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Cytochemical Detection of Mannose-Specific Receptors for Glycoproteins with Horseradish Peroxidase as a Ligand *

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Summary. Horseradish peroxidase (HRP), a glycoprotein rich in mannose groups, was used as a ligand to detect receptors for glycoproteins in formalin-fixed, frozen sections of rat liver. Specific binding of HRP occurred to surface membranes of sinusoidal cells but not to those of parenchymal cells. The binding sites were visualized after the peroxidatic reaction in erythrocytes had been suppressed by methanol-H_2O_2 and phenylhydrazine, the latter reagent also decreasing the nonspecific background adsorption of HRP. Several factors influencing the reaction were studied systematically. The specific binding of HRP to sinusoidal cells was greatly decreased or abolished when tissue blocks were fixed for longer than 1–2 h in a cold 4% formaldehyde solution and the frozen sections subsequently treated for 30 min in cold methanol. The specific binding of HRP increased when the concentration of HRP in the medium was increased from 10 μg/ml to 40 μg/ml, when the time of incubation with HRP was increased from 1 h to 4 h, or when the temperature of incubation with HRP was increased from 4° C to 22° C, or from 22° C to 37° C. The specific binding of HRP also increased when the pH of the incubation medium was increased from 7.0 to 10.0. Little or no specific binding of HRP was observed in the absence of added Ca^{++}. The binding of HRP was suppressed by 10 mM mannose or 0.004% mannan whereas the suppression of the binding reaction by galactose or galactan required 30–40 times higher concentrations.

Introduction

The endocytic uptake of horseradish peroxidase (HRP) by kidney and liver cells has been the subject of previous investigations (Straus 1964a, 1967; Graham and Karnovsky 1966). In previous work, the question has not been clarified as to whether the uptake of HRP occurs by fluid phase or by adsorptive (receptor-mediated) endocytosis. In order to study this question a cytochemical method was developed for the visualization in formalin-fixed, frozen sections of sites

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where HRP is bound to receptors specific for the mannose groups of glycoproteins. Optimal conditions for the specific binding of HRP were determined on the basis of the experiments described below. Most of these observations were made on the sinusoidal cells (Kupffer cells and endothelial cells) of the liver which, from previous work (Straus 1964a, 1967), are known to take up much more HRP in vivo than the parenchymal cells.

HRP is also bound nonspecifically to fixed tissue sections (Straus 1964b). It was therefore important to distinguish between specific and nonspecific adsorption of HRP, and to prevent the nonspecific adsorption of HRP.

Materials and Methods

Sprague-Dawley rats, 200–300 g in weight, were used. HRP, type VI, mannan, mannose and galactose were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA. Galactan was obtained from P-L Biochemicals, Milwaukee, Wisconsin, USA.

Recommended Procedure for the Specific Binding of HRP. The following procedure was adopted finally as optimal on the basis of the experiments described below.

Blocks of tissue, 2–3 mm thick, were fixed for 30–60 min at 4° C in a solution of 4% formaldehyde, also containing 7.5% sucrose, 0.02 M cacodylate buffer, pH 7.5, and 0.1% $CaCl_2$. The formaldehyde solution was freshly prepared from paraformaldehyde. The tissue was washed for 6–8 h in 100 ml cacodylate-buffered 15% sucrose solution and for 12–15 h in 100 ml cacodylate-buffered 30% sucrose solution at 4° C. The tissue was quenched in a dry ice-acetone mixture, and sections were cut at 4 µm in a cryostat. The sections, attached to an albuminized cover glass, were briefly melted by warming the cover glass with the finger, and then were allowed to dry onto the cover glass in the cryostat. Since the ability of the receptor to bind HRP decreased during storage of the sections in the cryostat, the tissue was usually tested on the same day, or 1–2 days after having been cut.

