapted from the rapid equilibrium approach to enzyme kinetics (Segal, 1975). The two ligands had similar binding affinities (~1 nM) and the analysis indicated the presence of two classes of binding sites on the

Abbreviations used: ASOR, asialo-orosomucoid; MDE medium, modified Dulbecco's Eagle medium; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Gal_{39}-AI-BSA, neoglycoprotein synthesized with 39 mol/mol of galactosyl residues attached to BSA via amidino linkages; IgG, immunoglobulin G; BSA, bovine serum albumin. Unless otherwise stated, all sugars are of D-pyranosyl structure.

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hepatocyte surface both at 2°C and at 37°C. The participation of both classes of binding sites in internalization was investigated by testing a variety of kinetic models, and it is proposed that both sites function in internalization at equal rates.

Experimental

Materials

Amino acids and vitamins used in the preparation of MDE medium were from Calbiochem. Hepes was from Research Organics (Cleveland, OH, U.S.A). All salts used were of reagent grade quality from J. T. Baker (Phillipsburg, NJ, U.S.A.). Crystal Violet, Trypan Blue and EGTA were from Sigma. The source and preparation of human ASOR (Connolly *et al.*, 1981) and Gal_{39}-AI-BSA (Lee *et al.*, 1976) have been described. The sheep anti-(rabbit hepatic lectin) antibody used, both as antiserum and as purified IgG, was that previously described (Connolly *et al.*, 1982). The hepatic lectin was purified from rabbit livers as described (Connolly *et al.*, 1981).

Preparation of rabbit hepatocytes

Rabbit hepatocytes were prepared and purified by the following modifications of the two-step collagenase technique as described by Seglen (1976). Male New Zealand White rabbits, 700–1200*g* (Bunnyville, Littlestown, PA, U.S.A.), were used in all procedures. The animals were anaesthetized with diethyl ether. After opening the abdominal cavity, 100 units of heparin (Eli Lilly) was given via the inferior vena cava. The perfusion buffers used were those previously described (Seglen, 1976), except that the first step perfusion buffer contained 0.1 mM-EGTA. A flow rate of 80 ml/min was required to produce morphologically intact hepatocytes. The collagenase perfusion time was 3 min using a collagenase (Sigma, type IV) concentration of 1 mg/ml. After collagenase perfusion, the cell suspension was cooled by the addition of MDE medium at 2°C. The whole liver cell suspension was passed sequentially through nylon mesh filters (TETKO Inc., Elmsford, NY, U.S.A.) of 100 μm and 35 μm pore size. Non-parenchymal cells and debris were removed by three centrifugations at 40*g* for 2 min. The pelleted cells were suspended again in fresh tissue culture medium after centrifugation. All operations after collagenase perfusion were performed at 2°C. Initial estimate of cell number, purity and morphological intactness was by a haemocytometer counting in the presence of 0.4% Trypan Blue. However, cell number could not be accurately determined by haemocytometer count because of the difficulty in discerning distinct hepatocytes occurring as clusters of 2–4 cells. Instead, nuclei counts (Patterson, 1979) were used to assess hepatocyte concentration. To determine that the average number of nuclei per hepatocyte is 1.27, cells were first plated on 35 mm tissue culture dishes (Flow Laboratories), coated with bovine collagen (Flow Laboratories) as previously described (Connolly *et al.*, 1982). After 2 h at 37°C, the cells had spread and hepatocytes with distinct borders could be visualized after staining with Wright's stain. A minimum of 2000 nuclei within hepatocytes were counted in each of five different preparations.

Assay of ligand binding to hepatocytes

The assay methods for binding of ^{125}I-labelled ligands to hepatocytes in suspension and as monolayers have been described (Connolly *et al.*, 1982). Non-specific binding was measured by subtracting the amount of cell-associated ^{125}I-labelled ligand under the above conditions but in the presence of 8 mM-EGTA. Identical results were obtained with a 100-fold molar excess of unlabelled ASOR was used instead of EGTA. Non-specific binding was less than 2% of the specific binding in both cases. When the cells were incubated under the usual assay conditions at 2°C, more than 98% of the bound ^{125}I-ASOR could be dissociated from the cells with 8 mM-EGTA, indicating that the ligand was bound to the cell surface and was not internalized under these conditions.

At 37°C, the amount of specific cell-associated ligand was defined as the sum of ligand that had been internalized and ligand that was bound to surface receptors. Surface ligand or internalized ligand could therefore not be determined directly. The amount of internalized ligand, however, could be obtained by first dissociating the surface-bound ligand by quickly diluting the 37°C cell suspension with an equal volume of 2°C medium containing 16 mM-EGTA, and incubating the suspension in an ice/water bath for 15 min. Since it took less than 15 s to reach a temperature of 10°C, further internalization probably did not occur (Weigel & Oka, 1982). Thus, internalized ligand, defined as that which could not be released by EGTA, could be determined. The amount of ligand bound to the surface was obtained from the difference between the total amount of cell-associated ligand, and that which was EGTA resistant.

Analysis of binding to hepatocytes

The process of binding and internalization of ligand by cells at 37°C was modelled after an enzyme–substrate reaction (Segal, 1975):

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \xrightarrow{k_3} L^* \qquad (1)$$

where R is the receptor, L is the free ligand, LR is the surface complex and L* is internalized ligand. L*

may also be L*R*, in which the receptor is still associated with the ligand.

This model assumes only that the amount of surface receptor is constant, regardless of the mechanism of regeneration of functional surface receptor. At 37°C, a steady state is quickly reached and the amount of ligand bound to surface receptors appeared to be reasonably constant (see Fig. 4) and thus these assumptions are justified. At 2°C, internalization does not occur; thus k_3 can be ignored and the binding parameters for the surface receptor can be obtained.

