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Unusual binding sites for horseradish peroxidase on the surface of cultured and isolated mammalian cells

Suppression of binding by certain nucleotides and glycoproteins, and a role for calcium

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Summary. Binding sites for horseradish peroxidase (HRP), with unusual properties, were detected on the surface of cultured and isolated cells after the cells (on cover slips) had been quickly dried, fixed in cold methanol, and post-fixed in a paraformaldehyde solution. The reaction for surface-bound HRP was suppressed by micromolar concentrations of glycoproteins such as invertase, equine luteinizing hormone (eLH) or human chorionic gonadotropin (hCG). The reaction was also suppressed by 20 mM CDP, UDP, GTP, NAD, and ribose 5-phosphate. Two to six times higher concentrations of GMP, fructose 1-phosphate, galactose 6-phosphate, mannose 6-phosphate, fructose 6-phosphate, and glucose 6-phosphate were required to suppress the binding reaction. AMP, ATP, heparin, mannan, and eight non-phosphorylated sugars showed relatively low competing potencies but fucoidin and α -lactalbumin were strong inhibitors. No addition of Ca^{2+} was required for the binding of HRP to the cell surface. However, calcium-depleted, inactive HRP did not compete with the binding of native (calcium-containing) HRP whereas H_2O_2 -inactivated HRP suppressed the binding. GTP, NAD, ribose 5-phosphate, and EGTA accelerated the release of previously-bound HRP from the cell surface whereas glycoproteins (invertase, eLH, and hCG) did not do so. Addition of Ca^{2+} to GTP, NAD, ribose 5-phosphate or to EGTA prevented the accelerated release of HRP from the cell surface. It is suggested that calcium, present either in the surface membrane or in HRP itself, is involved in the binding of HRP to the cell surface and in the inhibition of binding by GTP, NAD, and ribose 5-phosphate. It is also suggested that α -lactalbumin, GTP, UDP, and CDP compete with the binding of HRP to a glycosyltransferase on the cell surface.

Introduction

The occurrence of mannose-specific binding sites for horseradish peroxidase (HRP) in macrophages, fibroblasts, mast cells and endothelial cells of rat tissues has been demonstrated earlier by a cytochemical method (Straus 1981, 1983a, b, c). The binding of HRP to these sites required Ca^{2+} and was suppressed by mannose or mannan, as well as by glycoproteins such as invertase or glycoprotein hormones (Straus 1983a, c). The occurrence in liver sinusoidal cells and alveolar macrophages of mannose-specific receptors which require Ca^{2+} for the binding of glycoproteins

has also been reported by other investigators (Stahl et al. 1978; Schlesinger et al. 1978; Brown et al. 1978; Steer and Clarenburg 1979; Hubbard et al. 1979).

In the present cytochemical study, binding sites for HRP with unusual properties were detected at the surface of cultured and isolated cells. Certain phosphorylated sugars and nucleotides containing such sugars, in relatively low concentrations strongly inhibited or suppressed the binding of HRP as did glycoproteins and fucoidin, but mannose, mannan and eight nonphosphorylated sugars did not do so. Although added calcium was not required for the surface-binding of HRP, calcium in the surface membrane, or in HRP itself, seemed to be involved. Portions of this work have been presented at a recent meeting (Straus and Keller 1986).

Materials and methods

Reagents: The following reagents were purchased from the Sigma Chemical Company, St. Louis, MO: Horseradish peroxidase, types VI and VIII; guanosine 5'-triphosphate (GTP), lithium salt, type VII; cytidine 5'-diphosphate (CDP), sodium salt, grade III; uridine 5'-diphosphate (UDP), sodium salt, from yeast; guanosine 5'-monophosphate (GMP), disodium salt; adenosine 5'-triphosphate (ATP), disodium salt; adenosine 5'-monophosphate (AMP), sodium salt, type III; β -nicotinamide adenine dinucleotide (NAD), grade IIC; D-ribose 5-phosphate, disodium salt; D-fructose 1-phosphate, sodium salt; D-fructose 6-phosphate, disodium salt, grade I; D-galactose 6-phosphate, disodium salt; D-mannose 6-phosphate, disodium salt; D-glucose 6-phosphate, sodium salt; D-ribose; D-deoxyribose; L-fucose; D-arabinose; D-mannose; D-fructose; D-galactose; mannan; fucoidin; heparin, sodium salt, grade I; equine luteinizing hormone (eLH), from equine pituitaries; invertase, grade X; ribonuclease B (RNase B) from bovine pancreas; α -lactalbumin, grade II; diaminobenzidine tetrahydrochloride, grade II; and ethyleneglycol-bis(β -aminoethyl ether) N,N'-tetraacetic acid (EGTA).

Highly purified human chorionic gonadotropin (hCG) and asialo-gonadotropin (asialo-hCG) were obtained from the Center of Population Research of the National Institute of Child Health and Human Development of the NIH, through the courtesy of Dr. Robert Canfield, Columbia University. Highly purified equine luteinizing hormone was kindly given by Dr. H.D. Papkoff, University of California, San Francisco, California.

Cell lines. The following cells were obtained from the American Type Culture Collection, Bethesda, MD: Swiss mouse 3T3 cells (embryonic fibroblasts; CCL 92); L cells (transformed mouse connective tissue cells; CCL 1, NCTC clone 929); GH₃ cells (rat pituitary tumor cells; CCL 82.1); HeLa S₃ cells (human cervix tumor cells; CCL 2.2). WI-38 cells (human lung fibroblasts; CCL 75)

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and CHO-K1 cells (Chinese hamster ovary cells; CCL 61) were kindly provided by Dr. Nancy Owen from this department. HSWP cells (human skin fibroblasts), originally obtained from Dr. J.D. Regan, Oak Ridge National Laboratory, and Rat 1 cells (dermal fibroblasts), originally obtained from Dr. M. Villereal, Univ. of Chicago, were also provided by Dr. Nancy Owen. Mouse myeloma cells (line P3 $\times$ 63—Ag8653, GM 3570) and human skin fibroblasts (GM 0337) were obtained from the Institute for Medical Research, Camden, NJ, the latter cells kindly being provided by Dr. S. Detke from this department. Virus-transformed Swiss mouse 3T3 cells (SV40-3T3-101) were originally obtained from Dr. H. Green, Harvard University (see Underhill and Keller 1975), and SV40-1-3T3 cells were described by Underhill and Keller (1977).

