Synthesis and Characterization of *N*-Hydroxysuccinimide Ester Chemical Affinity Derivatives of Asialoorosomucoid That Covalently Cross-Link to Galactosyl Receptors on Isolated Rat Hepatocytes†

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ABSTRACT: We have developed chemical affinity reagents for the hepatic galactosyl receptor. Asialoorosomucoid (ASOR) was derivatized with five homobifunctional *N*-hydroxysuccinimide (NHS) ester cross-linkers. NHS/ASOR derivatives were synthesized, purified, and applied within 10 min to isolated rat hepatocytes at 4 °C. Specific binding of these ^{125}I-labeled derivatives was ~90% in the presence of either EGTA or excess ASOR. Specific cross-linking assessed by the resistance of specifically bound NHS/^{125}I-ASOR to release by EGTA, was 50–75% of the specifically bound ligand. The extent of specific cross-linking correlated with the average number of NHS groups per ASOR and was controlled by varying the molar ratio of cross-linker to ASOR during the synthesis. Cross-linking proceeded rapidly at 4 °C as a first-order process (k = 0.25 min^{-1}, $t_{1/2}$ = 2.8 min). After being cross-linked with any of the NHS/^{125}I-ASOR derivatives, cells were washed with EGTA, solubilized in Triton X-100, and analyzed by SDS–PAGE and autoradiography. Major bands were observed at $M_r \simeq$ 84K, 93K, and 105K corresponding to the expected size of 1:1 adducts between NHS/ASOR ($M_r \simeq$ 41.3K) and the three subunits of the receptor, $M_r \simeq$ 43K, 50K, and 60K. The three subunits, rat hepatic lectin (RHL) 1, 2, and 3, were labeled in the ratio of about 1.0:1.2:1.0, respectively. After cross-linking, a polyclonal goat antibody to the receptor immunoprecipitated up to 100% of the specifically cross-linked NHS/^{125}I-ASOR. Preimmune IgG immunoprecipitated <1% of the radiolabeled ligand. Cell surface receptors were cross-linked to NHS-ASOR, extracted with Triton X-100, immunoprecipitated with anti-orosomucoid–Sepharose, and subjected to Western blot analysis. By use of anti-sera specific for RHL 1 or RHL 2/3 (from K. Drickamer), cross-linked complexes of $M_r \simeq$85K or ~90–115K, respectively, were detected as were un-cross-linked native subunits. The ratio of free to cross-linked subunits was ~10:1 for RHL 1 and ~0.5:1 for RHL 2/3. We conclude that all three receptor subunits can cross-link to ligand. We propose a model in which the native receptor is a heterohexamer composed of four subunits of RHL 1 and two subunits of RHL 2 and/or RHL 3.

Mammalian hepatocytes possess a membrane glycoprotein that binds specifically to glycoproteins whose oligosaccharide chains terminate with galactose or *N*-acetylgalactosamine groups (Ashwell & Harford, 1982; Stockert, 1983; Schwartz, 1984; Breitfeld et al., 1985). After receptor binds to Gal[1]-terminated tri- or tetraantennary oligosaccharides, the receptor–ligand complex is internalized and dissociated, and the glycoprotein ligand is targeted for degradation in the lysosomes while the receptor returns to the cell surface to be reutilized. A single galactosyl receptor is capable of mediating thousands of rounds of ligand endocytosis (Warren & Doyle, 1981). This asialoglycoprotein or Gal receptor is a transmembrane glycoprotein of unknown subunit stoichiometry. In nonionic detergents the rat receptor is approximately 264 kDa as determined by gel filtration and sedimentation equilibrium analysis (Anderson et al., 1982). Radiation inactivation studies have determined that the ASOR binding domain is approximately 148 kDa in the presence and 105 kDa in the absence of detergent (Steer et al., 1981). In receptor preparations purified by ligand affinity chromatography, one major band and two minor bands are identified on SDS–polyacrylamide gels. These three subunits have been designated rat hepatic lectins (RHL) 1, 2, and 3 (Drickamer et al., 1984). The three separate subunits are, respectively, M_r = 41.5K, 49K, and 54K and are the products of two different genes (Drickamer et al., 1984; Holland et al., 1984). The RHL 2 and RHL 3 proteins differ only in the type and extent of posttranslational carbohydrate modification and show considerable sequence homology with RHL 1 (Halberg et al., 1987; Holland et al., 1984; Leung et al., 1985). The stoichiometry and subunit composition of the native rat receptor is unknown, and as analyzed by SDS–PAGE, the mole ratio for the RHL 1, 2, and 3 subunits varies respectively from 2.5:1:1 (Takahashi et al., 1985) to 8:1:1 (Drickamer, 1987).

We have recently shown that two subpopulations of functionally distinct Gal receptors and two different parallel pathways for ligand uptake, dissociation, and degradation (Weigel et al., 1986; Oka & Weigel, 1983, 1987; Clarke et al., 1987) exist in isolated rat hepatocytes. As a tool for the further study of these Gal receptor subpopulations and their characterization during recycling, we undertook development of a chemical affinity label for the Gal receptor using the chemistry of commercially available bifunctional NHS esters.

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[1] Abbreviations: Gal, galactosyl; ASOR, asialoorosomucoid; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; NHS, *N*-hydroxysuccinimide; NHS/ASOR, ASOR modified with any of the bifunctional NHS esters listed in Figure 1; NHS-ASOR, ASOR modified with DSS; NHS-S-S-ASOR, ASOR modified with DSP; RHL, rat hepatic lectin; PBS, phosphate-buffered saline. The bifunctional cross-linkers are defined in Figure 1.

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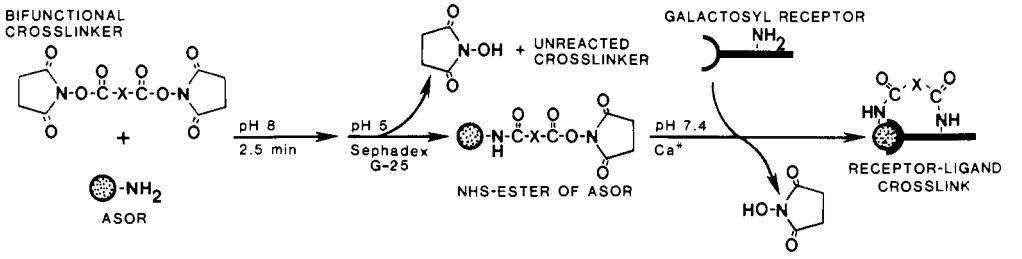
PROTOCOL FOR CROSSLINKING GALACTOSYL RECEPTORS AND NHS-ESTERS OF ASOR



BIFUNCTIONAL N-HYDROXYSUCCINIMIDE ESTER CROSSLINKERS

-X-	ABBREVIATION	CHEMICAL NAME
$-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$	DSS	DISUCCINIMIDYL SUBERATE
$-CH_2-CH_2-S-S-CH_2-CH_2-$	DSP	DITHIOBIS(SUCCINIMIDYL PROPIONATE)
$-O-CH_2-CH_2-\overset{\overset{O}{\|}}{S}-CH_2-CH_2-O-$	BSOCOES	BIS[2-(SUCCINIMIDOOXYCARBONYLOXY)ETHYL]SULFONE
$-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-O-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-$	EGS	ETHYLENE GLYCOLBIS(SUCCINIMIDYLSUCCINATE)
$-\overset{OH}{\overset{\|}{C}}H-\overset{OH}{\overset{\|}{C}}H-$	DST	DISUCCINIMIDYL TARTARATE

FIGURE 1: Scheme for cross-linking galactosyl receptors and NHS esters of ASOR. The synthesis and use of the NHS/ASOR chemical affinity derivatives are depicted. The cross-linker arm structures (X) and the names and abbreviations of the cross-linkers used in this study are below the schematic. The lengths of the linker arms are as follows: 11.4 Å, DSS; 12 Å, DSP; 13 Å, BSOCOES; 16.1 Å, EGS; 6.4 Å, DST.

Our goal was to label specifically Gal receptors on freshly isolated hepatocytes and then observe their fate during endocytosis and subsequent recycling of receptor. Baenziger and Fiete (1982) in an earlier study successfully developed photoactivatable glycopeptides for affinity labeling of lectins such as the Gal receptor. These reagents, however, had only about a 2% efficiency of covalent cross-linking to the Gal receptor. In a more recent study, Lee and Lee (1986) reported the preparation of a photolabeling reagent that cross-linked to Gal receptors with efficiencies ranging from 10–30%. All the subunits of Triton X-100 solubilized and purified rabbit and rat hepatic Gal lectins were labeled. The synthesis of this photolabile reagent required approximately 10 days.

We report here the synthesis and characterization of chemical affinity cross-linkers for the Gal receptor than can be prepared in less than 10 min. These derivatives label Gal receptors on live hepatocytes with greater than 90% specificity at an efficiency of cross-linking approaching 75% of the cell surface receptor–ligand complexes formed. We also propose a heterohexamer model for the native Gal receptor structure consisting of four subunits of RHL 1 and two subunits of RHL 2 and/or RHL 3. A preliminary report of these results has been presented (Herzig & Weigel, 1986).