Assuming that equilibrium can be attained between free ligand receptor outside the cell, the relationship between dissociation constant (K_d), the ligand–receptor complex (RL), unoccupied receptor (R), and free ligand (L) can be expressed as follows:

$$[RL] = \frac{[R]_{tot.}[L]}{K_d + [L]} \quad (2)$$

The ligand–receptor complex, [RL], was measured as a function of free ligand concentration, [L], and the values for $[R]_{tot.}$ and K_d were obtained directly from numerical analysis by non-linear least squares regression analysis using a program in Fortran (Johnson *et al.*, 1976) implemented on a Hewlett–Packard System 1000 computer, as previously described (Connolly *et al.*, 1981). Models involving two classes of sites were represented as the sum of two equations having the same form as eqn. (2). It should be noted that both the lectin and ligand are polyvalent, and thus the equilibrium state is probably more complicated than that described by eqn. (1). The value obtained for K_d in eqn. 2, therefore, represent an apparent binding constant. In order to avoid confusion regarding the mechanism of binding, the equilibrium constant obtained will therefore be referred to as $K_{app.}$.

Analysis of uptake

Since surface-binding data revealed the presence of two apparent classes of binding sites, it became important to determine if both of these classes of sites were involved in endocytosis of ligand. Three simple models were used to analyse the steady-state rate of internalization by rabbit hepatocytes.

Model 1. If only one class of binding sites is involved in endocytosis, the rate of endocytosis (v) can be expressed in the form of the Michaelis–Menten equation:

$$v = \frac{[L]\,V_{max.}}{K_{uptake} + [L]} \quad (3)$$

$V_{max.}$ is the maximum rate of endocytosis, and K_{uptake} is the constant analogous to K_m in enzyme kinetics.

Model 2. If both classes of binding sites (R and R′) are involved in endocytosis, and both classes internalize ligand at different rates, then in addition to eqn. (1), the process shown in eqn. (4) would occur:

$$L + R' \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} LR \xrightarrow{k'_3} L^* \quad (4)$$

The expression describing the rate of endocytosis for this process would therefore be:

$$v = \frac{[L]\,V_{max.,1}}{K_{uptake,1} + [L]} + \frac{[L]\,V_{max.,2}}{K_{uptake,2} + [L]} \quad (5)$$

Model 3. If two classes of binding sites are involved in binding, but the rate of internalization is the same for both classes such that:

$$L + R' \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR' \xrightarrow{k_3} L^* \quad (6a)$$

$$L + R \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} LR \xrightarrow{k_3} L^* \quad (6b)$$

and therefore, the rate of internalization would be described by the following expression:

$$v = \frac{[L]\,V_{max.,1}}{K_{uptake,1} + [L]} + \frac{[L]\,V_{max.,1}}{K_{uptake,2} + [L]} \quad (7)$$

All three models can be evaluated by analysis of the initial rate of uptake of ligand by hepatocytes using non-linear least squares regression (Johnson *et al.*, 1976).

Results

Binding at 2°C

When suspensions of freshly isolated rabbit hepatocytes were incubated for different periods of time at 2°C with ^{125}I-ASOR, binding of the ligand reached a maximum value after about 60 min (Fig. 1). If a 100-fold excess of unlabelled ASOR or Gal_{39}-AI-BSA was included in the incubation medium, binding was inhibited by greater than 98%. Man- or GlcNAc-neoglycoproteins were at least 1000-fold less effective as inhibitors (Connolly *et al.*, 1982). The binding specificity of the hepatocytes, therefore, appeared to be similar to that of the isolated lectin. Binding of ^{125}I-ASOR could also be inhibited by greater than 98% when 8 mM-EGTA was included in the incubation medium, indicating that binding to the hepatocytes was calcium-dependent, as is binding to the isolated lectin (Connolly *et al.*, 1981). When the cells were incubated with ^{125}I-ASOR for 100 min at 2°C, and then treated with 8 mM-EGTA, greater than 98% of the bound ligand was released from the cells within 10 min, indicating

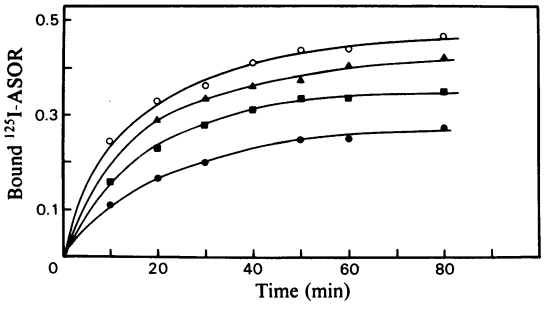

Fig. 1. *Time course of ^{125}I-ASOR binding to freshly isolated rabbit hepatocytes at 2°C*

Freshly isolated rabbit hepatocytes (2.5×10^6 cell/ml) were incubated in MDE medium at 2°C with ^{125}I-ASOR. Duplicate 100 μl samples were removed periodically, and the amount of bound ^{125}I-ASOR was determined by pelleting the cells through a layer of silicone/light mineral oil, as described in the Experimental section, and counting the radioactivity in the cell pellet. The initial concentrations of ^{125}I-ASOR in the 4 ml incubation mixture were: ○, 100 nM; ▲, 25 nM; ■, 12.5 nM; ●, 6.25 nM.