All cells, except the myeloma cells, were grown for 1–2 days on cover slips in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. The myeloma cells were grown for 1–2 days in suspension in the same medium (Stamatoglou and Keller 1983).

Cytochemical detection of binding sites for HRP on the surface of cultured or isolated cells. Sub-confluent cover slip cultures were washed in large volumes of phosphate-buffered saline (PBS) (0.15 M NaCl and 0.002 M phosphate buffer, pH 7.4). Most of the PBS was drained into filter paper, and the cells were then quickly dried in a desiccator over CaCl_2 and NaOH under reduced pressure (water pump). The dried cells were fixed for 45 min in ice-cooled methanol. They were then stepwise rehydrated and transferred to a modified Hank's balanced salt solution (HBSS) containing 136 mM NaCl, 5.3 mM KCl, 4.8 mM NaHCO_3 , 0.34 mM Na_2HPO_4 and 0.44 mM KH_2PO_4 . (Glucose was omitted because it may compete with the binding of HRP.) The cells were post-fixed in a solution of 1.5% formaldehyde (prepared from paraformaldehyde) in HBSS, for 90 min at room temperature. After thorough washing in HBSS, the cells (on cover slips) were incubated for 1 h at 37°C in a solution containing 20 $\mu\text{g}/\text{ml}$ HRP (Sigma, type VI) in 25 mM barbital buffer, pH 8.0–8.5 or in 10 mM 2-amino-2-methyl-1-propanol buffer, pH 8.5 (total volume 10.0 ml). When strongly acidic inhibitors (see below) were added to the incubation medium with HRP, the pH was further adjusted to 8.0–8.5 with NaOH or with 2-amino-2-methyl-1-propanol. After the incubation with HRP, the cells were washed several times with Tris-HCl buffered saline, pH 7.4, and were stained for peroxidase activity with 0.02% diaminobenzidine tetrahydrochloride (Graham and Karnovsky 1966), H_2O_2 (0.01%), and 0.01 M imidazole (neutralized), in 0.1 M Tris-HCl buffer, pH 7.4, for 10 min at room temperature (Straus 1982). Micro-modifications of the method were described previously (Straus 1983b, c) in which the volume of the incubation medium was decreased to 2.0 or 0.1 ml. These methods were applied when limited quantities of competitors were available (only 0.1 mg of highly purified eLH, for example). After washing in Tris-HCl buffered saline, the cover glasses with the cells were dehydrated through graded ethanol and xylene and embedded in Permount. For photomicrography, the nuclei were counterstained with 0.1% methyl green in physiological saline, and the cells were washed in physiological saline. Cultured cells grown in suspension were sedimented by low speed centrifugation and washed with PBS. The sediment containing the cells in a few drops of PBS was dispersed with the aid of a glass rod on cover slips and was dried and processed as mentioned above.

Suspensions of rat liver parenchymal cells, isolated by the method of Berry and Friend (1969) with modifications as described (Jurin and McCune 1985), were kindly given by Dr. Sylvia McCune from this department. Partially purified suspensions of lymphocytes were prepared by macerating, with the aid of a glass rod, popliteal lymph nodes from rats through a fine-meshed tissue capsule into PBS. The suspensions of liver cells and lymphocytes were sedimented by low speed centrifugation and the sediments were spread on cover slips and dried. Further processing was as mentioned above except that the isolated cells were incubated with 24 or 28 $\mu\text{g}/\text{ml}$ HRP for 1 h at 37°C, instead of with 20 $\mu\text{g}/\text{ml}$ as was used for the cultured cells.

Inhibition test with carbohydrate-containing antagonists. The procedure was the same as used previously (Straus 1983a, c). In brief, the cells were incubated with HRP in the presence of increasing amounts of the antagonists and were then stained for peroxidase activity. The gradual decrease and the final disappearance of the reaction for surface-bound HRP were observed by light microscopy (oil immersion). The concentration of the antagonist that abolished the surface reaction was taken as an approximate, inverse measure of the competing potency. The test was considered conclusive only if the cells, incubated in the absence of competitors, showed a strong surface reaction and a relatively weak background staining for HRP, and if all cells (on different cover glasses) had been processed under the same, standardized conditions.

To be conclusive, the competition test also requires that the activity of HRP does not decrease significantly during incubation of the cells for 1 h at 37°C since the intensity of the surface reaction is influenced by the concentration of HRP in the incubation medium (see below). It was found, unexpectedly, that commercial preparations of ribose 5-phosphate, and to a lesser extent, fructose 6-phosphate and ribose, caused a decrease in the activity of HRP. The decrease of HRP activity occurred in phosphate, cacodylate and modified HBSS buffer solutions but did not occur in barbital or aminopropanol buffer solutions. The addition of 100 mM EDTA or EGTA to HRP in phosphate, cacodylate or HBSS buffer solutions prevented the decrease of HRP activity thus suggesting that traces of heavy metals in the sugar preparations were responsible for these effects. In subsequent experiments, barbital or aminopropanol buffers were used exclusively for the competition experiments, and the constancy of the HRP activity was verified by spectrophotometry (Steinman and Cohn 1972).

Release of HRP from the binding sites. The method is similar to that used previously with liver sinusoidal cells (Straus 1983b). In brief, the cells were processed and incubated with HRP (20 $\mu\text{g}/\text{ml}$) for 1 h at 37°C as described above. After thorough washing in Tris-HCl buffer, pH 7.4, the cells were held for 2 h at 37°C in a barbital buffer-physiological saline mixture (1:1), pH 8.0, containing various inhibitors or EGTA, with or without added CaCl_2 , and were then stained for peroxidase activity. The intensity of the peroxidase reaction on the cell surface was compared on cells incubated with the inhibitors (or EGTA), and on cells incubated with buffer solution alone, with and without added Ca^{2+} . They were also compared with cells stained for peroxidase activity immediately after the incubation with HRP, without post-incubation.