Materials and Methods

Materials. Orosomucoid from Sigma Chemical Co. or a gift from Dr. M. Wickerhauser of the American Red Cross Plasma Derivatives Laboratory was desialylated as described previously (Weigel & Oka, 1982). Glutaraldehyde (25%, EM grade) was from Polysciences. ^{125}I-ASOR and ^{125}I protein A were prepared with Iodogen from Pierce Chemical Co. as described earlier (Weigel & Oka, 1982) with Na^{125}I from Amersham (10–20 mCi/μg iodine). The bifunctional NHS cross-linkers, DSS, DSP, DST, BSOCOES, and EGS (see Figure 1), were also from Pierce. Sephadex G-25, superfine, was from Pharmacia. BSA (clinical reagent grade, CRG-7) was from Armour Pharmaceutical Co., and protein A was from Genzyme. Collagenase (research grade) was from Serva. Phenylmethanesulfonyl fluoride, MES, leupeptin, pepstatin, CNBr-activated Sepharose 4B, and antibodies to orosomucoid, both a nephalometric grade raised in goats and an IgG fraction from rabbits, were from Sigma Chemical Co. The Gal receptor antisera developed in goat, was characterized and purified as described previously (McAbee & Weigel, 1987, 1988). Digitonin was from Sigma. Investigators should note, however, that over the last 2 years the purity of digitonin preparations from this source has decreased significantly so that 1.4% (w/v) stock solutions in absolute ethanol (Weigel et al., 1983) can no longer be prepared. We find that Kodak Chemical Co. (Rochester, NY) presently supplies the most suitable digitonin for the studies described here. All other materials were of reagent grade.

Media and Buffers. Medium 1/BSA is a modified Eagles' medium from Grand Island Biologicals Co. (GIBCO) supplemented with 2.4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.22 g/L $NaHCO_3$, and 0.1% (w/v) BSA, pH 7.4. Buffer 1 contains 143 mM NaCl, 6.8 mM KCl, and 10 mM HEPES, pH 7.4. BIC 10 is buffer 1 with 10 mM $CaCl_2$ while BIE 5 is buffer 1 with 5 mM EGTA. Hanks' balanced salt solution was prepared according to the GIBCO formulation. PBS contains 137 mM NaCl, 8 mM sodium phosphate, 2.7 mM potassium chloride, and 1.5 mm potassium phosphate, pH 7.4.

Cells. Isolated hepatocytes from male Sprague-Dawley rats (Harlan, Houston, TX) were prepared by a modified (Oka & Weigel, 1987) collagenase perfusion procedure (Seglen, 1973). The cells were stored on ice in medium 1/BSA and were >85% viable and single cells. Before use, cells were first incubated at 37 °C for 1 h to increase and stabilize the surface and total receptor content (Weigel & Oka, 1983a).

Determination of Receptor Activity. Ligand binding was determined by a centrifugation assay (Weigel, 1980a) at a

saturating concentration of ^{125}I-ASOR or related derivative (1.5 μg/mL) in BIC 10. Nonspecifically bound material was assessed by either of two ways: by binding radiolabeled ligand in the presence of a 50-fold excess of nonradiolabeled ASOR or by changing the binding buffer to an EGTA-containing buffer, BIE 5. After 1 h at 4 °C, cells were washed with 7 volumes of ice-cold Hanks, centrifuged, and aspirated prior to two further 10-min washes in either medium 1 containing 7.5 mM EGTA or Hanks. Specifically bound ligand was the total cell associated radioactivity minus the nonspecifically bound radioactivity. When ^{125}I-ASOR was used as the ligand, radioactivity remaining after washing with EGTA usually equaled the nonspecifically bound material. When NHS/^{125}I-ASOR derivatives were used, radioactivity remaining in the cell pellet after an EGTA wash represented both nonspecifically bound material plus material cross-linked to the hepatocytes. Specific cross-linking was defined as the percent of the specific cell-associated radioactivity (as assessed with excess ASOR) that was resistant to EGTA treatment.

In some instances cells were treated with 0.055% digitonin to gain access to the internal pools of receptor prior to measuring ligand binding activity (Weigel & Oka, 1983b). Digitonin-permeabilized cells fixed with glutaraldehyde still have active Gal receptors and can be frozen for later use. These digitonin-permeabilized and fixed cells, prepared as described previously (Weigel et al., 1983), were used for receptor binding studies in a modification of the above centrifugation assay using microfuge tubes.

Synthesis of Chemical Affinity Probes. Various molar ratios of cross-linker to the 12 amine groups of ASOR (Schmid et al., 1973) were used, and all concentrations of reactants were chosen on the basis of the final molar ratio desired. For a typical synthesis, 100 μg of ^{125}I-ASOR (2.5 nmol of ASOR, 30 nmol of amine) in 250 μL of 100 mM sodium borate, pH 8.0, was reacted with DSS at a 500:1 molar ratio of DSS to ASOR amine groups. In this example 5.53 mg (15 μmol) of DSS in 55.3 μL of dimethyl sulfoxide was added to the ^{125}I-ASOR solution. The final concentration of DSS cross-linker at this ratio of reactants was 18.1 mM. For the experiments presented, initial ASOR concentrations ranged from 5 to 12 μM, and all cross-linkers were prepared as 100 mg/mL stock solutions in dimethyl sulfoxide just prior to use. At the concentrations of cross-linker used, precipitation occurred immediately upon addition of cross-linker to the aqueous ^{125}I-ASOR solution. Precipitation of the cross-linkers depended on their concentration and intrinsic aqueous solubility and became a problem for the DSS cross-linker only at molar ratios ≥ 1000:1 at which point protein recovery decreased. After 2.5 min of reaction, with stirring at room temperature, the mixture was acidified to pH 5.6 by addition of 200 mM MES to 50 mM. Reacted and unreacted ^{125}I-ASOR were then separated from free cross-linker and small reaction products by gel filtration over a syringe column of Sephadex G-25. This was centrifuged to dryness in a 15-mL conical tube in a Sorvall GLC-1 table-top centrifuge at 2000 rpm for 2 min. The preswollen G-25 was first treated with BSA (1–2 mg/mL of resin) and then equilibrated with PBS, pH 7.4, or 10 mM MES, pH 4.1. The G-25 bed was five times the volume of the reaction mix and was set up in either a 1-, 3-, 5-, or 10-mL syringe depending on the sample volume. The recovery of ASOR and derivatized ASOR in the eluate was about 70%. Unreacted cross-linker and small reaction products were retained in the G-25 column at these volumes. For the experiments presented, the NHS esters of ASOR were synthesized at pH 8.0 and acidified to pH 5.6 to stop the reaction, and then a final pH of 6.4 or of 5.4 was achieved after the centrifugation over G-25 equilibrated with PBS or 10 mM MES, respectively. After centrifugation, the G-25 eluate, containing both reacted and unreacted ^{125}I-ASOR, was brought to a final concentration of ≃30 μg of protein/mL in either PBS or 10 mM MES. This eluate was ready for the receptor cross-linking reaction and was immediately applied to cell suspensions at a final concentration of ≃1.5 μg/mL.

Immunoprecipitations. Cells at ≤8 × 10^6 cells/mL were extracted for 1 h at 4 °C with 1% Triton X-100 in either BIC 10 or BIE 5 buffer containing 133 μM phenylmethanesulfonyl fluoride alone or also with 10 μM each of pepstatin and leupeptin. These samples were centrifuged at 12000*g* for 20 min, filtered through 0.45-μM cellulose–acetate filters, and diluted to 0.5% Triton X-100 with BIC 10 or BIE 5 as needed. The purified IgG fraction from either a preimmune polyclonal goat antiserum or an antiserum directed against the purified Gal receptor was used at a final concentration of 0.4 mg/mL. The cell extracts were incubated with antibody for 1 h at 4 °C prior to addition to a final concentration of approximately 1.5% (v/v) fixed *Staphalococcus aureus* cells (Pansorbin from Calbiochem) or 5% (v/v) protein A–Sepharose. The *S. aureus* cells were first incubated for 1 h with unlabeled cell extract and washed three times prior to use in immunoprecipitations. After another 1 h incubation, samples were centrifuged in a microfuge, and the pellets were resuspended in 1.25 mL of BIC 10 or BIE 5 containing 0.05% Triton X-100 and washed twice by centrifugation. The tube tips with pellets were cut off, and their radioactivity was determined. Immunoprecipitations were also performed with commercial antibodies to human orosomucoid that recognize the desialylated protein. Typically, a 1:100 dilution of the orosomucoid antibodies raised in goat immunoprecipitated 90% of the ^{125}I-ASOR in both control and cross-linked samples with either protein A–Sepharose or *S. aureus* cells as the precipitant. The orosomucoid antibody raised in rabbit was conjugated to CNBr-activated Sepharose 4B at a final concentration of 1.75 mg of antibody/mL of resin. A one-step immunoprecipitation with a final concentration of 2% (v/v) anti-orosomucoid–Sepharose was then possible.

Electrophoresis. SDS–PAGE was performed as described by Laemmli (1970). Cell samples were extracted as above and prepared for electrophoresis by dilution with a 4-fold concentrated Laemmli sample buffer. Reduced samples were treated with 11 mM dithiothreitol prior to boiling and were alkylated with 50 mM iodoacetamide after boiling. The samples were resolved by electrophoresis on 6.5% (w/v) acrylamide slab gels, either 14 × 16 cm (Hoefer vertical slab chamber) or 10 × 15 cm (Idea Scientific mini-slab stilts apparatus). After electrophoresis, gels stained with Coomassie Blue R-250 were dried between two cellophane sheets with a Hoefer Scientific Instruments slab gel dryer, Model SE 540. Dried gels were exposed to Kodak X-OMAT AR film at −60 °C either with or without a phosphor screen (Kodak X-Omatic No. 153-6051). Western blotting was by the method of Burnette (1981) with some buffer modifications (McAbee & Weigel, 1988). Transfer of proteins from SDS–polyacrylamide gels to Schleicher & Schuell nitrocellulose paper (BA 85, 0.45 μm) was in an Idea Scientific GENIE electrophoretic blotter. The protein blots were analyzed immediately at room temperature or after storage at 4 °C. Nonspecific binding was blocked by incubation for 1 h in 10 mM Tris-HCl and 154 mM NaCl, pH 7.4, containing 5% (w/v) BSA, 10% (w/v) nonfat milk solids, and 0.2% (v/v) Nonidet P-40. Primary antisera incubations were for 12 h with a 1:200 dilution of rabbit antisera specific for RHL 1 or RHL 2/3, which were

a generous gift of Dr. K. Drickamer. After being washed, ^{125}I-protein A was used at 5 μg/mL (8.9×10^6 cpm/mL) for 90 min to detect bound antibody. The Western blots were then washed, dried, and autoradiographed. The two antisera, previously characterized by Halberg et al. (1987), were confirmed to be specific for either RHL 1 or RHL 2/3 by Western blot analysis of purified Gal receptor with ^{125}I–protein A. Densitometric scans (Helena Laboratories, Quick Scan Jr) were performed only on film exposed without the phosphor screen or on film that had been preflashed with a xenon flash unit (Vivitar 125).