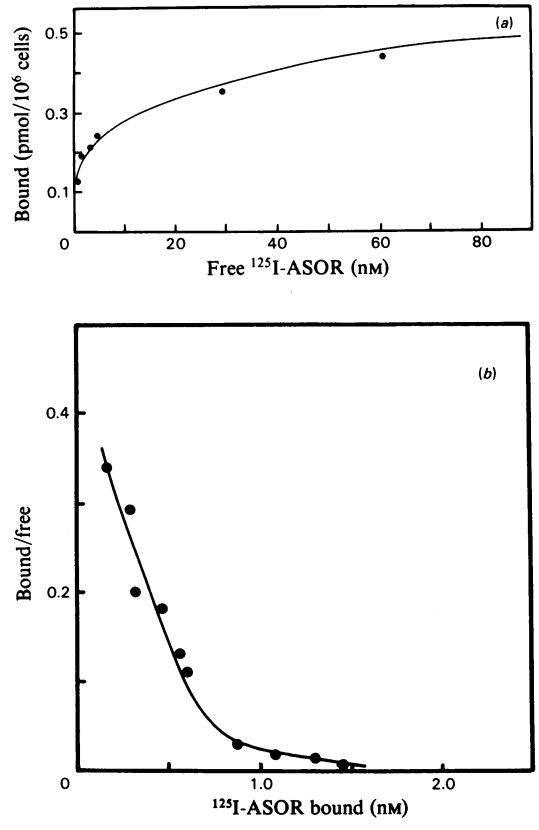

Fig. 2. *Binding of ^{125}I-ASOR to freshly isolated rabbit hepatocytes at 2°C*

Freshly isolated rabbit hepatocytes were incubated for 100 min at 2°C with different amounts of ^{125}I-ASOR, and assayed for specifically bound ligand. (*a*) The data were fitted directly to a two-site model of binding by non-linear least squares regression analysis. The solid line was the best fit curve for which the parameters are: $K_{app.} = 1.3 \times 10^{-9}$ M, 140000 sites/cell; $K_{app.} = 6.7 \times 10^{-8}$ M, 280000 sites/cell. (*b*) Scatchard plot. After grouping the data in (*a*) by inspection into high-affinity sites (bound ligand < 0.6 nM) and low-affinity sites (bound ligand > 0.6 nM), linear regression analysis yielded the following parameters: $K_{app.} = 2.0 \times 10^{-9}$ M, 190000 sites/cell; $K_{app.} = 3 \times 10^{-8}$ M, 220000 sites/cell. The correlation coefficients (linear regression) for the two lines were −0.955 and −0.979 for the high- and low-affinity sites, respectively.

that binding was to the cell surface, and that internalization could not occur to a measurable extent at this temperature.

In order to estimate the binding affinity of ligands to the hepatocyte receptor and the number of ligand binding sites present on the cell surface, freshly isolated rabbit hepatocytes were incubated for 100 min at 2°C with different amounts of ^{125}I-ASOR. The binding data were then analysed either by non-linear regression analysis (Connolly *et al.*, 1981; Johnson *et al.*, 1976) (Fig. 2*a*), or, as is shown in Fig. 2(*b*), by the graphic method of Scatchard (1949). Both methods of data analysis clearly indicated the presence of two apparent classes of binding sites with different affinities. The direct-fit method yielded apparent binding constants $K_{app.} = 3.0 \times 10^{-9}$ M and $K_{app.} = 6.7 \times 10^{-8}$ M, with 140000 and 280000 sites per cell for the high- and low-affinity classes of sites, respectively. Scatchard analysis yielded similar results, with apparent binding constants of $K_{app.} = 2.0 \times 10^{-9}$ M and $K_{app.} = 3 \times 10^{-8}$ M, with 190000 and 220000 high- and low-affinity sites per cell, respectively. Although the relative proportions of the two classes of sites differed according to the method of data analysis, the total number of sites estimated by the two methods were similar. The direct-fit method produced a total of 420000 sites/hepatocyte, and the Scatchard plot method produced 410000 sites/hepatocyte.

Binding data were also analysed for the neoglycoprotein, Gal_{39}-AI-BSA (Lee *et al.*, 1976). Fig. 3(*a*) shows that the binding isotherm for this ligand appears very similar to that obtained for ^{125}I-ASOR. The Scatchard plot shown in Fig. 3(*b*) indicates the presence of two apparent classes of sites, as was the case with ^{125}I-ASOR. The best parameters obtained by the direct-fit method for binding of ^{125}I-Gal_{39}-AI-BSA to the hepatocytes were $K_{app.} = 1.1 \times 10^{-9}$ M and $K_{app.} = 4.0 \times 10^{-8}$ M for 140000 and

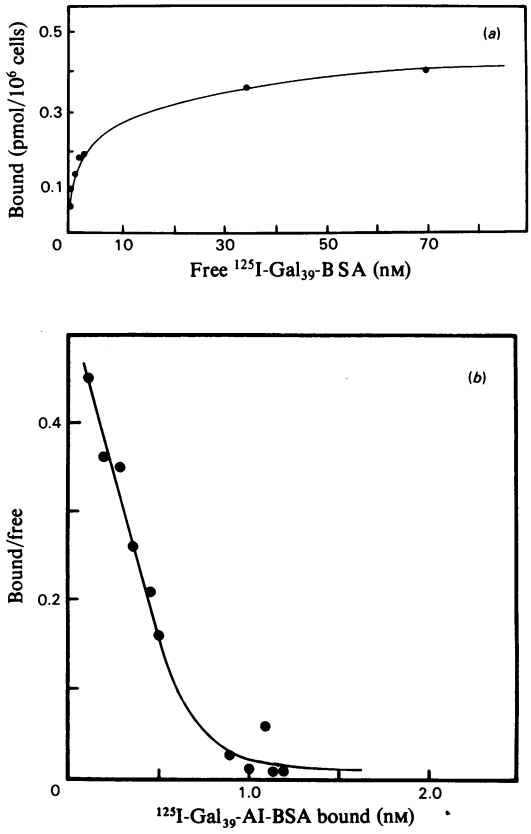

Fig. 3. *Binding of ^{125}I-Gal$_{39}$-AI-BSA to freshly isolated rabbit hepatocytes at 2°C*

The conditions for incubation and methods of data analysis were exactly as described in Fig. 2, except that the ligand used was ^{125}I-Gal$_{39}$-AI-BSA. (*a*) The parameters obtained from non-linear least squares regression analysis were $K_{app.} = 1.1 \times 10^{-9}$M, 140000 sites/cell; $K_{app.} = 4.0 \times 10^{-8}$M, 162000 sites/cell. (*b*) The parameters obtained from Scatchard plot analysis were $K_{app.} = 1.4 \times 10^{-9}$M, 180000 sites/cell for the high-affinity binding (bound ligand < 0.6 nM); $K_{app.} = 2.8 \times 10^{-8}$M, 220000 sites/cell for the low-affinity binding data (bound ligand > 0.6 nM).