Inactivation of HRP by H_2O_2 . Ten mg HRP, Sigma type VI, were dissolved in 45 ml distilled water, and 5 ml 3% H_2O_2 were added. After 24 h at room temperature, the solution was dialyzed repeatedly against 4 l distilled water at 4°C. NaCl was added to the dialysate to 0.9% concentration. The original peroxidase activity of 18,700 units/mg protein had decreased to 1.6 units/mg protein, after the treatment with H_2O_2 .

Depletion of calcium from HRP. Five to 10 mg HRP, Sigma type VI, were dissolved in 10–20 ml distilled water. A few drops of 0.1 N NaOH were added to bring the pH to 10.2. EGTA was added to make the concentration 1 mM, and the pH was readjusted to 10.2. After 18 h at room temperature, the solution was dialyzed several times in the cold room against 4 l of water which had been made slightly alkaline (pH 8–9) with barbital buffer. After the treatment with EGTA at pH 10.2, the peroxidase activity had decreased to 10%–20% of the original activity. No decrease of peroxidase activity was observed when HRP was held at pH 10.2 without EGTA, or when HRP was treated with EGTA at pH 7–8.

Results

Elaboration of the method

A systematic study was made in order to find conditions that resulted in a strong binding of HRP to the cell surface

and only a slight "background" adsorption of HRP. Based on these studies, conditions were selected for an optimal procedure (see Methods). Some of these observations are described in more detail.

Importance of quick drying and initial treatment with methanol. The initial treatment of dried cells with cold methanol was essential to attain a strong binding of HRP to the cell surface and to prevent a strong background adsorption of HRP. When PBS-washed cells were fixed for 90 min at 22° C in a 1.5% paraformaldehyde solution followed or preceded by the treatment with methanol *without having been first dried*, the reaction for surface-bound HRP was relatively weak and a strong background adsorption of HRP was observed to the cytoplasm and the nucleus. A similar staining pattern was observed when the cells, either dried or not dried, were fixed in the formaldehyde solution (with or without post-fixation with methanol) without having been treated with cold methanol *prior to the formaldehyde fixation*. An initial treatment of dried cells with cold ethanol or acetone resulted in stronger background adsorption of HRP than the initial treatment with cold methanol.

The speed of drying also influenced the preservation of the binding sites at the cell surface. When the cells were air-dried in a humid atmosphere or when the PBS had not been drained sufficiently from the cover slips, many cells showed a partial diffusion of the reaction product for HRP from the cell surface to the cytoplasm. For these reasons, the cells were quickly dried in a desiccator as indicated above.

Effects of the pH on the surface binding of HRP. Cultured cells, fixed as indicated above, were incubated with HRP (20 µg/ml) for 1 h at 37° C in 5 mM phosphate buffer, the pH varying between 6.2 and 7.6; in 5 mM barbital buffer, the pH varying between 7.5 and 8.5; or in 10 mM aminopropanol buffer, the pH varying between 8.5 and 10.0. The reaction for HRP at the cell surface was most intense at pH values between 7.5 and 9.0. For reasons mentioned above, barbital or aminopropanol buffers, pH 8.0–8.5, were used for the standard procedure.

Effects of the concentration of HRP and the temperature of incubation. The incubation of the cells with 20 µg/ml (0.5 µM) HRP, Sigma type VI (peroxidase activity approx. 400 units/ml) for 1 h at 37° C was selected because it resulted in a relatively strong and quick surface reaction and a relatively weak background staining for HRP. When the cells were incubated with 16 or 12 µg/ml HRP for 1 h at 37° C, the intensity of the surface reaction as well as the slight background staining were decreased as compared with the staining intensity observed with 20 µg/ml HRP in the incubation medium. On the other hand, the intensities of both the surface reaction and the background reaction increased when the concentration of HRP in the incubation medium was increased from 20 to 30–40 µg/ml. It was estimated from microscopic observations that the binding sites for HRP (in the methanol and formaldehyde-fixed cells) became saturated after 1 h incubation at 37° C when the incubation medium contained 20–30 µg/ml HRP.

The surface binding of HRP was stronger after 18–24 h incubation of the cells with 20 µg/ml HRP at 4° C than after 1 h incubation at 37° C. It was estimated from microscopic observations that the binding sites became saturated

after 18–24 h incubation at 4° C when the incubation medium contained 10–15 µg/ml HRP.

Since commercial preparations of HRP differ in purity and isoenzyme composition and since different cells probably contain different concentrations of the binding protein on the cell surface (see below), it is recommended to determine in each case the concentration of a particular HRP preparation that gives a strong surface reaction and a relatively weak background reaction.

Properties of the binding sites

Effects of trypsin, added Ca^{2+} and EGTA on the surface binding of HRP. The surface reaction was abolished when cultured cells had been treated with 2.5 µg/ml trypsin for 30 min at 22° C before incubating them with HRP.

The binding of HRP to the cell surface did not require the addition of Ca^{2+} to the incubation medium, and the addition of 1–2 mM Ca^{2+} did not increase the intensity of the surface reaction. However, the addition of 2.0–5.0 mM EGTA inhibited the binding of HRP to the cell surface, and the addition of an excess of Ca^{2+} together with EGTA reversed the inhibition (see also the effect of EGTA, and EGTA + CaCl_2 on the release of HRP, below).

Competition of H_2O_2 -inactivated HRP with the binding of native HRP to the cell surface. HeLa cells were incubated for 1 h at 37° C with 20 µg/ml HRP in the presence of increasing concentrations of H_2O_2 -inactivated HRP. The reaction for surface-bound HRP gradually decreased and was suppressed when 20–30 µg/ml H_2O_2 -inactivated HRP had been added. Thus, H_2O_2 -inactivated HRP was bound to the same sites as native HRP, and the binding sites were saturated at the same concentrations of H_2O_2 -inactivated HRP, approximately, as of native HRP.