General. Protein was determined by a dye-binding method (Bradford, 1976) or by the method of Lowry et al. (1953) with BSA as the standard. The fluorescamine assay using glycine as a standard was used for detection of primary amines (Udenfriend et al., 1972). Centrifugations of intact or permeable/fixed cell suspensions were at 800*g* or 1000*g*, respectively, for 3 min in a Beckman Model TJ-6 refrigerated table-top centrifuge. Permeable/fixed cells, immunoprecipitation samples, and samples for electrophoresis were centrifuged for 10 s in a Beckman Model B microfuge. ^{125}I radioactivity was determined on a Packard Multiprias 2 γ-counter.

Results

Synthesis of Chemical Affinity Probes. The synthesis of the chemical affinity probes is depicted schematically in Figure 1. Each of the bifunctional NHS cross-linkers listed below the protocol was examined for use in this reaction scheme. With its nonreactive alkyl linker arm, the NHS/ASOR ester synthesized with DSS is referred to as NHS-ASOR. Since DSP has a disulfide group, its derivative is called NHS-S-S-ASOR. These two derivatives were the most completely characterized. NHS esters have a half-life on the order of 10 min at pH 8.6, 4 °C, and of several hours at pH 7.0, 0 °C (Ji, 1979; Cuatrecases & Parikh, 1972; Lomant & Fairbanks, 1976). The extent of a 10-min reaction of NHS esters with amines at pH 8.0 is 4-fold greater than that of a reaction at pH 6.3 (Cuatrecasas & Parikh, 1972). Thus, ASOR was reacted at pH 8.0 with a large excess of bifunctional NHS cross-linker for 2.5 min at room temperature. Acidification to pH 5.6 effectively stopped the reaction and stabilized the NHS esters of ASOR for subsequent purification prior to addition to cells.

Since ASOR binding is Ca^{2+} dependent, chelation of the Ca^{2+} with EGTA prevents specific receptor–ASOR interactions and disrupts receptor–ASOR complexes already formed. Thus, cell-associated radioactivity not released by EGTA treatment measures the degree of covalent cross-linking of the NHS/^{125}I-ASOR to the cell surface. The efficiency of this NHS/^{125}I-ASOR cross-linking is equivalent to the percentage of specifically bound ligand that is cross-linked (i.e., that remains after EGTA treatment). Specific cross-linking is thus defined as (the total bound radioactivity that is EGTA resistant minus the radioactivity that is due to nonspecific binding) divided by (total bound radioactivity in the absence of EGTA minus nonspecifically bound radioactivity).

Stability of Affinity Derivatives. The extent of cross-linking to cells was inversely proportional to the age of the NHS/ASOR derivative. The stability of NHS-^{125}I-ASOR was assessed by its ability to cross-link to permeabilized/fixed hepatocytes over the course of several days when stored at 4 °C in buffers at either pH 6.4 or pH 5.4 (Figure 2). When the G-25 column was equilibrated in 10 mM MES, pH 4.1, the NHS-ASOR eluated at pH 5.4 and could be stored at 4 °C in this same buffer. Efficient cross-linking of this chemical

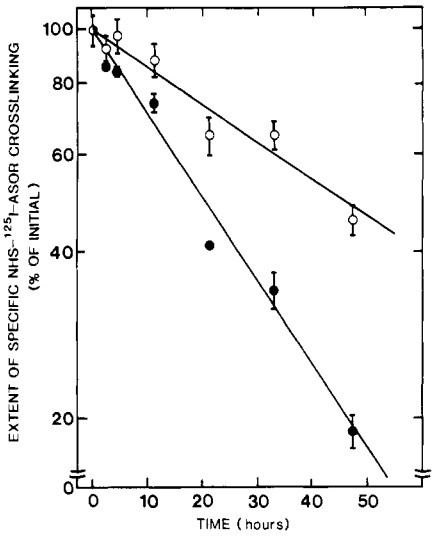


FIGURE 2: Effect of pH on the stability of NHS-^{125}I-ASOR. NHS-^{125}I-ASOR, synthesized at a molar ratio of 500:1 (DSS:ASOR-amine), was divided in half and centrifuged over two separate Sephadex G-25 columns equilibrated in either PBS (●) or 10 mM MES, pH 4.1 (○). The pH of these eluates was respectively 6.4 and 5.4. At the indicated times after the initial addition of DSS to ASOR, the proteins were added to permeable/fixed hepatocytes at pH 7.4. Permeable/fixed hepatocytes were used in order to have a uniform cell preparation throughout the course of the experiment. Total, nonspecific, and EGTA-resistant binding of the NHS-^{125}I-ASOR were determined in triplicate. The greatest specific cross-linking observed for the pH 6.4 (38.1%) and pH 5.4 (40.0%) eluates were normalized to 100%.

affinity derivative to the Gal receptor was still obtained up to 6 h after its synthesis and purification. On the basis of least-squares linear regression analysis of semilog plots, the $t_{1/2}$ was 43.5 h at pH 5.4 versus a $t_{1/2}$ of 19.8 h at pH 6.4. Thus, the NHS ester lifetime was demonstrably enhanced when the protein derivative was kept at a lower pH. Nonetheless, these derivatives were usually applied to cells within 10 min of the addition of the cross-linker to the ASOR solution.

Reaction Conditions. The purpose of the reaction of ASOR with the bifunctional cross-linker was to obtain an NHS ester of the protein, not ASOR cross-linked to itself. Therefore, both a very short reaction time and a very large molar excess of cross-linker relative to the free amino groups of ASOR were used in the synthesis of the derivative. Optimum cross-linking efficiency of the resulting NHS derivative to freshly isolated hepatocytes was achieved at a molar ratio of cross-linker to free amine groups of 500:1 for NHS-ASOR or at 2000:1 for NHS-S-S-ASOR (Figure 3). On the basis of these results, an experiment was performed to examine the extent of derivatization of the 12 amino groups of ASOR. The extent of specific cross-linking to the cells correlated with the number of amino groups derivatized on the protein (Table I). Optimal cross-linking for the NHS-ASOR derivative occurred at a molar ratio of 500:1 (DSS:ASOR-amine) and gave 65.1% specific cross-linking. At this ratio of reactants, approximately 70% of the accessible amine groups was derivatized. This corresponded to an average of approximately eight NHS esters on each ASOR molecule. The NHS-S-S-ASOR exhibited a similar correlation of ASOR derivatization and cellular cross-linking (not shown).

Specificity of NHS-ASOR Cross-Linking to the Cell Surface. The strategy of chemical affinity cross-linking rests upon three premises: (1) that the derivatized ligand is recognized by Gal receptors with appropriate specificity and affinity, (2) that the derivatized ligand is not nonproductively

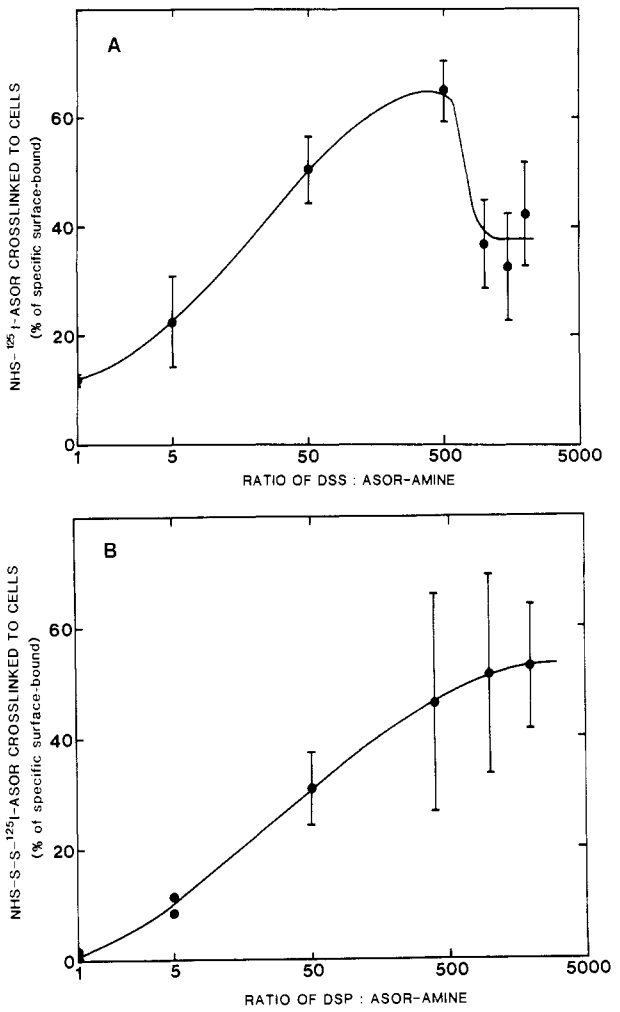


FIGURE 3: Effect of synthesis conditions on reactivity of NHS/^{125}I-ASOR affinity reagents. The molar ratio of cross-linker to the free amino groups on ^{125}I-ASOR was varied from 1:1 to 2000:1 during synthesis of the derivative. The purified derivative was then allowed to bind to freshly isolated hepatocytes, and the specific cross-linking was determined as described under Materials and Methods. Each point is the average of several cell binding experiments ($n \geq 3$) using DSS as the bifunctional cross-linker in the synthesis of NHS-^{125}I-ASOR (A) or using DSP to synthesize NHS-S-S-^{125}I-ASOR (B). The variation reflects differences in both derivative and hepatocyte preparations.