The cover glass with the frozen sections was immersed for 30 min in ice-cooled absolute methanol. It was then transferred through ice-cooled graded methanol to phosphate-buffered saline (PBS) and to 0.03% H_2O_2 in PBS at room temperature, and was held there for 30 min. After washing in PBS, the sections were transferred to a solution of 0.1% phenylhydrazine-HCl containing 0.35% NaCl and 0.06 M phosphate buffer, pH 7.5, for 1 h at 37° C. After washing in PBS, the sections were held in a 0.03 M 2-amino-2-methyl-1-propanol buffer solution, pH 9.5–10.0, for 15 min at room temperature. They were then incubated for 2 h at 37° C, or for 3–4 h at 22° C, in a solution of 0.004% HRP in physiological saline, also containing 0.03 M 2-amino-2-methyl-1-propanol buffer, pH 9.5–10.0, and 0.01% $CaCl_2$. (The $CaCl_2$ was dissolved in 0.05 M glycine buffer, pH 9.0, and 0.1 ml of a 1% solution was added to 9.9 ml of the incubation mixture.) After thorough washing in PBS, the sections were stained for peroxidase activity in a solution containing 0.04% diaminobenzidine (DAB)-tetrahydrochloride, 0.015% H_2O_2 (Graham and Karnovsky 1966), and 0.05 M cacodylate buffer, pH 7.4, for 10 min at room temperature. After washing in PBS, the sections were counterstained with methyl green, and were transferred through graded ethanol, ethanol-xylene (1:1) and xylene to Permount.

A control section was treated in the same way except that the exposure to HRP was omitted. This section showed the extent of inhibition of endogenous peroxidase activity by methanol-H_2O_2 and phenylhydrazine. The specificity of the binding reaction was tested by omitting Ca^{++} from the incubation mixture with HRP, or by adding 10 mM mannose or 0.004% mannan to the incubation mixture with HRP. No significant binding of HRP to specific receptors should occur after such treatments (see below).

Results

The results were obtained by observing the intensity of the reaction for HRP adsorbed to the endothelial cells and Kupffer cells (specific reaction; see below) as well as the nonspecific adsorption of HRP to the background and to cell

membranes (see below). Serial sections from the same block were used in individual experiments and were processed under identical conditions except that the factor under study was varied.

Influence of the Time of Fixation with Formaldehyde on the Specific and Nonspecific Binding of HRP

Specific Binding of HRP. Blocks of the liver, 2–3 mm thick, were fixed in cold, 4% formaldehyde solutions for 15 min, 30 min, 45 min, 60 min, 90 min, and for 2 h, 3 h, 4 h, 5 h, and 24 h, respectively. The frozen sections obtained from this tissue were treated successively with cold methanol, H_2O_2, phenylhydrazine and HRP (see below), and were stained for peroxidase activity with DAB and H_2O_2.

A strong binding of HRP to the sinusoidal cells was observed only if the tissue had been fixed for less than 1–2 h in the formaldehyde solution. The staining was much decreased after 2–3 h of fixation and no significant staining occurred after 4–5 h. In most subsequent experiments, the tissue was fixed for 30–60 min in the 4% formaldehyde solution.

Nonspecific Binding of HRP. The nonspecific adsorption of HRP appeared as background staining and membrane staining in all liver cells (parenchymal cells and sinusoidal cells). It was significant only when the treatment of the tissue sections with phenylhydrazine was omitted (see below). The nonspecific binding of HRP to cell membranes became more marked in tissue that had been fixed for longer than 4–5 h in the cold 4% formaldehyde solutions. However, a strong adsorption of HRP to the background was observed also in weakly-fixed tissue.

Inhibition of Endogenous Peroxidases by Successive Treatment with Methanol and H_2O_2

The specific binding of HRP to sinusoidal cells was more clearly distinguishable (in nonperfused liver) after the strong peroxidatic reaction of hemoglobin in erythrocytes of the sinuses was inhibited. In the standard procedure, the formalin-fixed, frozen sections were held for 30 min in ice-cooled methanol (Straus 1971, 1980). They were then transferred to PBS containing 0.03% H_2O_2, for 30 min at room temperature (Streefkerk 1972). After this treatment, the peroxidatic reaction of hemoglobin in erythrocytes was much decreased but was not yet abolished. The treatment with methanol also improved the fixation of the tissue.

Inhibition by Phenylhydrazine of Endogenous Peroxidases and of Nonspecific Binding of HRP

In the usual procedure (see Recommended Procedure above) the formalin-fixed, frozen sections of the liver, after having been treated with methanol and H_2O_2, were held for 1 h at 37° C and pH 7.2 in a solution of 0.1% phenylhydrazine (Straus 1972, 1980) and were then exposed to HRP (40 μg/ml). In such sections,

the endogenous peroxidase reaction in erythrocytes (and in other cells) was abolished. In addition, the nonspecific adsorption of HRP to the tissue section was greatly decreased.