162000 sites/hepatocyte, respectively. By Scatchard plot analysis, the binding constants were $K_{app.} = 1.4 \times 10^{-9}$M and 2.8×10^{-8}M, with a stoichiometry of 180000 and 220000 sites/hepatocyte, respectively. The total number of sites was 302000 by the direct-fit method, and 400000 by Scatchard plot analysis.

Table 1 summarizes the binding data for both ^{125}I-ASOR (three separate experiments) and ^{125}I-Gal$_{39}$-AI-BSA (two separate experiments). The number of high affinity sites estimated for both ligands was not significantly different. Approx.

Table 1. *Binding of ^{125}I-ASOR and ^{125}I-Gal$_{39}$-AI-BSA to rabbit hepatocytes at 2°C*

Binding of ^{125}I-ASOR and ^{125}I-Gal$_{39}$-AI-BSA to rabbit hepatocytes was measured as a function of ligand concentration as shown in Figs. 1 and 2. The data were analysed by non-linear least squares regression analysis using a two-site model for binding. The apparent dissociation constants, $K_{app.}$, and the number of ligand molecules bound per hepatocyte were determined for the two ligands. The data are expressed as the mean ± S.D. of three experiments for ^{125}I-ASOR or two experiments for ^{125}I-Gal$_{39}$-AI-BSA.

	ASOR	Gal$_{39}$-AI-BSA
K_1 (nM)	1.7 ± 0.58	0.8 ± 0.4
n_1 (sites/cell)	122000 ± 20000	136000 ± 12000
K_2 (nM)	190 ± 130	25 ± 22
n_2 (sites/cell)	344000 ± 81000	204000 ± 62000
n_{total} (sites/cell)	466000 ± 70000	341000 ± 49000

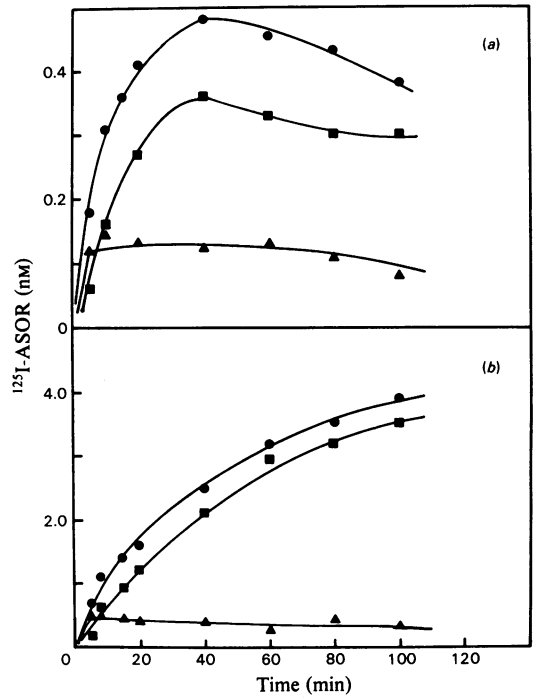

Fig. 4. *Time course of cell surface binding and internalization of ^{125}I-ASOR to rabbit hepatocytes at 37°C*

Freshly isolated rabbit hepatocytes (1×10^7 cells/ml) were warmed to 37°C for 5 min, and then added to 4.5 ml of medium (37°C) containing, in final concentration, either 0.625 nM-^{125}I-ASOR (*a*) or 6.25 nM-^{125}I-ASOR (*b*). Samples were removed at various times and assayed for total cell associated ligand (●), internalized ligand (■), and surface ligand (▲) as described in the Experimental section.

120000–140000 molecules could be bound to the high-affinity class of sites in both instances. There appeared to be about 70% more low-affinity ^{125}I-ASOR binding sites than the corresponding ^{125}I-Gal$_{39}$-AI-BSA binding sites. Because of the degree of precision in the estimation of the number of low-affinity sites, this difference is of questionable significance. It is possible, however, that steric factors could reduce the accessibility of some of the low affinity sites to the BSA derivative.

Binding at 37°C

When hepatocytes were incubated with ^{125}I-ASOR for different periods of time at 37°C (Fig. 4), the ligand was bound much more rapidly than at 2°C (Fig. 1). In contrast with the results obtained at 2°C, a large portion of ligand could not be released by treating the cells with EGTA. The resistance to EGTA release was probably due to the internalization of ligand. Within 5 min or less, the amount of cell-surface-associated ligand appeared to reach a maximum value that gradually decreased with time. The amount of internalized ligand as defined by EGTA resistance increased linearly for about 20 min at both of the initial ^{125}I-ASOR concentrations tested. At 0.625 nM-^{125}I-ASOR (Fig. 4*a*), the amount of bound and internalized ligand reached a maximum value by 40 min and then began to decline. In an experiment with a higher initial concentration, 6.25 nM-^{125}I-ASOR (Fig. 4*b*), the ligand continued to be internalized by the cells for at least 100 min, and a decrease in the amount of cell-associated ligand was not observed during the course of the experiment. The rate of internalization was linear for at least 10 min in both cases.

The process of binding and internalization was studied as a function of ligand concentration, and was analysed according to a model analogous to an enzyme–substrate reaction. Also, because the amount of surface-bound ligand, as determined by the EGTA release technique, had appeared to reach a steady state by 10 min, the number of surface receptors and their apparent binding affinities could be estimated at 37°C. The equations and methods of data analysis were adapted from the rapid equilibrium approach to enzyme kinetics (Segal, 1975), as described in the Experimental section.

The amount of ^{125}I-ASOR bound to the hepatocyte cell surface after 10 min at 37°C, as a function of free ^{125}I-ASOR, is shown in Fig. 5(*a*). These data revealed the presence of two classes of binding sites with apparent binding constants $K_{app.} = 1.9 \times 10^{-9}$ M (210000 sites/cell) and $K_{app.} = 1.3 \times 10^{-7}$ M (420000 sites/cell). These apparent affinity constants are very close to the values obtained at 2°C for both intact hepatocytes (Table 1) and the isolated lectin (Connolly *et al.*, 1981). The total of 630000 ^{125}I-ASOR sites/cell is about 35% higher than the estimate obtained at 2°C, indicating that more receptors are present on the surface at 37°C than at 2°C.