hydrolyzed or reacted prior to coming in contact with an amine group on the receptor, and (3) that the receptor will have amine groups available to react with the derivatized ligand. In support of the first premise, the saturation binding curves of derivatized and underivatized ASOR were virtually identical (Figure 4). Nonspecific binding was, if anything, lower with the NHS-^{125}I-ASOR then that observed with underivatized ^{125}I-ASOR. The slight increase in the specific binding with the derivatized ASOR may be due to the additional purification step by centrifugation over G-25 just prior to use. Thus, derivatization of ASOR did not interfere with the specificity of binding to receptor. The major difference was seen in the effect of EGTA treatment, as was expected. In this experiment, approximately 35% of the specifically bound NHS-^{125}I-ASOR (Figure 4A) and nearly 58% of the specifically bound NHS-S-S-^{125}I-ASOR (Figure 4B) cross-linked to the cell surface. EGTA treatment of cells with bound underivatized ^{125}I-ASOR stripped the ligand to nonspecific levels only 1–2% higher than those assessed in the presence of excess ASOR (Figure 4A). Since the derivatized ASOR interacts irreversibly and covalently cross-links to the cells, one cannot

Table I: Effect of Cross-Linker Concentration on Derivatization of ASOR Amino Groups[a]

molar ratio of cross-linker to ASOR amine groups	specific cross-linking (%)[b]	amino groups derivatized (%)	av number of NHS groups/ ASOR
0:1	0	0	0
5:1	22.6 ± 8.5	30.8 ± 17.5	2.2
50:1	50.5 ± 6.2	59.0 ± 10.2	6.2
250:1	51.4	70.2 ± 7.6	7.8
500:1	65.1 ± 5.9	69.7 ± 0.4	8.3

[a] NHS-ASOR was synthesized at various molar ratios of DSS to ASOR amino groups as in Figure 3. After gel filtration to remove unreacted cross-linker, reactive NHS groups on the protein were quenched with ethanolamine (0.2 M, 10 min, 24 °C). The protein solution was subjected to several sequential centrifugations over an Amicon Centricon apparatus of M_r 30 000 cutoff to remove the ethanolamine. After analysis for protein, the remaining free amino groups in each sample were analyzed by the fluorescamine assay in the presence of 1% SDS. SDS had no effect on a glycine control in this latter assay, yet it increased the reactivity of ASOR by over 2-fold. The number of amino groups detected in the 0:1 sample was set equal to 12 and was defined as 0% derivatized. [b] Taken from Figure 3.

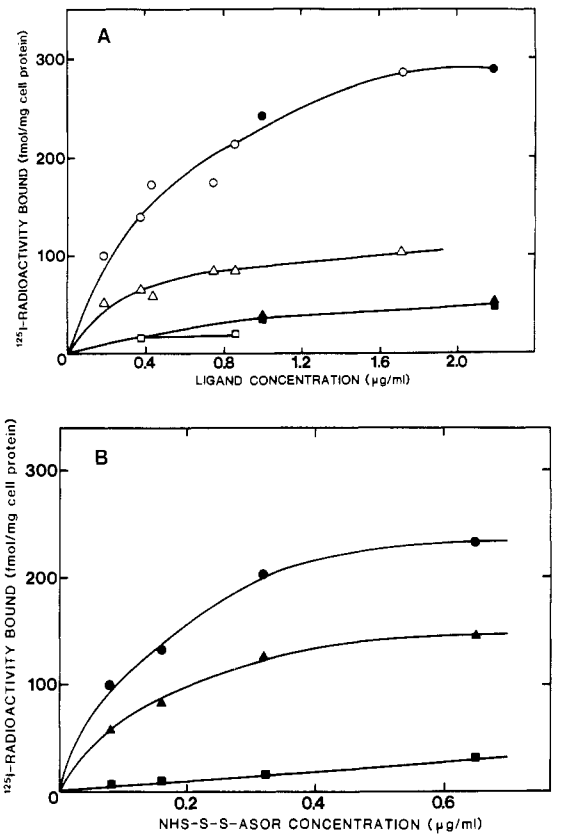


FIGURE 4: Saturation binding of NHS/^{125}I-ASOR derivatives to hepatocytes. Different concentrations of iodinated ASOR, NHS-ASOR, and NHS-S-S-ASOR synthesized at ratios of 1000:1 and 2000:1, respectively, were allowed to bind to freshly isolated hepatocytes for 1 h at 4 °C and total (●, ○) and nonspecifically (■, □) bound radioactivity were determined. Covalently attached ligand (▲, △) was assessed by washing with EGTA. Panel A depicts results with ^{125}I-ASOR (closed symbols) and NHS-^{125}I-ASOR (open symbols) while panel B shows NHS-S-S-^{125}I-ASOR (closed symbols). The average of triplicate determinations in a typical experiment is shown.

determine its binding affinity. Presumably, this is identical with that of ASOR; $K_d \simeq 10^{-9}$ M (Weigel, 1980a).

Kinetics of Cross-Linking between ASOR and Hepatocytes. We also examined the kinetics of cross-linking, as opposed to the kinetics of binding, between NHS-^{125}I-ASOR and Gal receptor (Figure 5). After 1 min of NHS-^{125}I-ASOR binding to hepatocytes, a large excess of unlabeled ASOR was added

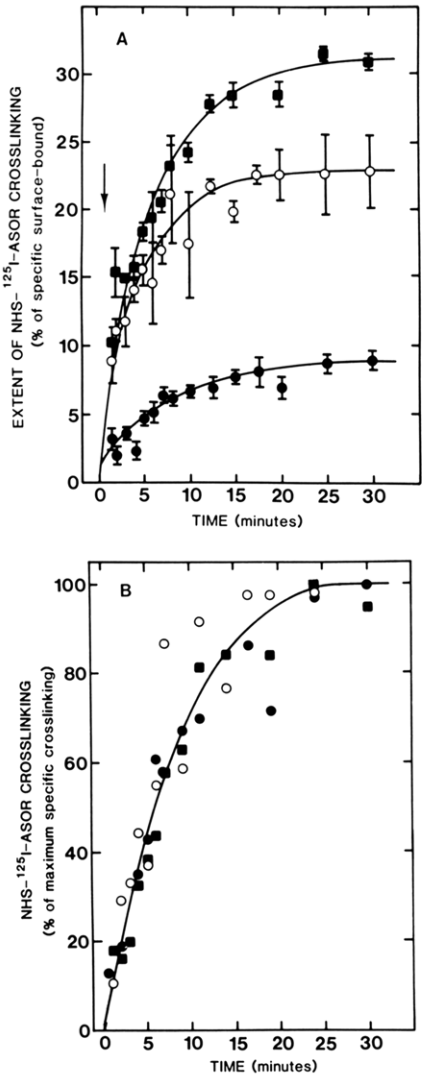


FIGURE 5: Kinetics of NHS-^{125}I-ASOR cross-linking to hepatocytes. (A) NHS-^{125}I-ASOR was synthesized at a 5:1 (●), 50:1 (○), or 500:1 (■) molar ratio of cross-linker to ASOR-amine groups. Cells were allowed to bind these NHS-^{125}I-ASOR derivatives in BIC 10 at a saturating concentrations (~1.5 μg/mL) for 1 min, at which time nonradiolabeled ASOR was added in 100-fold excess. At various times, triplicate 1.0-mL samples were added to 3.5 mL of medium 1/BSA at 4 °C containing 100 mM glycine both to quench the remaining unreacted NHS esters and to dilute the derivatized ^{125}I-ASOR and minimize any further binding. After centrifugation in Hanks, the cell pellet was resuspended and incubated with 7.5 mM EGTA in medium 1 for 10 min and then centrifuged. The supernatant was removed, and both supernatant and pellet were assessed for radioactivity. The nonspecific binding during the time course was assessed on cells that were in the presence of 100 mM glycine prior to addition of the NHS-^{125}I-ASOR. (B) The above data are replotted with the amount of cross-linking expressed as a percentage of the final extent of cross-linking at 30 min. The amount of cross-linking present at 1 min, upon the addition of excess ASOR, has been subtracted.

to stop further binding of the radioactive ligand. Bound radiolabeled ligand did not increase appreciably after this addition (≤10%) throughout the experimental time course (data not shown). Cell samples taken at various times after this ASOR addition and then washed with EGTA, therefore, assessed the ability of the NHS-^{125}I-ASOR bound within the first minute to cross-link to the cell surface. By measurement of both the released NHS-^{125}I-ASOR molecules in the EGTA supernatant and the cell-associated radioactivity, the kinetics of cross-linking were directly assessed for each sample. Three different molar ratios for the synthesis of NHS-^{125}I-ASOR were examined (Figure 5A). The percent of the total bound