Lack of competition of calcium-depleted HRP with the binding of native HRP to the cell surface. The treatment of HRP with EGTA at pH 10.2 resulted in a loss of 80%–90% of the peroxidase activity (see Methods). This change was probably correlated with a loss of calcium from HRP (Haschke and Friedhoff 1978; Ogawa et al. 1979). The effects of calcium-depleted HRP on the binding of native HRP were tested in two ways. In one type of experiment, an aliquot of EGTA-treated HRP containing 300 µg protein was added to 10 ml of the incubation medium containing the standard concentration of native HRP (200 µg/10 ml). In spite of the excess of calcium-depleted HRP in the incubation medium, the reaction for HRP on the surface of HeLa cells was not suppressed suggesting that calcium-depleted HRP did not compete with the binding of native HRP. It should be noted that the residual peroxidase activity of the EGTA-treated preparation that was added in this experiment to the incubation medium was approximately 10% of the original activity or 62 units/ml. This amount of peroxidase was too low to give a reaction at the cell surface when tested alone. In another experiment, HeLa cells were incubated with a relatively large amount of EGTA-treated HRP (1.0 mg/10 ml). The residual peroxidase activity added with this preparation was 20% of the original activity or 400 units/ml, close to that used for the standard procedure. In spite of a four-fold excess of calcium-depleted, inactive HRP in the incubation medium, the residual, native HRP was bound, in part, to the surface

of HeLa cells thus showing again that the calcium-depleted HRP did not compete significantly with the binding of the native HRP.

Release of HRP from the binding sites. After HRP had been bound to the surface of HeLa cells by the usual procedure (see Methods), the cells (on cover slips) were incubated for 2 h at 37° C with various inhibitors (see below) or with EGTA, with or without added CaCl₂, and were then stained for peroxidase activity. During incubation in buffer solutions, pH 6–8, alone, little HRP was released from the cell surface over a period of 2 h, as estimated from the intensity of the microscopically observed surface reaction. (The release at a more acid pH could not be tested because HRP lost some of its activity during incubation at pH 4–5.) The addition of 0.5–1.0 mM EGTA to the buffer solution, pH 8.0, as well as the addition of 40 mM NAD, 40 mM ribose 5-phosphate, or 30 mM GTP (see below) greatly accelerated the release of HRP from the cell surface. The accelerated release of HRP was prevented when 5 mM CaCl₂ was added with the inhibitors. Inhibitors which did not contain phosphate groups such as eLH, invertase or RNase B (Table 1) did not accelerate the release of HRP from the cell surface.

Comparative observations on the binding of an acidic and a basic isoenzyme of HRP. HRP, Sigma type VI, which mostly contains basic isoenzymes with a pI close to 10.0 (Pino 1985) may be bound to anionic sites in cells (Bök 1972; Pino 1985). It was of interest, therefore, to compare its binding with that of an acidic isoenzyme, Sigma type VIII, with a pI reported to be 3.6 (Pino 1985).

In parallel experiments, GH₃, HeLa, SV3T3, SV40-1, and L-cells were incubated for 1 h at 37° C with HRP, Sigma types VI and VIII. The enzyme activities of both isoenzymes in the incubation medium, as measured with o-dianisidine in the presence of serum albumin (Straus 1983b), had been made equal and to correspond to the standard concentration. After the incubation with HRP, the binding of the acidic isoenzyme to the cell surfaces was somewhat weaker than that of the basic isoenzyme. However, when the concentration of the acidic isoenzyme was made 40% higher in the incubation medium than the standard concentration, a strong binding to the surfaces of all 5 cell types was also observed with the acidic isoenzyme. The addition of 20 mM ribose 5-phosphate to the incubation medium with HRP (see below) abolished the surface binding of both isoenzymes. These observations suggest that the acidic isoenzyme is bound to the same sites at the cell surface as the basic isoenzyme.

Inhibition of the surface binding of HRP by various carbohydrates, or compounds containing carbohydrates

The competing potencies of various carbohydrates, or compounds containing carbohydrates, in the binding of HRP to the surfaces of GH₃, HeLa, SV3T3, SV40-1, and L cells were estimated by the procedure indicated in Materials and methods. As may be seen from Table 1 and is illustrated, in part, in Figs. 1–10, two glycoprotein hormones (eLH and hCG), as well as GTP, NAD, and ribose 5-phosphate showed relatively high competing potencies. Ribose 5-phosphate (20 mM) also suppressed the binding of HRP to the

Table 1. Approximate concentrations of antagonists that suppress the cytochemical reaction for surface-bound HRP on cultured cells

	GH ₃ Cells (mM)	HeLa Cells (mM)	SV3T3 Cells (mM)	SV40-1 Cells (mM)	L- Cells (mM)
Guanosine 5'-triphosphate	15	15	15	15	15
Uridine 5'-diphosphate	20	20	NT*	20	20
Cytidine 5'-diphosphate	20	20	NT	20	30
Nicotinamide adenine dinucleotide	20	20	20	20	20
D-Ribose 5-phosphate	20	20	20	20	20
Guanosine 5'-monophosphate	40	40	40	40	40
D-Fructose-1-phosphate	40	40	40	40	40
D-Galactose 6-phosphate	50	50	50	50	50
D-Fructose 6-phosphate	70	70	70	70	70
D-Mannose 6-phosphate	120	120	120	120	120
D-Glucose 6-phosphate	120	120	120	120	120
Adenosine 5'-monophosphate	250	200	200	250	250
Adenosine 5'-triphosphate	200	250	150	150	250
D-Ribose	150	150	150	150	NT
Deoxy-D-Ribose	200	200	200	200	NT
L-Fucose	250	250	300	250	300
D-Arabinose	250	250	300	250	300
D-Mannose	300	300	300	300	350
D-Glucose	300	300	250	250	350
D-Fructose	400	400	400	400	NT
D-Galactose	400	400	400	400	400

	GH ₃ Cells (%)	HeLa Cells (%)	SV3T3 Cells (%)	SV40-1 Cells (%)	L- Cells (%)
Fucoidin	0.02	0.02	0.02	0.02	0.02
Mannan	0.35	0.35	0.35	0.35	0.35
Heparin	>1.5	>1.5	>1.5	>1.5	>1.5
Luteinizing Hormone ^a	0.004	0.004	0.003	0.004	0.004
Luteinizing Hormone ^b	0.025	0.025	NT	NT	0.025
Chorionic gonadotropin	0.05	0.05	0.03	0.05	0.06
Asialo-chorionic gonadotropin	0.013	0.025	0.025	0.035	0.035
α-Lactalbumin	0.04	0.04	NT	0.04	0.04
Invertase	0.06	0.06	0.06	0.07	0.07
Ribonuclease B	0.1	0.1	0.07	0.1	NT
Ovalbumin	0.5	0.4	0.4	0.5	NT