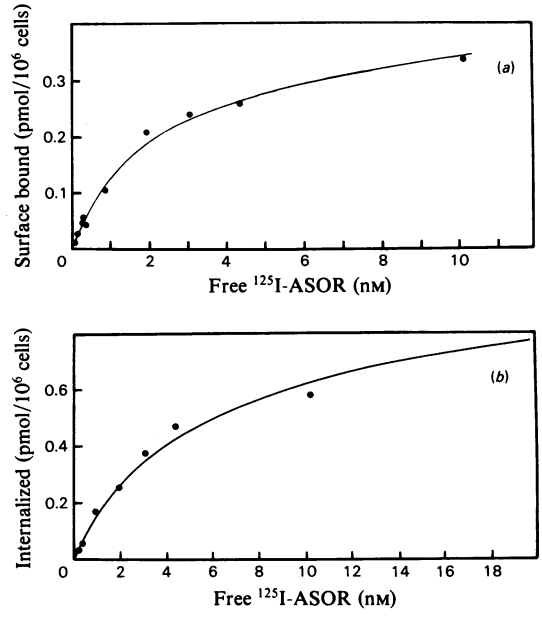

Fig. 5. *Steady-state binding and internalization of ^{125}I-ASOR to rabbit hepatocytes at 37°C*

Freshly isolated hepatocytes in 0.25 ml of medium (1×10^7 cells/ml) were warmed to 37°C for 5 min, and then added to 0.75 ml of medium (37°C) containing various amounts of ^{125}I-ASOR. After 10 min, the amount of ligand specifically bound to the cell surface and internalized ligand were determined by the EGTA release technique. (*a*) Cell surface binding: the data were fitted by non-linear least squares regression analysis to a two-site binding model. The line was generated by a computer program using the following parameters: $K_{app.} = 1.9 \times 10^{-9}$ M, 210000 sites/cell; $K_{app.} = 1.3 \times 10^{-7}$ M, 420000 sites/cell. (*b*) Internalization: the same data were then fitted to model 2 (see the text) which assumes two kinetic classes of binding sites having different values of $V_{max.}$.

Rate of internalization

The steady-state rate of internalization at 37°C was measured as a function of free ^{125}I-ASOR concentration (Fig. 5*b*). Because the binding data (Figs. 2, 3 and 5) indicated that there were two classes of binding sites, it was possible that: (1) only one class of sites was involved in the process, in which case only one kinetic constant and one $V_{max.}$ would be revealed by kinetic analysis; (2) that both classes of sites were involved in the process, in which case two sets of values for K_{uptake} and $V_{max.}$ would be obtained; or (3) both classes of sites were involved in binding, but the rates of internalization

were equal for both classes of sites once ligand was bound. In this case, two values for K_{uptake}, but only one value for $V_{max.}$, would be obtained.

The binding data were evaluated by all three models. Unfortunately, it was not possible to distinguish between the three models on the basis of this analysis alone. All three models produced good fits to the data based on visual examination of the computer-generated curves. An example is shown in Fig. 5(*b*), in which the uptake data were fitted according to model 2. The fits for models 1 and 3 were similar. The statistical analysis of the data also were similar for each of the models. A summary of this analysis is presented in Table 2. Although it would seem that model 2 is slightly better than the other two models based on the values of the square root of variance, the difference was not sufficient to choose this model over the other two, especially when experimental error in the data and the flexibility inherent in the curve-fitting procedure were taken into account.

Therefore, an independent method of measuring the rate of internalization by rabbit hepatocytes was used to determine the internalization rate constant, k_3, and the half-life for surface residency of ligand. In the experiment shown in Fig. 6, the amount of internalized ligand was measured as a function of time using the EGTA release technique. It is seen that initially an exponential decrease in the amount of surface-bound ligand (i.e., internalization of ligand) occurred as a function of time. This apparently first-order process could therefore be described by the following expression:

$$[RL]_t = [RL]_0 e^{-k_3 t} \quad (8)$$

where $[RL]_0$ is the amount of ligand bound to the cell surface at the beginning of the experiment, and k_3 is the rate constant for internalization. From the data shown in Fig. 6, a $t_{\frac{1}{2}} = 7.1 \pm 0.6$ min (six determinations) was obtained for ^{125}I-ASOR. The rate constant for internalization was found to be $k_3 = 0.098\,min^{-1}$. The rate and extent of internalization of ^{125}I-Gal$_{39}$-AI-BSA was indistinguishable from the rate of internalization of ^{125}I-ASOR (Fig. 6).

Only 80–82% of the ligand that was bound initially to the cell surface (Fig. 6) was internalized. Since only 80% of the cells were judged morphologically intact by Trypan Blue dye exclusion at the end of the experiment, the most likely explanation for this result was that 20% of the ligand was bound by the dead cells, in which no internalization took place. In three experiments, the extent of internalization varied from 65% to 85%, and correlated