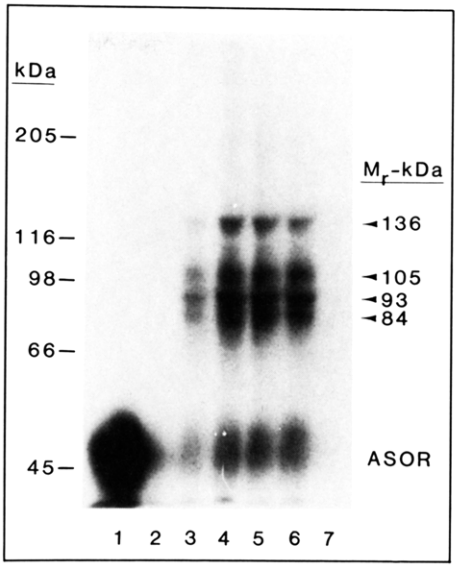


FIGURE 6: Autoradiographic analysis of cross-linked products using NHS-^{125}I-ASOR prepared under different synthetic conditions. Cells were incubated at 4 °C for 60 min with ^{125}I-ASOR or with different preparations of NHS-^{125}I-ASOR synthesized with molar ratios of DSS:ASOR-amine groups from 5:1 to 2000:1. The NHS-^{125}I-ASOR cell samples were then treated with EGTA, and the ^{125}I-ASOR samples were washed with Hanks or with EGTA. All samples were extracted with Triton X-100 and normalized for DNA content. Equal cell equivalents of each sample were subjected to SDS–PAGE, dried, and autoradiographed. The autoradiogram shows specifically bound ^{125}I-ASOR washed with HANKS (lane 1) or with EGTA (lane 2). The NHS-^{125}I-ASOR-labeled cell extracts (at the indicated ratios) were in lanes 3 (5:1), 4 (50:1), 5 (500:1), and 6 (2000:1). The binding of NHS-^{125}I-ASOR (synthesized at a ratio of 500:1) in the presence of excess unlabeled ASOR is shown in lane 7. Calculated masses based on coelectrophoresed standards are depicted to the right.

ligand that was specifically cross-linked was time dependent and proportional to the average number of NHS groups per ASOR molecule. When these data were expressed as a percentage of the final extent of cross-linking at 30 min, the three different NHS-ASOR derivatives showed identical cross-linking kinetics (Figure 5B). Thus, the absolute rate of cross-linking was independent of the molar ratio of cross-linker used for synthesis of the derivative. Cross-linking proceeded as an apparent first-order, essentially intramolecular, process with a rate constant of 0.25 min^{-1} and $t_{1/2}$ of 2.8 min (derived from a semilog plot; $r = -0.92$).

Analysis of Cross-Linking between NHS/ASOR Derivatives and Hepatocytes. Cells that had been cross-linked to NHS-^{125}I-ASOR were treated with EGTA, extracted, and subjected to SDS–PAGE and subsequent autoradiography (Figure 6). Three major species of approximately 84, 93, and 105 kDa were detected that corresponded to the expected cross-link products between the ~41-kDa NHS-ASOR molecule and each of the three RHL subunits of the Gal receptor. The RHL 1, 2, and 3 subunits usually migrate as proteins of 43, 50, and about 60 kDa in our reduced SDS–PAGE system. In addition, a larger species at 136 kDa was detected, suggesting a possible cross-link either between two modified ASOR molecules and one receptor polypeptide or between two receptor polypeptides and one ASOR molecule. When an excess of nonderivatized ASOR was present, no cross-linking products were seen (lane 7, Figure 6) which demonstrates the high specificity of this NHS-^{125}I-ASOR chemical affinity derivative. No differences in the labeling patterns from 84 to 105 kDa were seen among the NHS-^{125}I-ASOR derivatives synthesized at different molar ratios of DSS cross-linker (Figure 6, lanes 3–6). This indicates that the increased cross-linking efficiency at the higher molar

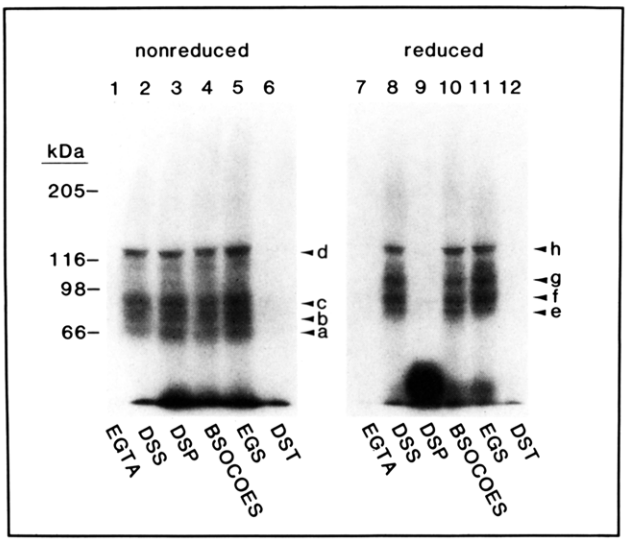


FIGURE 7: Analysis of cross-linking products using different NHS/^{125}I-ASOR affinity derivatives. Cells were incubated for 60 min at 4 °C with ^{125}I-ASOR derivatized at a reactant ratio of 1000:1 with each of the bifunctional NHS ester cross-linkers listed in Figure 1. After the cells were washed with EGTA, Triton X-100 extracts were prepared, and equal protein samples were analyzed by SDS–PAGE and autoradiography. Samples in lanes 1–6 on the autoradiogram were not reduced whereas those in lanes 7–12 were reduced and alkylated. The cross-linkers used for the ^{125}I-ASOR derivatization were DSS (lanes 1, 2, 7, and 8), DSP (lanes 3 and 9), BSOCOES (lanes 4 and 10), EGS (lanes 5 and 11), and DST (lanes 6 and 12). Lanes 1 and 7 are samples with the DSS/^{125}I-ASOR derivative allowed to bind to cells in the presence of EGTA and are the nonspecific binding controls. Mass determinations are to the left. The calculated masses (kDa) are (a) 66, (b) 76, (c) 97, (d) 132, (e) 82, (g) 103, and (h) 132.

ratios is not due to an increased access of the chemical affinity probe to different cell surface proteins. However, the relative incorporation of ^{125}I-ASOR into the 136-kDa species increased as the number of NHS groups/ASOR increased. On the basis of densitometry scans, the percent of radiolabel incorporated into this band changed from 5.5% for the 5:1 ratio of reactants to 13.4% for the 500:1 ratio and reflected the increased derivatization of ASOR.

Use of the Reaction Scheme with Other Bifunctional NHS Cross-Linkers. All of the commercially available bifunctional NHS esters listed in Figure 1 were tested for their ability to derivatize ASOR and to cross-link ASOR to isolated hepatocytes. To maximize cross-linking, the intracellular receptors were made accessible by treatment with 0.055% digitonin so that both surface and internal Gal receptors could be cross-linked to the ASOR derivatives. These cell samples were then extracted and subjected to SDS–PAGE and autoradiography (Figure 7). From the marked similarity of the labeling patterns it can be seen that all but one of the NHS/^{125}I-ASOR derivatives tested apparently cross-linked to the same proteins and were successful in this cross-linking protocol. In each case the covalent cross-link products formed corresponded to the expected products between ASOR and each of the three Gal receptor subunits. In normal synthesis buffer, DST did not cross-link, possibly due to the formation of a complex between the vicinal hydroxyl group in the linker arm with borate. After the synthesis buffer was changed to 50 mM Tris-HCl, pH 8.0, DST-^{125}I-ASOR then also cross-linked to cells and gave similar labeling patterns as assessed by autoradiography (not shown). The NHS-S-S-^{125}I-ASOR derivative showed the typical cross-link pattern in nonreduced SDS-PAGE but, as expected, showed only the ^{125}I-ASOR band after reduction in the presence of dithiothreitol (lane 9, Figure 7).

Table II: Immunoprecipitation of ^{125}I-ASOR Derivatives after Binding to Hepatocytes[a]

		percent of radioactivity immunoprecipitated			
		anti-orososmucoid Ab[b]		anti-Gal receptor Ab[c]	
ligand	experiment	+Ca^{2+}	+EGTA	+Ca^{2+}	+EGTA
ASOR	1	73.6	95.2	34.4	2.3
	2	53.9	81.3	37.9	2.2
	3	68.4	77.7		6.9
NHS-ASOR	1	61.7	90.8	102.9	72.2
	2	26.7	86.6	85.5	74.5
	3	67.8	91.7	98.6	53.2
	4	54.7	89.1	87.9	55.0
NHS-S-S-ASOR	2	28.1		57.5	60.2
	3	35.6	44.8	88.6	56.6

[a] Hepatocytes were allowed to bind radioiodinated ligand for 60 min at 4 °C. Cells with bound derivatized ^{125}I-ASOR were washed with EGTA, and cells with bound ASOR were washed with Hanks prior to extraction. Immunoprecipitations were done in duplicate in either BIC 10 or BIE 5 as indicated with either anti-orosomucoid antibody or the IgG fraction of an anti-Gal receptor antisera, with fixed *S. aureus* cells as the precipitant. [b] Values are expressed as a percent of the total radioactive ligand present. [c] Values are expressed as a percent of the specifically cross-linked radioactive ligand that is present except in the case of ASOR. For ASOR, the values are expressed as a percent of the specifically bound ligand present.