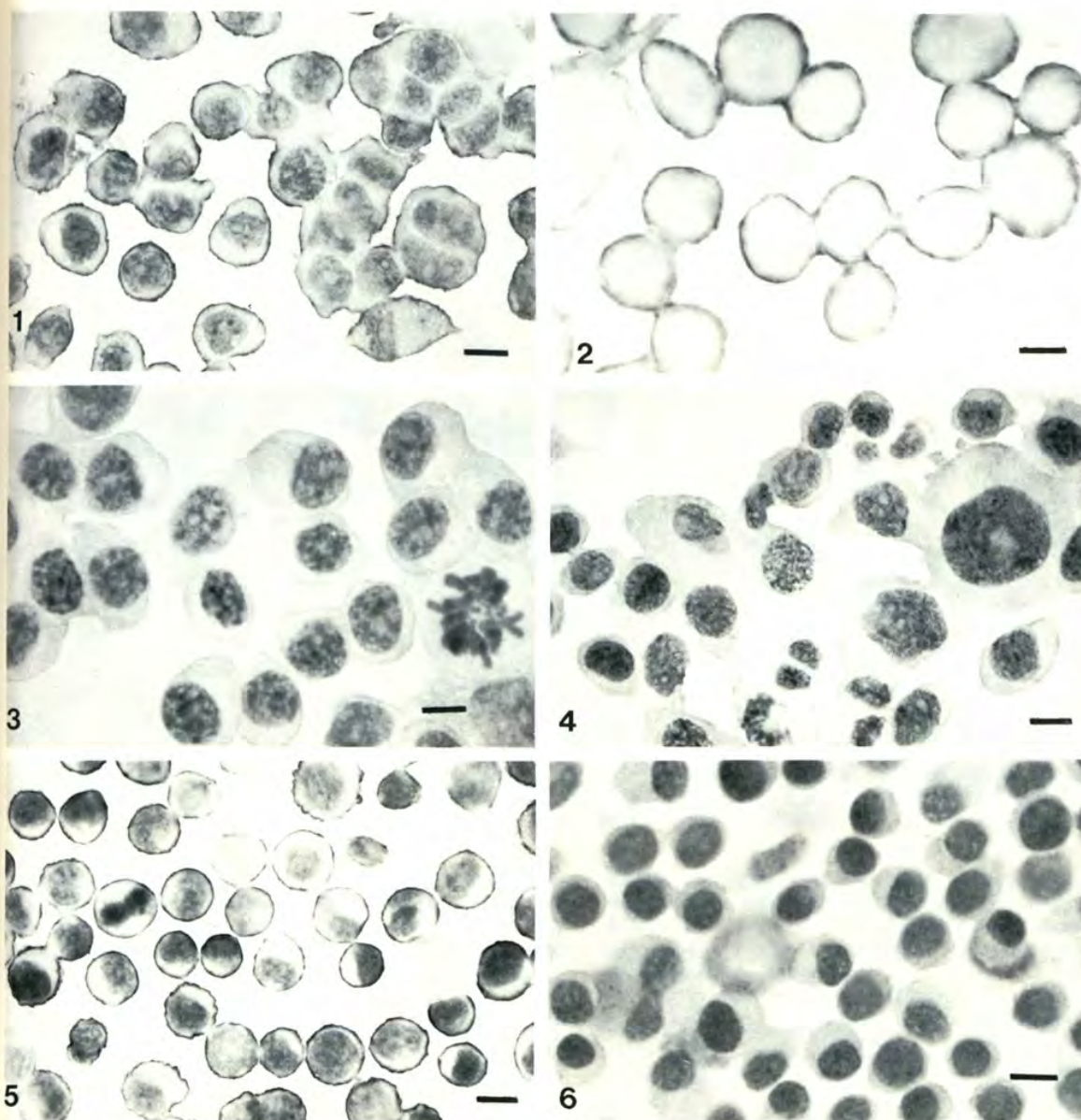
* Not tested

^a Partially purified preparation

^b Highly purified preparation

surface of the cell types mentioned below in the section on comparative observations but not listed in Table 1. Five other phosphorylated sugars and GMP showed intermediate competing potencies whereas ATP, AMP, heparin, and eight non-phosphorylated sugars showed relatively low competing potencies. However, fucoidin was a strong inhibitor. The suppressing concentrations of the inhibitors often have the same values (Table 1) because 3–4 different cell lines were usually incubated in parallel, in solutions containing HRP together with the same serial dilutions of the inhibitors.

After most of the experiments had been completed and



Figs. 1 and 2. Reaction for surface-bound HRP on GH₃ cells. Note in Fig. 1 that the surface reaction is abolished where the surfaces are in contact. **Fig. 3.** The surface reaction in GH₃ cells is suppressed by 10 mM GTP. **Fig. 4.** The surface reaction in GH₃ cells is suppressed by 20 mM ribose 5-phosphate. **Fig. 5.** Reaction for surface-bound HRP in L-cells. **Fig. 6.** The surface reaction in L cells is suppressed by highly purified, 0.06% hCG. The nuclei were counter-stained with methyl green except in Fig. 2

following the very recent report by Runyan et al. (1986), the effects of α -lactalbumin, UDP, and CDP on the binding of HRP were tested. UDP, CDP and α -lactalbumin were found to be relatively strong inhibitors of the reaction for surface-bound HRP (Table 1).

Comparative observations on the reaction for surface-bound HRP in various cell lines

The reaction for surface-bound HRP showed the highest intensity in sub-confluent cultures of the following cell lines: GH₃ cells; HeLa cells; SV40-3T3 cells; SV40-1-3T3 cells; and L cells. The reaction was of intermediate intensity

in the following cell lines: Swiss mouse 3T3 cells; Chinese hamster ovary cells; WI-38 cells; Rat 1 cells; and mouse myeloma cells. The following cell lines showed only a weak surface reaction: HSWP cells and GM 0337 cells.