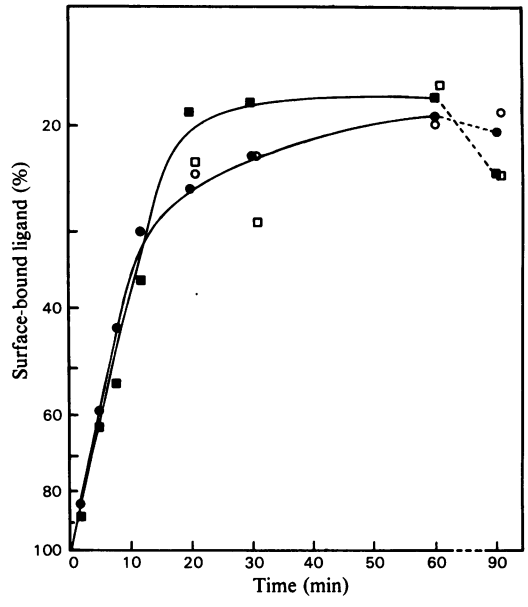

Fig. 6. *Rate of internalizaton at 37°C of ^{125}I-ASOR and ^{125}I-Gal$_{39}$-AI-BSA bound to the surface of hepatocytes*
Freshly isolated hepatocytes were incubated with 50nM-^{125}I-ASOR (●) or 25nM-Gal$_{39}$-AI-BSA (■) for 30min at 2°C. The free ligand was removed by centrifuging the cells and aspirating off the medium. After an additional wash with cold medium, 8ml of medium at 37°C was added. Samples were removed at various times after warming and assayed for total cell-associated and internalized ligand. The data were corrected for the amount of ligand that was internalized, degraded, and released (see Fig. 7). Cell viability, as determined by Trypan Blue dye exclusion, was 94% at the beginning of the experiment, and 80% after completion of the 90min incubation at 37°C. After 15min incubation at 37°C with labelled ligands, a 2ml sample of the incubation mixture was removed, and unlabelled ASOR (○) or Gal$_{39}$-AI-BSA (□) were added to final concentrations of 5μg/ml. These samples were then further incubated and treated as described above.

Table 2. *Kinetic parameters obtained using different models of endocytosis of ^{125}I-ASOR*
The steady-state rate of internalization of ^{125}I-ASOR was determined as described in the legend to Fig. 3, and the data were analysed by non-linear least squares regression analysis according to the models described in the Experimental section. The values for $K_{uptake,1}$, $K_{uptake,2}$, $V_{max.,1}$ and $V_{max.,2}$ were obtained for each model, as well as the square root of the variance.

Model	$K_{uptake,1}$ (nM)	$K_{uptake,2}$ (nM)	$V_{max.,1}$ (molecules/min per cell)	$V_{max.,2}$ (molecules/min per cell)	Square root of variance
1	7.7	—	70000	—	0.098
2	2.6	23	30000	43000	0.068
3	5.5	660	59000	—	0.085

Table 3. *Effect of receptor site occupancy on the rate of internalization by rabbit hepatocytes*

Freshly isolated rabbit hepatocytes (1×10^7 cells per ml, 95% intact by Trypan Blue dye exclusion) were incubated for 60 min in an ice/water bath with ^{125}I-ASOR at initial concentrations of either 0.2 nM, 20 nM, or 400 nM to attain low, medium, and high receptor site occupancies, respectively. After removing the free ligand by washing the cells, warm (37°C) medium was added. The total cell-associated ligand and internalized ligand were measured at different times using the EGTA release technique, as described in the legends of Figs. 6 and 7.

Sites occupied (%)	$t_{\frac{1}{2}}$ (min)	Extent of internalization (%)	Ligand released after 60 min (%)
2	7.5	78	27
26	7.5	82	30
89	7.5	80	29

well with the percent of Trypan Blue-negative cells at the end of the experiment. In all cases, however, the rate could be described by a single exponential with $t_{\frac{1}{2}}$ values ranging from 6 to 8 min.

In the experiment shown in Fig. 6, whe total receptor site occupancy was about 70%, and thus it can be estimated that approximately equal numbers of high- and low-affinity sites were occupied. Because model 2 would require two different rates of endocytosis, depending on whether ligand was bound to the high- or low-affinity class of sites, it can be concluded that these data do not support this model.

A single rate constant for the internalization of surface-bound ligand would be expected if binding to the low-affinity class of sites did not lead to internalization, as would be the case if internalization occurred according to model 1, or if ligand that was bound to either classes of sites was internalized at the same rate, as in model 3. To distinguish between these possibilities, the rates of internalization were compared at different receptor site occupancies (Table 3). At 2% occupancy, ligand should be bound primarily to high-affinity sites. At 26% occupancy, most of the high affinity and some of the low affinity sites should be filled, and at 89% occupancy, all of high and most of low affinity sites should be saturated. The apparent rate constants and extents of internalization were identical in all three cases. Furthermore, the percentage of radioactivity released by the cells after 60 min was the same in all three cases. The released material was degraded rather than dissociated ligand (see the next section). Models 1 and 2 must therefore be rejected on the basis of these results. It appears that both classes of binding sites on the hepatocyte cell surface can function in internalization, and can do so with equal efficiency.

At $V_{max.}$ of internalization, the surface receptor sites must be fully occupied, and therefore, according to model 3:

$$V_{max.} = k_3[LR] \quad (9)$$

where k_3 is the rate constant for internalization shown in eqn. (1), and [LR] is the concentration of ligand bound to the receptor on the hepatocyte cell surface. Using $[LR] = 6.3 \times 10^5$ sites/cell (Fig. 5*a*) and $V_{max.} = 59000$ molecules $\cdot$ min^{-1} $\cdot$ cell^{-1} (model 3, Table 2), $k_3 = 0.094$ min^{-1} was obtained. The residency time for each ligand on the cell surface, 7.4 min, was calculated from the half-life for internalization ($t_{\frac{1}{2}} = 0.693/k_2$). This calculated value agrees with the values obtained by the EGTA release technique (Table 3).

Fate of the ligand after binding to rabbit hepatocytes at 37°C

The incubation medium used in the experiment shown in Fig. 6 was analysed for the presence of degraded ligand using trichloroacetic acid precipitation (Figs. 7*a* and 7*b*). Degraded ligand did not appear in the medium for 25 min, but accumulated linearly thereafter. The extents of degradation of ^{125}I-ASOR (Fig. 7*a*) and ^{125}I-Gal$_{39}$-AI-BSA (Fig. 7*b*) were the same, with 22–26% of the original bound ligand appearing in the medium as trichloroacetic acid-soluble material by 90 min. The addition of excess unlabelled ASOR or Gal$_{39}$-AI-BSA, after the labelled ligand was internalized (arrows in Figs. 7*a* and 7*b*), did not change the rate of ligand degradation. However, a 10% decrease in the total cell-associated ligand, and a co-ordinate increase in the trichloroacetic acid-precipitable ligand in the medium, did occur upon addition of unlabelled ligand (open symbols, Figs. 7*a* and 7*b*).