Specificity of NHS-ASOR Cross-Linking to Gal Receptors. The previous results show that NHS-^{125}I-ASOR cross-links predominantly to three proteins on the hepatocyte surface and cross-linking is blocked by a 50-fold excess of ASOR or the presence of EGTA. Experiments with antibody to the receptor confirmed that the derivatized ASOR cross-linked specifically to the Gal receptor (Table II). Cells were either treated with NHS-^{125}I-ASOR and then EGTA prior to extraction or treated with ^{125}I-ASOR and washed with Hanks and extracted as a control. Cell extracts were immunoprecipitated with either anti-orosomucoid antisera or anti-Gal receptor antisera in buffer plus EGTA or Ca^{2+}. As expected the anti-orosomucoid antibodies immunoprecipitated radiolabeled ASOR optimally in a Ca^{2+}-depleted buffer, whereas the Gal receptor antibody immunoprecipitated ^{125}I-ASOR optimally when it was complexed to receptor in the presence of Ca^{2+}. With Ca^{2+} present, 85–100% of the specifically bound NHS-^{125}I-ASOR and up to 30% of the specifically bound ^{125}I-ASOR were coimmunoprecipitated by the anti-Gal receptor antibody. Since immunoprecipitation was performed several hours after the initial ligand binding, some dissociation of receptor–^{125}I-ASOR complexes may have occurred. Also, since the polyclonal anti-Gal receptor Ab competes for ^{125}I-ASOR binding, some of the noncovalently bound ligand may have been displaced. In the absence of Ca^{2+}, ^{125}I-ASOR was not immunoprecipitated by antireceptor antibody (2.2–6.9%), whereas recovery in the NHS-^{125}I-ASOR and NHS-S-S-^{125}I-ASOR extracts ranged from 53.2 to 74.5% (Table II). The variability is probably because these experiments were performed on separate days with different hepatocyte and derivatized ASOR preparations. The immunoprecipitation of the specifically cross-linked receptor–ligand complexes increased with increasing concentration of anti-Gal receptor antibody (data not shown). Cells were cross-linked with NHS-^{125}I-ASOR, washed with EGTA, and then extracted with 1% Triton X-100 in BIC 10. Virtually all (92 ± 3%) of the specifically cross-linked NHS-^{125}I-ASOR was immunoprecipitated by the immune but not the preimmune IgG (0.65 ± 0.52%). Since the antibodies to the Gal receptor immunoprecipitate the radioactive NHS/ASOR even in the absence of Ca^{2+}, we conclude

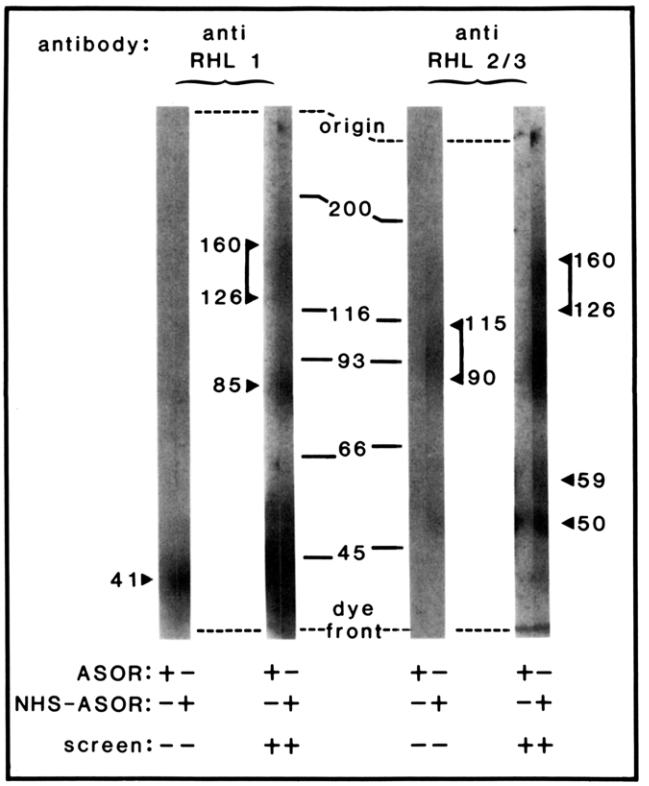


FIGURE 8: Western blot analyses of RHL subunit–ASOR complexes using antisera specific for RHL 1 or for RHL 2/3. Cells were incubated for 60 min at 4 °C with ASOR or NHS-ASOR synthesized at a reactant ratio of 500:1. Both samples were washed with EGTA and then extracted with 1% Triton X-100 in BIE 5. The extracts were immunoprecipitated with rabbit anti-orosomucoid–Sepharose and then underwent Western blot analysis as described under Materials and Methods. Rabbit anti-RHL 1 or anti-RHL 2/3 antiserum was used as the primary reagent, and ^{125}I-protein A was the detecting agent. Autoradiography was for 10 days either with or without a phosphor screen and preflashed film to give two different exposures. Masses (in kDa) for bands discussed in the text are indicated by arrowheads, and molecular masses (kDa) of coelectrophoresed standards are depicted in the middle of the figure.

that covalent cross-links must have formed between Gal receptors subunits and the NHS/ASOR derivatives.

NHS-ASOR Cross-Linking to the Three Gal Receptor Polypeptides. Gal receptor antibody immunoprecipitated three major cross-link products as seen in SDS–PAGE analysis of cell extracts. Western blot analyses were done with antisera specific for RHL 1 or RHL 2/3 (a generous gift of Dr. K. Drickamer) to confirm that these three products represent NHS-ASOR cross-linked to each Gal receptor subunit (Figure 8). Hepatocytes containing surface-bound ASOR or NHS-ASOR were washed with EGTA, extracted, and immunoprecipitated with anti-orosomucoid–Sepharose. Only ligand remaining after EGTA treatment could thus be immunoprecipitated, and only material cross-linked to the ligand or in complex with the cross-linked products should be coprecipitated. As expected, the ASOR control samples were negative; apparent development seen at higher exposures was due solely to spillover from adjacent lanes (Figure 8). However, in the NHS-ASOR sample three characteristics were observed. First, antiserum specific for RHL 1 or for RHL 2/3 detected bands of ~85 kDa and of ~90–115 kDa, respectively. This verified that NHS-ASOR indeed cross-linked to RHL 1 and to RHL 2/3 and not just to proteins of similar size. Multimeric cross-link products in the region of 126–160 kDa were also visualized by both antibodies. The second observation was that native, un-cross-linked subunits (indicated by the arrowheads at 41, 50, and 59 kDa) were coimmunoprecipitated with the cross-linked receptor–ASOR complexes by the anti-orosomucoid–Sepharose. This indicates that native, un-cross-linked subunits exist in a complex with the cross-linked subunits. Finally, the ratio of free, native subunits to cross-linked subunits differed greatly for RHL 1 versus RHL 2/3. By densitometry the ratio of free to cross-linked RHL 1 was ~10:1 whereas the ratio of free to cross-linked RHL 2/3 was only ~0.5:1. We conclude from these results that receptor subunits on the cell surface that are available to cross-link with NHS-ASOR are themselves in complex with other receptor subunits. In these complexes free RHL 1 predominates over free RHL 2/3.

Table III: Ratio of Incorporation of NHS-^{125}I-ASOR into the Three Gal Receptor Subunits[a]

method	Gal receptor subunit		
	RHL 1	RHL 2	RHL 3
densitometry	1.00 ± 0.04	1.15 ± 0.12	0.95 ● 0.09
band excision	1.00	1.23	0.91
gel fractionation	1.00	1.25	1.00
average	1.00	1.21	0.95

[a] Hepatocytes were allowed to bind NHS-^{125}I-ASOR at 4 °C for 60 min, washed with EGTA, extracted, and then subjected to a reduced SDS–PAGE analysis, dried, and autoradiographed. Data from densitometry scans are an average of three separate cell surface binding experiments. In one experiment the gel portions corresponding to the three main autoradiographic bands were excised after densitometric scanning, and ^{125}I radioactivity was determined directly with a γ spectrometer. In this same cell binding experiment after nonreduced SDS–PAGE analysis and autoradiography, another lane from the dried gel was fractionated into 2-mm slices. Radioactivity in each fraction was determined, and the fractions corresponding to the autoradiographic bands were summed. The data are presented as a relative ratio of radiolabeled ASOR incorporation into RHL 1, 2, and 3, with radioactive incorporation into RHL 1 arbitrarily set to 1.00.

Extent of ^{125}I-ASOR Cross-Linking to the Three Gal Receptor Polypeptides. From inspection of autoradiograms such as Figure 7, it appeared that radioactive NHS/ASOR incorporated equally into all three cross-linked complexes. To quantify the ratio of ASOR cross-linked to the RHL 1, 2, and 3 proteins, we did the following: (i) densitometry scans of the developed autoradiograms, (ii) direct quantitation of radioactivity in excised gel bands, and (iii) gel slicing, with determination of radioactivity in each slice and summation of the fractions corresponding to the appropriate molecular weights. Each approach indicated that the NHS-^{125}I-ASOR affinity probe reacted with RHL 1, RHL 2, and RHL 3 subunits on the cell surface in the ratio of about 1.0:1.2:1.0 (Table III). We also found that this affinity probe interacted similarly with intracellular receptors; it gave identical patterns of cross-linking and similar ratios of ligand incorporation.[2]

Discussion

Many investigators have added homo- or heterobifunctional cross-linkers to cells or membranes containing bound ligand to cross-link the ligand and receptor of interest (Pilch & Czech, 1978; Friesel et al., 1986). In these cases, however, molecules over the entire cell surface were cross-linked, and cell viability and function may be compromised. We have developed a chemical affinity label for the Gal receptor as a tool to elucidate its structure and functional itinerary. To our knowledge no one has systematically exploited such chemical cross-linkers to prepare and purify a ligand derivative prior to addition to cells. The major advantage of this approach is the ability to study the processing of covalently linked receptor–ligand

[2] M. C. S. Herzig and P. H. Weigel (unpublished results).

complexes by otherwise unperturbed live cells. The methodology developed here should be applicable in other receptor–ligand systems as well.