Independent of the cell type tested, the reaction for surface-bound HRP was much decreased or abolished when cells became confluent. Adjacent cell surfaces were not stained, or were stained only weakly, whereas the "free" surfaces of the same cells were stained strongly (see, for example, Figs. 1 and 7). These effects may be related to the decreased penetration of the reagents to confluent surfaces. Only the reaction on "free" surfaces was used in the comparison of staining intensities.

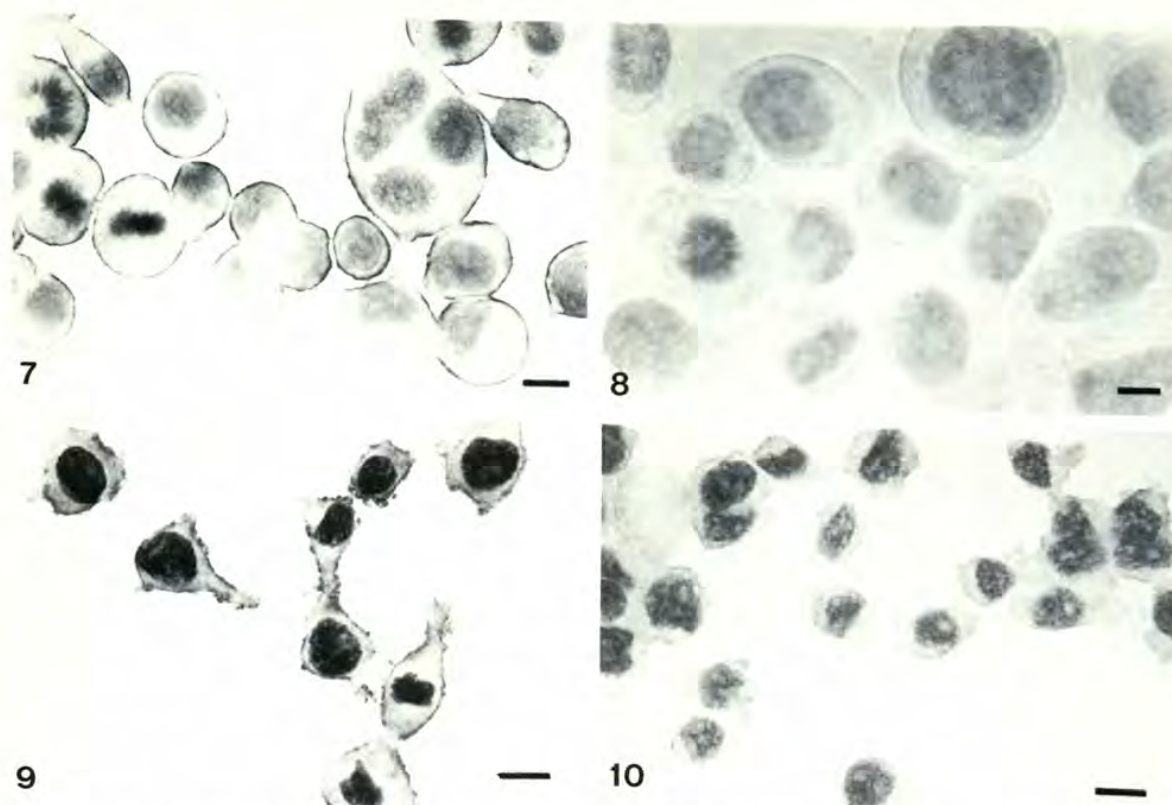


Fig. 7. Reaction for surface-bound HRP in HeLa cells. Note that the surface reaction is not seen where the surfaces are in contact. **Fig. 8.** The surface reaction in HeLa cells is suppressed by partially purified, 0.006% eLH. **Fig. 9.** Reaction for surface-bound HRP in SV3T3-40-1 cells. **Fig. 10.** The reaction for surface-bound HRP in SV3T3-40-1 cell is suppressed by 20 mM NAD

Table 2. Approximate concentrations of antagonists that suppress the cytochemical reaction for surface-bound HRP on isolated rat hepatocytes and lymphocytes

	Hepatocytes	Lymphocytes
Uridine 5'-diphosphate	20 mM	20 mM
Cytidine 5'-diphosphate	20 mM	20 mM
Guanosine 5'-triphosphate	20 mM	20 mM
Nicotinamide adenine dinucleotide	20 mM	20 mM
Guanosine 5'-monophosphate	40 mM	60 mM
Ribose 5-phosphate	50 mM	30 mM
Luteinizing hormone ^a	0.003%	0.003%
α -Lactalbumin	0.04%	0.06%
Fucoidin	0.04%	0.04%

^a Partially purified preparation

Observations on the reaction for surface-bound HRP in isolated rat hepatocytes and lymphocytes

Rat hepatocytes and lymphocytes were studied in less detail than the cultured cell lines listed in Table 1. Nevertheless, as may be seen from Table 2 and from Figs. 11–14, the data were sufficient to conclude that the binding sites for HRP on the surface of hepatocytes and lymphocytes have similar properties as those on the surface of cultured cells (Table 1).

In order to intensify the surface reaction to the level

observed with transformed cells, the lymphocytes and hepatocytes had to be incubated with a higher concentration of HRP than used for the transformed cells (see Methods). As was mentioned above, non-transformed cells seem to contain lower concentrations of the binding protein than transformed cells. Since the reaction for surface-bound HRP on lymphocytes was relatively weak (Fig. 13), the estimation of inhibitory potencies (Table 2) was less accurate for lymphocytes than for the other cells.

Discussion

Before these results are discussed, the special type of fixation used in these experiments has to be pointed out. Isolated cells, or cells grown on cover slips, were quickly dried, treated with cold methanol, and were then post-fixed in a formaldehyde solution. The initial treatment of dried cells with cold methanol was essential to obtain a strong binding of HRP to the plasma membrane and to prevent a significant "background" adsorption of HRP. These observations suggest that the HRP-binding protein may be washed out of the cell surface during conventional types of fixation. Although the fine structure of the cells was not well preserved after the fixation of dried cells with methanol, the preservation was sufficient to study the binding of HRP to the cell surface.