Effect of anti-(hepatic lectin) antibody on ^{125}I-ASOR binding to hepatocyte monolayers

Antibody was prepared against the isolated lectin. The IgG fraction caused 50% inhibition of ASOR binding by the soluble binding assay (Connolly *et al.*, 1981) at a concentration of about 20 μg/ml. The anti-(hepatic lectin) IgG completely inhibited uptake of ^{125}I-ASOR by cultured rabbit hepatocytes at a concentration of 90 μg/ml (Table 4). The inhibitory effect of the anti-(hepatic lectin) IgG was quickly reversible (Table 5). If cells that had been preincubated at 37°C for 30 min with 90 μg of anti-(hepatic lectin) IgG/ml were then incubated for an additional 10 min in the absence of antibody, binding activity was restored to 82% of its original value. When the antibody was not removed, however, binding activity was completely inhibited.

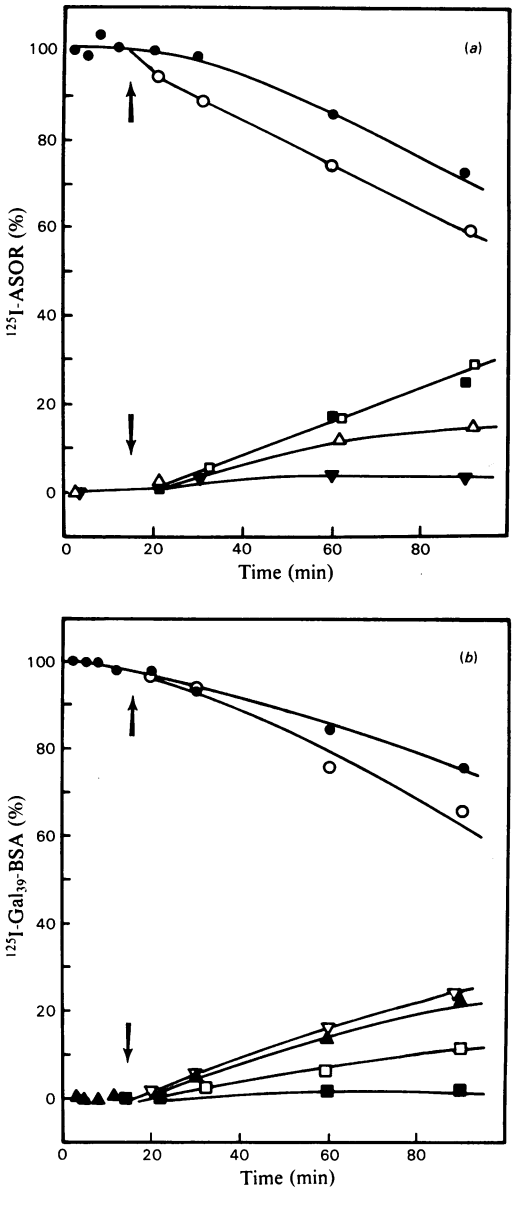

Fig. 7. *Time course of degradation of ^{125}I-ASOR and ^{125}I-Gal$_{39}$-AI-BSA by rabbit hepatocytes at 37°C*
The supernatant solutions from the ^{125}I-ASOR and ^{125}I-Gal$_{39}$-AI-BSA incubations with hepatocytes described in Fig. 6 were analysed by using trichloroacetic acid precipitation. Samples (100 μl) of the supernatant solutions in 500 μl Microfuge tubes were diluted with 100 μl of 30% (w/v) trichloroacetic acid, vortexed, and allowed to incubate for 30 min at room temperature. The samples were then centrifuged for 1 min in an Eppendorf centrifuge, the supernatants were removed, and 100 μl of the supernatants was counted for radioactivity. The pellets were washed once, and counted for radioactivity. (*a*) ●, total cell-associated ^{125}I-ASOR; ■, acid-soluble radioactivity in the supernatant; ▼, acid-precipitable radioactivity in the supernatant. The corresponding open symbols represent samples to which unlabelled ASOR was added to a final concentration of 5 μg/ml after 15 min incubation, as indicated by arrows. (*b*) ●, total cell-associated ^{125}I-Gal$_{39}$-AI-BSA; ▲, acid-soluble radioactivity in the supernatant; ■, acid-precipitable radioactivity in the supernatant. The corresponding open symbols represent samples to which unlabelled Gal$_{39}$-AI-BSA was added to a final concentration of 5 μg/ml after 15 min incubation, as indicated by the arrows.

Table 4. *Effect of anti-(hepatic lectin) IgG on binding of ^{125}I-ASOR to rabbit hepatocyte monolayers*
Rabbit hepatocytes (10^6 cells/culture well) were incubated for 24 h in six-well, 35 mm Linbro dishes, the medium was removed, and replaced with 2 ml of medium containing either anti-(hepatic lectin) IgG or pre-immune IgG obtained from the same animal. After 10 min, 100 μl of medium containing 0.5 μg of ^{125}I-ASOR was added to each well, and the incubation was continued for an additional 10 min at 37°C. After removing the medium and washing the monolayer with phosphate-buffered saline, the cells were solubilized in 2% Triton X-100 and assayed for radioactivity.

IgG added (μg/ml)	^{125}I-ASOR bound (% of control) with: Immune IgG	Pre-immune IgG
9	80	n.d.
18	60	n.d.
90	0	100

Table 5. *Reversibility of anti-(hepatic lectin) inhibition of ^{125}I-ASOR binding to rabbit hepatocyte monolayers*
Rabbit hepatocytes were cultured for 24 h (10^6 cells/well), the medium was removed, and the cells were incubated for 10 min with 2 ml of fresh medium containing 90 μg/ml either of anti-(hepatic lectin) IgG or of pre-immune IgG from the same animal as control. The medium was removed, and 2 ml of medium containing 0.5 μg of ^{125}I-ASOR/ml with or without antibody was added. After an additional 10 min incubation at 37°C, the amount of cell-associated radioactivity was determined by solubilizing the washed monolayers in 2% Triton X-100.