We chose to develop a chemical affinity as opposed to a photoaffinity reagent due to the generally low specific cross-linking efficiencies of photoaffinity labels (Das & Fox, 1979; Bayley & Knowles, 1978). The abundance of commercially available bifunctional NHS esters also guided our choice of reagent. ASOR has 12 free amine groups not required for recognition by the Gal receptor and was a suitable candidate for use with these cross-linkers. The chemistry of NHS esters is well-known (Ji, 1979; Cuatrecasas & Parikh, 1972; Lomant & Fairbanks, 1976). These esters react predominantly with primary amino groups to form an amide bond (aminolysis) but can also react to a less extent with the sulfhydryl group of cysteine and the imidazole group of histidine. These products are unstable and hydrolyze under physiological conditions. The entire synthesis and purification is rapid and simple and results in a product that specifically cross-links ASOR to Gal receptors with efficiencies up to 75% of the specifically bound ligand. Cells treated at 4 °C with NHS-ASOR or NHS-S-S-ASOR are capable of internalizing these covalent ASOR–receptor complexes at 37 °C (Herzig & Weigel, 1986).

The high cross-linking efficiencies observed with the various NHS/ASOR derivatives may be a function of the ligand. In addition to 12 amino groups, human ASOR also has 5 complex carbohydrate chains (Schmid et al., 1973) that provide the actual recognition signal for the Gal receptor. It is likely that, no matter which of these carbohydrate chains becomes involved in the receptor–ASOR interaction, there is a nearby NHS ester available for cross-linking to a receptor amino group. This would also explain the increased cross-linking efficiency as the number of NHS esters per ASOR was increased to a maximum of ~8. Baenziger and Fiete (1982) previously developed a photoaffinity reagent that in purified lectin extracts incorporated 2% of the specifically bound radioactivity. Recently Lee and Lee (1986) developed a photoaffinity label with high affinity for purified rabbit and rat Gal/GalNAc receptors that gave unusually high cross-linking efficiencies of up to 30%. This latter reagent, a modified triantennary glycopeptide of asialofetuin, is attached at or very near a galactose binding site, since the photoreactive group was at C_6 on a terminal Gal. Synthesis of this reagent required about 10 days. These investigators were the first to report that all three subunits of the purified rat receptor and both subunits of the purified rabbit receptor are able to bind ligand. Lee and Lee (1987) also reported a differential covalent incorporation of affinity label into each of the three Gal receptor subunits in isolated rat hepatocytes. RHL 2 incorporated 51% of the radiolabel, while RHL 1 and RHL 3 incorporated respectively 22% and 27% of the radiolabel. However, a significant amount of RHL 2 labeling was seen with this photoaffinity reagent even in the absence of Ca^{2+}. With EGTA present, labeling of RHL 2 was about 40% of that seen with Ca^{2+} present. This probe may therefore be less specific than ASOR. In this same study the receptor was iodinated using lactoperoxidase modified with GalNAc so that it would affinity label the Gal receptor. After 40 min of incubation, incorporation of the ^{125}I into receptor was predominantly into RHL 2 and RHL 3 (53% and 41%, respectively). Only after 90 min was radiolabel appreciably incorporated (19%) into RHL 1. From these data the authors concluded that RHL 2 is topologically prominent on the hepatocyte membrane and that RHL 1 is less accessible to aqueous reagents.

Earlier cell surface labeling experiments with lactoperoxidase (Schwartz et al., 1981) also indicated uneven labeling of the three receptor subunits in isolated rat hepatocytes; 90% of the incorporated ^{125}I appeared in RHL 2 and 3. Takahashi et al. (1985) also radioiodinated primary rat hepatocyte cultures by the lactoperoxidase procedure and reported the ratio of ^{125}I incorporation to be 1:2:3 for the RHL 1, 2, and 3 subunits. In contrast, our chemical affinity probe labeled all three subunits of the Gal receptor on intact freshly isolated hepatocytes in approximately equal ratios of 1.0:1.2:1.0 for RHL 1, 2, and 3. RHL 2 was more readily labeled but not to the degree seen with the other procedures. Some of the differences among all these results may be explained by accessibility. In lactoperoxidase labeling, only the tyrosyl residues exposed and accessible to this enzyme can be labeled. Since RHL 2/3 but not RHL 1 have a Tyr at their C-terminal end (Halberg et al., 1987) and this domain is extracellular, it could be the reason that lactoperoxidase preferentially labels these subunits. This result can no longer be solely interpreted to mean there is more RHL 2/3 on the cell surface compared to RHL 1. The difference between our results and those of Lee and Lee (1987) is more difficult to explain and may be a reflection of the organization of the three subunits into native receptor complexes as proposed below. Their affinity reagent is a modified glycopeptide of approximately 3 kDa while ours is a modified ASOR molecule of approximately 41 kDa. Hardy et al. (1985) have shown that the affinity of the receptor for either a triantennary glycopeptide or ASOR is approximately the same but that the number of binding sites differs greatly. The smaller glycopeptide ligand shows approximately three times the number of binding sites. The apparent greater specificity of the NHS-ASOR ligand may also have a role in the differing results.

Recently, Halberg et al. (1987) used 1,5-difluoro-2,4-dinitrobenzene to cross-link the Gal receptor in detergent solution and crude membranes in the absence of Ca^{2+}. Cross-linking detergent-solubilized receptor resulted in the formation of separate polypeptide homooligomers, culminating with a hexamer. Each subunit was capable of binding galactose. Cross-linking in isolated rat liver microsomes resulted in formation of only dimers and trimers of RHL 1 or RHL 2/3. They concluded that RHL 1 and RHL 2/3 are present separately in independent molecular complexes [e.g., $(RHL\ 1)_6$ or $(RHL\ 2/3)_6$]. The chicken and the rat receptors both show an interesting requirement for clusters of sugar residues for cell binding to synthetic culture surfaces (Weigel et al., 1979; Weigel, 1980b) and for optimal binding and endocytosis of ligand by the receptor (Kuhlenschmidt et al., 1984; Lee et al., 1983, 1984; Bezouska et al., 1985). Therefore, some form of multimer arrangement of these hepatic lectin subunits is not unexpected.

Our results do not exclude the possibility that homooligomers of RHL 1 and RHL 2/3 exist, although the equal labeling with NHS-ASOR and the different ratios of free to cross-linked subunits suggest that RHL 1 oligomers would be much larger than RHL 2/3 oligomers. However, in preliminary experiments (Herzig & Weigel, 1989) we observed that, in the absence of ligand, antiserum specific for RHL 1 coprecipitated RHL 2/3 in Triton X-100 extracts of whole cells. The converse was also true. This indicates that heterooligomeric complexes exist. Furthermore, each of these antisera inhibited identically and almost completely ^{125}I-ASOR binding to hepatocytes, a result not predicted by a homooligomeric model for the subunits. As this paper was being submitted, Bischoff et al. (1988) reported results supporting

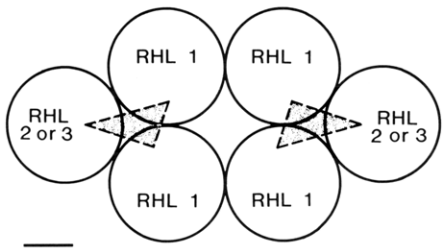


FIGURE 9: Model for the subunit composition and stoichiometry of the rat hepatic gal receptor. The C-terminal 150 amino acid sugar binding regions of the RHL 1, 2, and 3 subunits (M_r = 18 000) are shown viewed from above the membrane. Assuming a globular shape and $\bar{v}$ = 0.734 cm^3/g, their diameters were calculated to be 35 nm with the formula $r = (3\ M\bar{v}/4\pi N)^{1/3}$. The shaded triangles represent the geometry of the proposed trimeric ligand binding domain where each vertex is a single Gal binding site (Lee et al., 1984). The bar represents 15 Å. The RHL 2 and/or RHL 3 subunits in each heterohexamer are not coplanar with the four RHL 1 subunits and are further removed from the membrane bilayer. Three receptors isotypes are possible as discussed in the text.

a heterooligomeric model. The human hepatic Gal receptor also is the product of two genes and consists of two polypeptides H1 and H2 (Speiss & Lodish, 1985). The smaller polypeptide H1 is more abundant than H2. Using chemical cross-linking and a cross-immunoprecipitation approach with specific antisera, these investigators found that H1 and H2 polypeptides associate to form an oligomeric complex in HepG2 cells.

Understanding the arrangement of the native rat Gal receptor is complicated by the unknown number of subunits per native receptor, the stoichiometry of the three RHL subunits in a native receptor, and the existence of two functionally distinct receptor populations (Weigel et al., 1986; Weigel, 1987). We propose the following model for the structure of the rat Gal receptor (Figure 9) on the basis of our data and the current literature. We suggest that there are discrete types of Gal receptors present in the native state as heterohexamers composed of four RHL 1 subunits and two subunits of RHL 2 and/or RHL 3. Each subunit would contribute a binding site for a single sugar residue. Each hexamer would have two potential binding domains for tri- or tetraantennary oligosaccharide ligands. Each ligand binding domain would be a heterotrimer composed of two RHL 1 subunits and one RHL 2 or RHL 3 subunit. These domains are arranged at the vertices of a right triangle of sides 15, 22, and 25 Å as proposed by Lee et al. (1984).