The binding sites for HRP on the surface of cultured and isolated cells detectable by this procedure were found to have quite different specificities toward carbohydrates

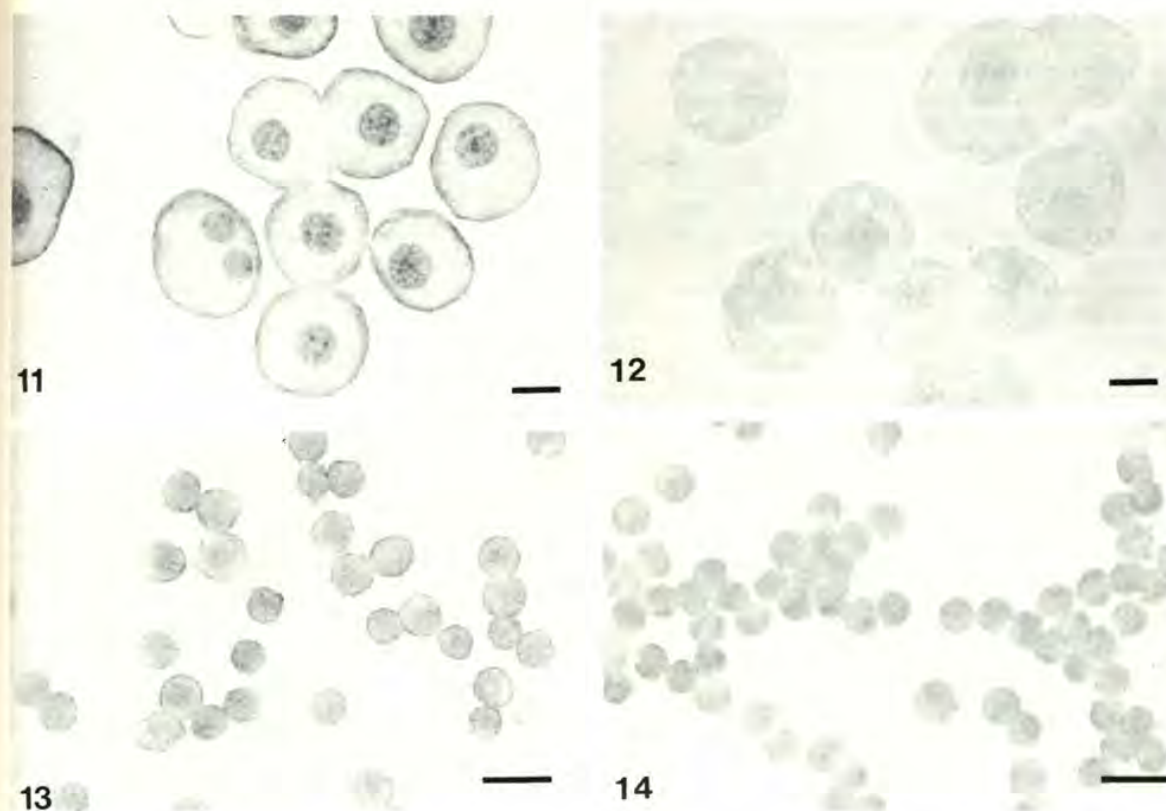


Fig. 11. Reaction for surface-bound HRP in isolated, rat liver parenchymal cells. **Fig. 12.** The surface reaction in rat liver cells is suppressed by 20 mM GTP. **Fig. 13.** Reaction for surface-bound HRP in rat lymphocytes. **Fig. 14.** The surface reaction in rat lymphocytes is suppressed by 0.04% fucoidin. The magnification is $\times 600$ – 700 in Figs. 1–12, and $\times 970$ in Figs. 13 and 14. Bar = 10 μ m

from the binding sites for HRP in rat tissues studied previously (Straus 1981, 1983a, b, c). For example, the binding reaction for HRP in cells of rat tissues was suppressed by relatively low concentrations of mannan, D-mannose, and L-fucose (Straus 1983a), whereas the binding reaction on the surface of cultured cells was not.

As indicated in Table 1, the binding of HRP to the surface of cultured cells was suppressed by 20 mM UDP, CDP, GTP, NAD, and ribose 5-phosphate. Two to six times higher concentrations of GMP and of five phosphorylated sugars were required to suppress the surface reaction. AMP, ATP and eight non-phosphorylated sugars showed relatively low competing potencies. The binding of HRP to the surface of the cultured cells, listed in Table 1, was also suppressed by micromolar concentrations of eLH and hCG. The suppressing concentrations of these glycoprotein hormones were several times lower than those required to suppress the binding of HRP to mannan-specific lectins in cells of rat tissues (Straus 1983c, Tables 1 and 2). The enhanced competing potency of the partially purified, commercial eLH preparation over the highly purified eLH preparations obtained from Dr. Papkoff (Table 1), suggests that a contaminating glycoprotein in the commercial preparation is responsible for the exceptionally high competing potency. The observation that polyvalent ligands (glycoproteins) show much higher affinities than monovalent ligands (sugar phosphates) has also been reported for other glycoprotein receptors (Stahl et al. 1978; Sly and Fischer 1982). As may also be seen from Tables 1 and 2, the binding

of HRP to the surfaces of five cell lines, as well as to the surfaces of rat hepatocytes and lymphocytes, was suppressed by similar concentrations of the same antagonists, suggesting that HRP was bound to the same type of surface protein in these cells.

The reaction for surface-bound HRP appeared to be more intense in transformed or established cell lines such as GH₃, HeLa, SV40-3T3, SV40-3T3-1, and L cells than in non-transformed, fibroblast-like cell lines such as HSWP and GM 0337 cells. More non-transformed cell lines have to be tested before it can be decided whether or not the binding of HRP to the surface of these cells is decreased.