Pretreatment	IgG in incubation with ^{125}I-ASOR	^{125}I-ASOR bound (%)
Immune IgG	+	0
Immune IgG	–	82
Pre-immune IgG	+	136
Pre-immune IgG	–	124

Discussion

It is now recognized that endocytosis mediated by the hepatic lectin involves a series of processes leading to transport of ligands to lysosomes in hepatic parenchymal cells (LaBadie *et al.*, 1975). The intricacies of intracellular ligand routeing have been further emphasized by the recent observations on exocytosis of synthetic glycosides (Connolly *et al.*, 1982) and asialo-transferrin, type 3 (Regoeczi *et al.*, 1982). In this study, we have investigated the initial steps in endo- and exocytosis, binding and internalization. By controlling temperature and concentration of ligand and calcium, we were able to study the kinetic and steady state properties of binding and internalization of two structurally distinct ligands, ASOR and Gal_{39}-AI-BSA. Isolated rabbit hepatocytes were used so that direct comparisons of binding parameters could be made with purified rabbit hepatic lectin which we have previously studied in detail (Connolly *et al.*, 1981; Lee, 1982; Lee *et al.*, 1982). In addition, parallels could be drawn between the present investigation and other studies utilizing isolated rat hepatocytes (Weigel & Oka, 1982). The experiments with anti-(hepatic lectin) IgG demonstrate that the purified lectin obtained from whole rabbit liver and studied *in vitro* is the same protein that is involved in the endocytosis of asialoglycoproteins in intact hepatocytes. Interestingly, the inhibitory effect of this antibody was quickly reversible. These results are in contrast to those of Stockert *et al.* (1980), who showed that a single passage through the perfused rat liver of antibody directed against the rat receptor was sufficient to inhibit its function for up to 3h. It is possible that different factors regulate the turnover time of surface receptor and the bound antibody in isolated hepatocytes than in the perfused liver.

Analysis of binding parameters of ^{125}I-ASOR and ^{125}I-Gal_{39}-AI-BSA to the hepatocytes (Figs. 2 and 3, Table 1) indicated that the apparent affinities of the two ligands were similar and that the total number of sites for the two ligands differ only slightly. Considering the vast difference in the carbohydrate structures of the two ligands, these similarities in the binding parameters are noteworthy. It appears that if sufficient number of Gal residues are clustered on BSA effective binding and internalization results.

Many of the ligand-binding properties observed for the lectin present on the cell surface of hepatocytes were also observed with the isolated lectin (Connolly *et al.*, 1981). For instance, both species displayed calcium-dependent, high-affinity binding to both ASOR and Gal_{39}-AI-BSA. However, the ratios of high-affinity to low-affinity sites were different for the two species of lectin. The ratio of high-affinity to low-affinity sites was about 1:1 for the isolated lectin, but was about 1:3 for the lectin *in situ*. So overall, the isolated lectin bound ASOR 'tighter' than did cells. A similar trend has been observed using rat hepatocytes (Weigel & Oka, 1982).

The reported values for the apparent high-affinity binding constant for rat hepatocytes were variable, but generally, $K_d > 10^{-8}$ M (Weigel, 1980; Schwartz *et al.*, 1980; Baenziger & Fiete, 1980; Steer & Ashwell, 1980). These values are somewhat higher than values obtained in this study for rabbit hepatocytes at 2°C. The total number of ASOR binding sites per cell reported for the rat hepatocyte is also quite variable (40000–750000), but the values obtained here, 466000 sites/cell at 2°C and 630000 sites/cell at 37°C, are certainly within this range. Previous studies with rat hepatocytes have shown that freshly isolated cells increased the number of surface receptors upon incubation at 37°C (Weigel, 1980; Steer & Ashwell, 1980). This was interpreted as either replacement of receptors that had been damaged during isolation (Steer & Ashwell, 1980), or as the clearance of endogenous inhibitors by the cells (Weigel, 1980). It is possible that the difference between the 2°C value and the 37°C value in the present study is also a result of these factors. It should be emphasized that neither serum nor BSA were included in the medium used in the present studies, and thus the potential effects of these agents on the measurement of binding site number were not a factor (Schwartz *et al.*, 1980).

The amount of cell-associated ligand that became resistant to EGTA treatment increased as a function of time at 37°C, but not at 2°C, indicating that the ligand was not being internalized from the cell surface at 2°C. By 10min, the amount of ligand on the surface had reached an apparent steady state at 37°C (Fig. 4), and internalization of ligand over this period was nearly linear. This enabled kinetic analysis of the system. The kinetic analysis of data obtained at 37°C was based on a number of assumptions regarding the state of the system. First, the derivation of the equations for the determination of $K_{app.}$ were based upon the assumption that a rapid equilibrium was established between the free ligand and the surface receptor. Because it is known that the 'off rate' for bound ligand, k_{-1}, is very slow (Weigel, 1980), this condition may not have been totally satisfied. Furthermore, the determination of K_{uptake} assumed that the free ligand concentration does not change appreciably during the measurement of the initial rate of internalization. However, for lower ligand concentrations, accurate assessment of the initial rate becomes difficult because up to 16% of the added ligand became bound during the course of the experiment. Nevertheless, the binding parameters obtained at 37°C by this analysis closely resembled those observed at 2°C. Two apparent classes of ^{125}I-ASOR binding sites were evident at

37°C with apparent binding constants of $K_{app.} = 1.9$ and 130nM.

The dual nature of binding sites raised the question as to whether both classes of sites are involved in internalization. The results obtained indicate that the low-affinity sites function as well as the high-affinity sites (model 3). The low-affinity binding, either with cells or isolated lectin, could be due to the heterogenous carbohydrate structure of the ligands. Alternatively, low-affinity binding could result from steric hindrance between ligands bound to adjacent sites. The use of small ligands of well-defined structure, as well as more refined data analysis, should help in resolving these issues.

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