This model reconciles many experimental observations. (i) The structurally different Gal receptor populations (RHL 1)$_4$(RHL 2)$_2$, (RHL 1)$_4$(RHL 3)$_2$, and (RHL 1)$_4$(RHL 2)(RHL 3) may explain the two functionally different pathways for ligand dissociation and degradation (Weigel, 1987). (ii) The reported mass of the native receptor is 264 ± 16 kDa (Anderson et al., 1982). The mass of the three proposed heterohexamers would be about 268, 279, and 288 kDa. Each of the three Gal receptor subunits is able to bind galactose (Lee & Lee, 1986; Halberg et al., 1987), and the ASOR binding domain of the native receptor is 104 kDa (Steer et al., 1981). Since no single subunit approaches this molecular mass, several subunits must participate in ASOR binding. We propose that an ASOR binding domain consists of the C-terminal domains from three subunits. If the cytoplasmic and/or transmembrane domains of each subunit can be irradiated without altering ASOR binding ability, then the size of the remaining trimer ($M_r \simeq$ 110K) is in close agreement with the results of Steer et al. (1981). (iii) The two complete trimeric sugar binding sites can be simultaneously occupied by small glycopeptide ligands, but large ligands bound to one domain sterically preclude binding to the adjacent one (Hardy et al., 1985). (iv) In the presence of some nonionic detergents there is a doubling of the valency of the affinity-purified receptor even when immobilized on Sepharose (Ray et al., 1986). Presumably, the hexamer is distorted by the detergent to allow the second binding domain to also bind ligand. (v) The mole ratio of RHL 1, 2, and 3 in affinity-purified receptor preparations will be 4:1:1. (vi) Expression of fully active receptor (i.e., two complete ligand binding sites, each built from three subunits) requires the genes for both RHL 1 and RHL 2/3 (McPhaul & Berg, 1986). (vii) Immunoprecipitation of Gal receptor from cell extracts with antiserum specific for either RHL 1 or RHL 2/3 coprecipitates the other subunit(s). (viii) chemical or photoaffinity labeling of native receptors labels all three subunits but with different efficiencies depending on the size of the ligand; RHL 2 and 3 are more exposed above the membrane and are more accessible than RHL 1 for radioiodination by lactoperoxidase. (ix) This same model also applies to other mammalian Gal receptor systems. The rabbit Gal receptor consists of two polypeptides (Hudgin et al., 1974; Kawasaki & Ashwell, 1976), and the human Gal receptor has two subunits that are the products of different genes (Speiss & Lodish, 1985).

The proposed model does not agree with the homooligomer organization suggested by Halberg et al. (1987), although their data are quite compelling. Their model on the other hand is difficult to reconcile with the results of McPhaul and Berg (1986), the findings presented here, and the studies by Lee and Lee (1987) that RHL 1 is not preferentially labeled compared to RHL 2 and RHL 3. Since homooligomers of RHL 1 should be about four times as abundant as those of RHL 2 or RHL 3, then RHL 1 should be preferentially labeled in total cell membranes or detergent extracts. Perhaps the discrepancy is due to the different natures of the cross-linkers used. In the studies by Lee and Lee (1987) and those presented here, modified ligands were used to cross-link to receptor under ligand binding conditions. Halberg et al. (1987) used a bifunctional cross-linking reagent in the absence of ligand or Ca^{2+}. Interpretation of the cross-linking studies may also be complicated by reorganization of receptor subunits after detergent extraction as evidenced by different cross-linking products in membranes versus extracts (Halberg et al., 1987). Also, in our model the RHL 2/3 subunit extends above the plane of the RHL 1 subunits. The very short cross-linking agent 1,5-difluoro-2,4-dinitrobenzene (3 Å) might only be able to cross-link the homooligomeric RHL 1 species unlike the longer cross-linkers (11.4–16 Å) used here. Deciding between these two models will require further investigation.

Added in Proof

After submission of our manuscript, Sawyer et al. (1988) reported results that corroborate our conclusion that the three RHL subunits can exist as a heterooligomer on the cell surface. They were able to immunoprecipitate both RHL 1 and RHL 2/3 from lactoperoxidase-labeled primary hepatocyte culture with antibody directed against unique peptide sequences of either the RHL 1 or the RHL 2/3 subunits.

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References

Anderson, T. T., Freytag, J. W., & Hill, R. L. (1982) *J. Biol. Chem. 257*, 8036–8041.

Ashwell, G., & Harford, J. (1982) *Annu. Rev. Biochem. 51*, 531–554.
Baenziger, J. U., & Fiete, D. (1982) *J. Biol. Chem. 257*, 4421–4425.
Bayley, H., & Knowles, J. R. (1978) *Biochemistry 17*, 2414–2419.
Bezouska, K., Taborsky, O., Kubrycht, J., Pospisil, M., & Kocourek, J. (1985) *Biochem. J. 227*, 345–354.
Bischoff, J., Libresco, S., Shia, M. A., & Lodish, H. F. (1988) *J. Cell Biol. 106*, 1067–1074.
Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Breitfeld, P. P., Simmons, C. F., Strous, G. J. A. M., Geuze, H. J., & Schwartz, A. L. (1985) *Int. Rev. Cytol. 97*, 47–95.
Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.
Clarke, B. L., Oka, J. A., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 17384–17392.
Cuatrecasas, P., & Parikh, I. (1972) *Biochemistry 11*, 2291–2299.
Das, M., & Fox, C. F. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 165–193.
Drickamer, K. (1987) *Kidney Int. 32*, S167–S180.
Drickamer, K., Mamon, J. F., Binns, G., & Leung, J. O. (1984) *J. Biol. Chem. 259*, 770–778.
Friesel, R., Burgess, W. H., Mehlman, T., & Maciag, T. (1986) *J. Biol. Chem. 261*, 7581–7584.
Halberg, D. F., Wager, R. E., Farrell, D. C., Hildreth, J., IV, Quesenberry, M. S., Loeb, J. A., Holland, E. C., & Drickamer, K. (1987) *J. Biol. Chem. 262*, 9828–9838.
Hardy, M. R., Townsend, R. R., Parkhurst, S. M., & Lee, Y. C. (1985) *Biochemistry 24* 22–28.
Herzig, M. C. S., & Weigel, P. H. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 45*, 1901.
Herzig, M. C. S., & Weigel, P. H. (1989) *J. Cell Biol.* (in press), Abstract.
Holland, E. C., Leung, J., & Drickamer, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7338–7342.
Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., & Morell, A. G. (1974) *J. Biol. Chem. 249*, 5536–5543.
Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.
Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.
Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., Roseman, S., & Lee, Y. C. (1984) *Biochemistry 23*, 6437–6444.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, R. T., & Lee, Y. C. (1986) *Biochemistry 25*, 6835–6841.
Lee, R. T., & Lee, Y. C. (1987) *Biochemistry 26*, 6320–6329.
Lee, R. T., Lin, P., & Lee, Y. C. (1984) *Biochemistry 23*, 4255–4261.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnarp, J., Haraldsson, M., & Lonn, H. (1983) *J. Biol. Chem. 258*, 199–202.
Leung, J. O., Holland, E. C., & Drickamer, K. (1985) *J. Biol. Chem. 260*, 12523–12527.
Lomant, A. J., & Fairbanks, G. (1976) *J. Mol. Biol. 104*, 243–261.
Lowry, O. H., Roseborough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
McAbee, D. D., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 1942–1945.
McAbee, D. D., & Weigel, P. H. (1988) *Biochemistry 27*, 2061–2069.
McPhaul, M., & Berg, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8863–8867.
Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3383.
Oka, J. A., & Weigel, P. H. (1983) *J. Biol. Chem. 258*, 10253–10262.
Oka, J. A., & Weigel, P. H. (1987) *J. Cell. Phys. 133*, 243–252.
Ray, D. A., Oka, J. A., & Weigel, P. H. (1986) *Biochemistry 25*, 6097–6103.
Sawyer, Sanford, & Doyle (1988) *J. Biol. Chem. 263*, 10534–10538.
Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M., & Nanno, S. (1973) *Biochemistry 12*, 2711–2724.
Schwartz, A. L. (1984) *Crit. Rev. Biochem. 16*, 207–233.
Schwartz, A. L., Marshak-Rothstein, A., Rup, D., & Lodish, H. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3348–3352.
Seglen, P. O. (1973) *Exp. Cell Res. 82*, 391–398.
Spiess, M., & Lodish, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6465–6469.
Steer, C. J., Kempner, E. S., & Ashwell, G. (1981) *J. Biol. Chem. 256*, 5851–5856.
Stockert, R. J. (1983) in *Receptors and Recognition Series B* (Cuatrecasas, P., & Roth, T., Eds.) Vol. 15, pp 169–185, Chapman and Hall, London.
Takahashi, T., Nakada, H., Okumura, T., Sawamura, T., & Tashiro, Y. (1985) *Biochem. Biophys. Res. Commun. 126*, 1054–1060.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–872.
Warren, R., & Doyle, D. (1981) *J. Biol. Chem. 256*, 1346–1355.
Weigel, P. H. (1980a) *J. Biol. Chem. 255*, 6111–6120.
Weigel, P. H. (1980b) *J. Cell Biol. 87*, 855–861.
Weigel, P. H. (1987) in *Vertebrate Lectins* (Olden, K., & Parent, B., Eds.) pp 65–91, Van Nostrand-Reinhold, New York.
Weigel, P. H., & Oka, J. A. (1982) *J. Biol. Chem. 257*, 1201–1207.
Weigel, P. H., & Oka, J. A. (1983a) *J. Biol. Chem. 258*, 5089–5094.
Weigel, P. H., & Oka, J. A. (1983b) *J. Biol. Chem. 258*, 5095–5102.
Weigel, P. H., Schnaar, R. L., Kuhlenschmidt, M. S., Schmell, E., Lee, R. T., Lee, Y. C., & Roseman, S. (1979) *J. Biol. Chem. 254*, 10830–10838.
Weigel, P. H., Ray, D. A., & Oka, J. A. (1983) *Anal. Biochem. 133*, 437–449.
Weigel, P. H., Clarke, B. L., & Oka, J. A. (1986) *Biochem. Biophys. Res. Commun. 140*, 43–50.