The possibility of artifacts must be considered when applying cytochemical methods. The possibility may exist, for example, that the basic glycoprotein, HRP, Sigma type VI, may be bound to acidic sites at the cell surface. Acidic substances, such as GTP, NAD, and sugar phosphates, may then suppress the binding of the glycoprotein by neutralizing the basic groups in HRP. Such an interpretation is not supported by the observations reported above. These showed that an acidic isoenzyme of HRP was also bound to the cell surface and that its binding was suppressed by ribose 5-phosphate. In addition, several acidic substances such as AMP, ATP, and heparin showed relatively low competing potencies to the binding of HRP thus suggesting that the strongly competing substances, listed in Table 1, were bound to specific sites. Another type of artifact which may occur during cytochemical reactions is that of diffusion. Courtoy et al. (1983), while studying the ultrastructur-

al localization of extracellular matrix antigens by an immunoperoxidase method, observed that the DAB reaction product for HRP diffused over a distance of several hundred nanometers and was then adsorbed to cell membranes. The binding of HRP to the surface of cultured cells cannot be explained by the diffusion of the DAB reaction product from the cytoplasm because the cytoplasm was not stained significantly. However, the DAB reaction product may have diffused from the cytoplasmic to the external surface of the plasma membrane.

The release of previously-bound HRP from the cell surface was greatly accelerated by EGTA, as well as by GTP, ribose 5-phosphate and NAD. The addition of Ca^{2+} to EGTA or to the inhibitors prevented the accelerated release of HRP from the cell surface. Calcium-depleted HRP did not compete with the binding of native HRP to the cell surface whereas H_2O_2 -inactivated HRP did do so. It may be concluded that calcium ions are involved in the binding of HRP as well as in the inhibition of binding by phosphate-containing compounds. It should be noted that the original binding of HRP to the cell surface did not require the addition of Ca^{2+} but that the binding was inhibited by EGTA. HRP itself contains calcium (Haschke and Friedhoff 1978; Ogawa et al. 1979), and this intrinsic calcium, or the calcium in the surface membrane, may be involved in the binding of HRP.

Inhibitors of binding that did not contain phosphate (such as glycoproteins), did not accelerate the release of HRP from the cell surface. Although this observation is not understood, it may be related to studies by Guarnaccia et al. (1982) and Guarnaccia and Schnaar (1982) on surface lectins involved in the adhesion of chicken hepatocytes. The investigators reported that an original weak binding of hepatocytes to an extracellular sugar was "strengthened" and became sugar-resistant during incubation at 37°C . The latter modification was shown to be correlated with the phosphorylation of an extracellular sugar attached to a polyacrylamide membrane (Brandley and Schnaar 1985; see below).

In spite of some similarities, the binding sites for HRP described above seem to be different from the phosphomannosyl receptor for lysosomal enzymes (for review, see Sly and Fischer 1982) and from ligatin (Jakoi et al. 1976, 1981). For example, the binding of ligands to the phosphomannosyl receptor is inhibited more by mannose 6-phosphate than by ribose 5-phosphate (Sando and Neufeld 1977), whereas the inverse competing potencies were found for the surface "receptor" for HRP (Table 1). Fucoidin, an inhibitor of the binding of HRP, did not inhibit the binding of ligands to the phosphomannosyl receptor in alveolar macrophages (Shepherd et al. 1984). The binding of ligands to ligatin is strongly inhibited by mannose 6-phosphate and, in some tissues, by glucose 1-phosphate (Jakoi et al. 1981), whereas these phosphorylated sugars showed relatively low competing potencies to the binding of HRP (data on glucose 1-phosphate not shown).

The inhibition of the binding of HRP to lymphocytes and other cells by fucoidin (a sulfated polymer of fucose) and phosphorylated sugars shows analogies with the inhibition of the adhesion of lymphocytes to the surface of endothelial cells by fucoidin (Stoolman and Rosen 1983; Stoolman et al. 1984). Brandley et al. (1985) reported that rat lymphocytes adhere to fucoidin immobilized on polyacrylamide gels and that the binding is specifically inhibited by

free fucoidin and by certain phosphorylated sugars. The inhibition of binding by fucoidin has been observed only in these cases and in the present experiments (Tables 1 and 2).

Brandley and Schnaar (1985) also found that chicken hepatocytes specifically adhere to and phosphorylate acetylglucosamine immobilized on a polyacrylamide gel. They suggested that enzymes on the cell surface may transfer phosphate to carbohydrates in glycoproteins and that this process may be involved in the modulation of adhesion or endocytosis. Perhaps, the inhibition of the binding of HRP to the cell surface by GTP, NAD, and ribose 5-phosphate is related to the function of these (hypothetical) enzymes.

A similar hypothesis is suggested by a very recent report by Runyan et al. (1986). These investigators observed that the migration of neural crest cells through an extracellular matrix containing glycoproteins was strongly inhibited by α -lactalbumin. This protein is known to bind specifically to galactosyltransferase and to alter its specificity (Schanbacher and Ebner 1970). A role of glycosyltransferases in cell adhesion had first been suggested by Roseman (1970) and supported by experiments of Shur and of other investigators (reviewed by Pierce et al. 1980). Recently, glycosyltransferases were shown, by immunocytochemical methods, to occur on the surface of cultured cells (Shaper et al. 1985) and on the surface of absorptive intestinal cells (Roth et al. 1985). The involvement of glycosyltransferases in adhesion was supported by experiments of Rauvala et al. (1983) according to which skin fibroblasts adhered to fucosyltransferase immobilized on polystyrene plates and the adhesion was inhibited by asialoglycoproteins.

Following the report by Runyan et al. (1986), we tested the effects of α -lactalbumin, UDP and CDP on the binding of HRP to the surfaces of four cultured cell lines and of liver cells, and found these substances to be strongly inhibitory (Tables 1 and 2). The possibility may be considered, therefore, that HRP was bound to a glycosyltransferase on the cell surface, and that UDP, CDP, GTP, NAD, and ribose 5-phosphate inhibited the binding because of their affinities for the same sites.

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Note added in proof: After completion of the experiments, it was observed that relatively high concentrations of added CaCl_2 intensify the reaction for surface-bound HRP. For an intensified reaction, it is recommended that 10-20 mM CaCl_2 be added to the incubation medium with HRP.