Cytochemical Observations on Mannose-Specific Binding Sites for Horseradish Peroxidase in Liver Sinusoidal Cells

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Summary. Paraformaldehyde-fixed, frozen sections of the liver of rats were processed for the detection of mannose-specific binding sites of horseradish peroxidase (HRP) by a method reported previously, with some modifications resulting in a more intense binding reaction. Before staining for peroxidase activity, the sections were held in buffered solutions of physiological saline at different temperatures and pH's, and in the presence or absence of added Ca$^{2+}$, mannose or galactose. The gradual decrease and final disappearance of the binding reaction were observed. The release of HRP from the binding sites as determined by the disappearance of the cytochemical reaction was 50–100 times faster at 22°C than at 4°C and was 5–10 times faster at 37°C than at 22°C. The release was approximately twice as fast at pH 7.0 than at pH 9.0 and 20–30 times faster at pH 6.0 than at pH 7.0. The release of HRP was 10–15 times faster in the absence of 1 mM Ca$^{2+}$ in the buffer solution and was approximately 100 times faster in the presence of 0.1 M D-mannose as compared to 0.1 M D-galactose. Pretreatment of the sections with trypsin abolished the binding reaction whereas neuraminidase, phospholipases A$_2$ and C, and chondroitinase ABC were without effect. An acidic isoenzyme of HRP, Sigma type VIII, was bound more intensely and more widely to liver sinusoidal cells than another acidic isoenzyme, Sigma type VII, a basic isoenzyme, Sigma type IX, and the routinely used preparation, Sigma type VI. The effect of the temperature on the binding reaction was re-examined with an improved procedure. In contradistinction to the previous finding, strong binding of HRP after 2–4 h incubation at 4°C was observed.

Introduction

In a previous report (Straus 1981) a new method was described for the cytochemical detection of mannose-specific binding sites of horseradish per-
oxidase (HRP) in paraformaldehyde-fixed, frozen sections. The development of this method was based on observations of binding sites for HRP in liver sinusoidal cells. In subsequent work, binding sites for HRP were also demonstrated in macrophages, fibroblasts, and mast cells of various tissues and the affinities of various carbohydrates and glycoproteins toward these sites were estimated (Straus 1983).

In the present experiments, other properties of the binding sites for HRP in liver sinusoidal cells were studied. The release of HRP from the binding sites was investigated at different temperatures and pH's, and in the presence or absence of added Ca\(^{2+}\), mannose or galactose. The effects of some lytic enzymes on the cytochemical binding reaction were tested. In other experiments, the affinities of different isoenzymes of HRP to the lectin-like binding sites were studied, and the effects of the temperature on the binding reaction were re-investigated with an improved method.

**Materials and Methods**

*Animals and Reagents.* Sprague-Dawley rats, 200–300 g in weight, were used. The following reagents were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA: HRP, types VI, VII, VIII, and IX; 3,3'-diaminobenzidine tetrahydrochloride; trypsin type III (from bovine pancreas); neuraminidase type X (from Clostridium perfringens); chondroitinase ABC (from Proteus vulgaris); hyaluronidase type VI-S (from bovine testis); phospholipase A\(_2\) (from Naja naja venom); phospholipase C type XII (from Clostridium perfringens); D-mannose and D-galactose.

*Method for the Cytochemical Detection of Lectin-Bound HRP.* The previously described method (Straus 1981) was modified as follows: before the incubation with HRP, the sections were washed with Tris-HCl buffered saline, pH 7.4 [instead of phosphate-buffered saline (PBS), previously] and after incubation with HRP, were washed with Tris-HCl buffered saline, pH 7.4, also containing 1 mM Ca\(\text{Cl}_2\) (instead of PBS without Ca\(\text{Cl}_2\), previously). Imidazole was added during staining for peroxidase activity (see below). In brief, small blocks of liver were fixed for 1 h in a cold, 4% paraformaldehyde solution containing 0.1% Ca\(\text{Cl}_2\). Frozen sections, attached to 22 mm square cover glasses, were processed in 10-ml micro-staining dishes through cold methanol, \(\text{H}_2\text{O}_2\), and phenylhydrazine in order to suppress the endogenous peroxidases and to decrease the nonspecific background adsorption of HRP. Between these treatments, the sections were rinsed in large volumes (600 ml, approximately) of Tris-HCl buffered saline, pH 7.4. The sections were then incubated for 1 h at 37° C in 10 ml of physiological saline containing 40 \(\mu\)g/ml HRP, Sigma type VI, 0.03 M 2-amino-2-methyl-1-propanol buffer, pH 9.0, and 1 mM Ca\(\text{Cl}_2\). They were washed thoroughly with large volumes of Tris-HCl buffered saline, pH 7.4, containing 1 mM Ca\(\text{Cl}_2\). The sections were then stained for peroxidase activity with diaminobenzidine (DAB), \(\text{H}_2\text{O}_2\) (Graham and Karnovsky 1966) and 0.01 M imidazole (Straus 1982). For details, see the previous report (Straus 1981).

*Test of the Stability of the Receptor-HRP Complexes.* Serial sections from the same block of liver were treated successively with methanol, \(\text{H}_3\text{O}_2\), and phenylhydrazine as indicated above and were then incubated for 1 h at 37° C with HRP, Sigma type VI (40 \(\mu\)g/ml) at pH 9.0. Unbound HRP was washed off thoroughly with large volumes of Tris-HCl buffered saline, pH 7.4. The wash fluid contained 1 mM Ca\(\text{Cl}_2\) when the sections were post-treated in buffer solution containing added Ca\(\text{Cl}_2\) (see below). However, HRP was washed off with ice-cooled Tris-HCl buffered saline, pH 7.4, without Ca\(\text{Cl}_2\), when the sections were post-treated with buffer solutions without added Ca\(\text{Cl}_2\). After washing off the HRP, the sections were held for periods lasting for a few minutes to several weeks in physiological saline buffered with 0.03 M cacodylate at pH 6.0 or 7.0, or with 0.03 M 2-amino-2-methyl-1-propanol at pH 9.0. Different sections were held in these solutions at 4°, 22° and 37° C, respectively,