Influence of the Concentration of HRP and of the Time of Incubation on the Specific and Nonspecific Binding of HRP

Formalin-fixed, frozen sections of the liver were treated with methanol and H_2O_2 *but were not treated with phenylhydrazine.* They were then exposed at room temperature to 0.001%, 0.002%, 0.004%, and 0.008% HRP, respectively, in physiological saline also containing cacodylate buffer, pH 7.4, and 0.01% $CaCl_2$ (see below) for 1 h, 2 h, 3 h, and 4 h, respectively. The specific binding of HRP to sinusoidal cells increased with increasing concentrations of HRP in the medium and with increasing times of incubation. However, the nonspecific adsorption of HRP also increased with these parameters.

In parallel experiments, sections from the same block, treated with methanol and H_2O_2 were *incubated with phenylhydrazine* as indicated above and were then exposed to increasing concentrations of HRP for 1–4 h at 22° C. As above, the specific binding of HRP to sinusoidal cells increased with increasing concentrations of HRP in the medium and with increasing times of incubation. However, the nonspecific adsorption of HRP showed only a slight increase. Thus, after the treatment with phenylhydrazine, the specific reaction could be intensified by increasing the concentration of HRP in the medium and by increasing the time of exposure to HRP without causing a strong background adsorption. A slight background adsorption of HRP appeared when tissue sections were incubated at 22° C with solutions of HRP containing 40 µg/ml for longer than 4 h, or with 80 µg/ml for longer than 2 h.

Influence of the Temperature of Incubation on the Specific and Nonspecific Adsorption of HRP

Formalin-fixed, frozen sections of the liver were treated with methanol and H_2O_2, with or without subsequent treatment with phenylhydrazine. They were then exposed to solutions of 0.004% HRP for 1–4 h at 4° C, 22° C (room temperature), and 37° C, respectively. Both the specific binding of HRP (observed after treatment with phenylhydrazine) and the nonspecific binding of HRP (in sections not treated with phenylhydrazine) increased with the increase of temperature of incubation. Only little specific or nonspecific adsorption of HRP was observed after exposure of the sections to HRP for 4 h at 4° C.

In most of the following experiments, the tissue sections were exposed to solutions containing 40 µg HRP/ml, for 2 h at 37° C or for 3–4 h at 22° C. Figures 1 and 2 show a section of liver after treatment with methanol-H_2O and phenylhydrazine exposed to HRP (40 µg/ml) for 2 h at 37° C (see Recommended Procedure, above).

Effect of the pH on the Specific Reaction

Formalin-fixed, frozen sections from the same block of liver were treated with methanol, H_2O_2, and phenylhydrazine as usual. They were then exposed to

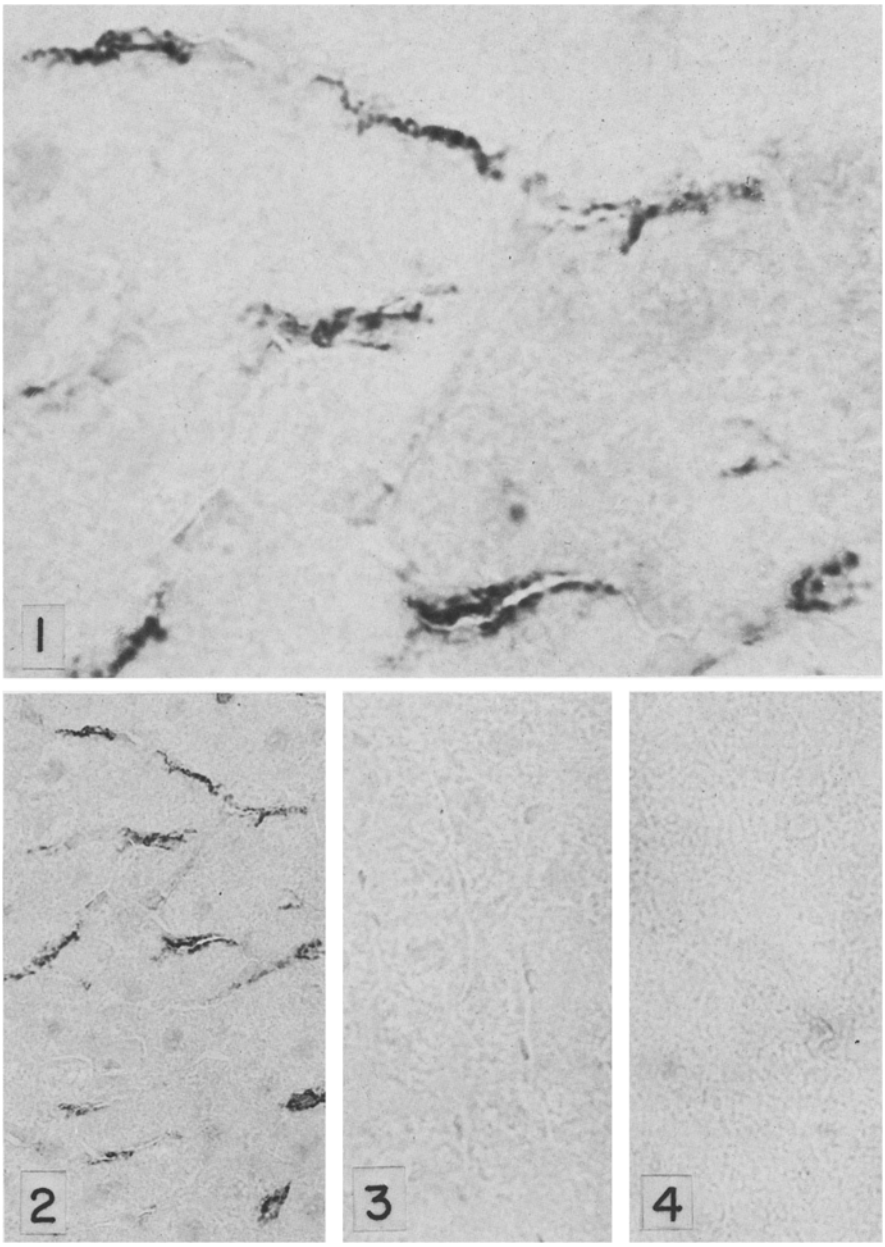

Fig. 1. Specific binding of HRP to surface membranes and to granules adjacent to membranes of liver sinusoidal cells. × 1,600

Fig. 2. Same section as shown in Fig. 1 at lower magnification for comparison with Figs. 3 and 4. × 560

Fig. 3. Lack of specific binding of HRP in the absence of added Ca^{++} from the incubation medium with HRP. The section was from the same block as that shown in Figs. 1 and 2. × 560

Fig. 4. Suppression of specific binding of HRP by the addition of 10 mM mannose to the incubation medium with HRP. The section was from the same block as those shown in Figs. 1–3. × 560

HRP (40 μg/ml) for 4 h at 22° C in physiological saline, buffered with 0.03 M cacodylate buffer at pH 6.2, 6.5, 6.8, 7.1, and 7.5, respectively; with 0.01 M barbital buffer at pH 7.8, 8.2, 8.6, and 9.0, respectively; and with 0.03 M 2-amino-2-methyl-1-propanol buffer at pH 9.0, 9.5, 10.1, and 10.5, respectively.

The specific binding of HRP to sinusoidal cells decreased when the sections were exposed to HRP at a pH below 6.7. No significant binding of HRP was observed at pH 6.2. However, the binding reaction increased when the pH in the incubation medium was increased from 7.0 to 10.0. The reaction was decreased at pH 10.5. In most subsequent experiments, incubation with HRP was applied at pH 9.5–10.0.

Effect of Ca^{++} on the Specific Reaction

Little or no binding of HRP to the sinusoidal cells was observed when no $CaCl_2$ was added to the incubation mixture with HRP (Fig. 3). Different concentrations of $CaCl_2$, varying between 0.018 and 4.5 mM, were added to the incubation mixture and the effects on the binding reaction were compared. No significant binding of HRP occurred with 0.018 mM $CaCl_2$. Only weak binding was observed with 0.09 mM $CaCl_2$. Maximal binding of HRP occurred with 0.9 mM and 1.8 mM (0.01% and 0.02%) $CaCl_2$, and one of these concentrations was used for the standard procedure.

Suppression of the Specific Binding of HRP to Sinusoidal Cells by Mannan, Mannose, Galactan, and Galactose

Formalin-fixed, frozen sections of the liver were treated in the usual way with methanol, H_2O_2, and phenylhydrazine, and were then exposed for 2 h at 37° C to solutions of HRP (40 μg/ml) containing 1.25, 2.5, 5.0, 10, or 20 mM mannose or 25, 50, 100, 200, 300, or 400 mM galactose, respectively. Other sections from the same block were exposed to solutions of HRP (40 μg/ml) containing 0.0005, 0.001, 0.002, 0.004, or 0.008% mannan or 0.01, 0.02, 0.04, 0.08, 0.16, or 0.32% galactan, respectively.

The specific reaction in sinusoidal cells was suppressed when the HRP solution contained 10 mM mannose (Fig. 4) or 0.004% mannan (or higher concentrations). The suppression of the reaction by galactose or galactan required 30–40 times higher concentrations than the suppression of the reaction by mannose or mannan. The effects of competition by other carbohydrates on the binding of HRP, and the effects of competition by some glycoproteins, will be reported later.

Discussion

HRP is a glycoprotein rich in mannose and N-acetylglucosamine groups (Clarke and Shannon 1976). Rodman et al. (1978) observed that the clearance rate of HRP from the blood was greatly decreased by the simultaneous injection of mannan. The investigators concluded that receptors specific for the mannose

or N-acetylglucosamine groups of glycoproteins were involved in the uptake of HRP. Kawasaki et al. (1978) have isolated a mannose-specific binding protein from liver. It was also known that mannose-specific receptors for glycoproteins were located in sinusoidal cells (Brown et al. 1978; Schlesinger et al. 1978; Steer and Clarenburg 1979) and that HRP in vivo is taken up predominantly by the endothelial cells and Kupffer cells (Straus 1964a, 1967). As is shown in the present report, HRP is bound to mannose-specific receptors of sinusoidal cells also in fixed tissue sections. The binding required Ca^{++} and was supressed by 0.004% mannan or by 10 mM mannose. The suppression of the binding reaction by galactan or galactose required 30–40 times higher concentrations. It is therefore probable, that HRP is bound in fixed tissue sections to the same receptor to which it is also bound in vivo, prior to its endocytosis by Kupffer cells and endothelial cells.

The binding of HRP to a lectin-like receptor in sinusoidal cells could be distinguished more clearly after the strong peroxidatic reaction of erythrocytes in the sinuses had been suppressed. As was known from previous work, methanol inhibits HRP by splitting off the heme group (Straus 1971, 1974), and methanol followed by dilute H_2O_2 inhibits endogenous peroxidases (Streefkerk 1972) by denaturing them (Straus 1976). Phenylhydrazine also inhibits the cytochemical reaction for endogenous peroxidases (Straus 1972). Therefore, by treating tissue sections successively with methanol, H_2O_2, and phenylhydrazine, it was possible in the present work to suppress the reaction for endogenous peroxidases including the weak endogenous peroxidase in Kupffer cells (Fahimi 1970).

As was reported previously (Straus 1964b), HRP is bound nonspecifically to cells in fixed tissue sections, and the nonspecific adsorption increases with increasing concentrations of HRP in the medium. In the present experiments, the concentration of HRP in the medium was relatively high (40 μg/ml, in most cases). It was therefore necessary to decrease the nonspecific binding of HRP before the specific binding of HRP to lectin-like receptors could be recognized clearly. As in recent immunocytochemical work (Straus 1980), the nonspecific adsorption of HRP was greatly decreased by pretreating the tissue sections with phenylhydrazine.

The ability of the receptors in sinusoidal cells to bind HRP did not survive fixation of tissue blocks for longer than 2–3 h in a cold 4% formaldehyde solution. In order to obtain a strong binding reaction, the tissue was usually fixed for only 30–60 min. Subsequently, the frozen sections were immersed for 30 min in ice-cooled methanol. As was already mentioned, the treatment with methanol had the double effect of improving the fixation and inhibiting endogenous peroxidases. Since the fixation was not strong, the tissue was not well preserved. However, it was sufficient to distinguish the binding of HRP to the surface membranes and to long extensions of endothelial cells as well as to the surface membranes of Kupffer cells. HRP was also bound to granules (phagosomes ?) adjacent to the membranes (Fig. 1). Because artifacts of diffusion and adsorption are possible in weakly-fixed tissue, the fine-structural localization of the receptor should be determined more accurately when a better fixation of the tissue has been achieved.

Receptors specific for the terminal galactose in glycoproteins are known to be located in parenchymal cells but not in sinusoidal cells of the liver (Ashwell and Morell 1974). Hardonk and Scholtens (1980) described a cytochemical method for the detection of galactose-specific receptors in the parenchymal cells using dog alkaline phosphatase as a ligand. In contradistinction to the galactose-specific receptors, mannose-specific receptors for glycoproteins are known to occur in both hepatic and extra-hepatic tissues (Schlesinger et al. 1980). It will be interesting to establish by the above described method which extra-hepatic cells contain mannose-specific receptors for glycoproteins (work in progress).

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Note Added in Proof

In current experiments, the cytochemical reaction for peroxidase with DAB and H_2O_2 is intensified considerably by adding imidazole (Straus 1980). With this modified assay, the concentrations of mannose, mannan, galactose or galactan required to suppress the cytochemical reaction for the specific binding of HRP had to be 2–3 times higher than in the experiments without imidazole described above.