

## Binding of Purified and Radioiodinated Capsular Polysaccharides from *Cryptococcus neoformans* Serotype A Strains to Capsule-Free Mutants

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Strains 6, 15, 98, 110, and 145 of *Cryptococcus neoformans* serotype A vary in capsule size, animal virulence, and susceptibility to in vitro phagocytosis. The isolated capsular polysaccharides (CPSs) differ in monosaccharide composition ratios and molecular size, as determined by gel filtration. The purpose of this investigation was to characterize the binding of CPSs to capsule-free mutants of *C. neoformans* and to examine CPSs from these strains for differences in their ability to bind, to determine whether such differences might explain the variation in the pathobiology of these strains. CPSs were partially periodate oxidized, tyraminated, iodinated with <sup>125</sup>I, and used in binding studies with two capsule-free mutants of *C. neoformans*, strain 602 and Cap59. Binding was specific for yeast species and for polysaccharide and was saturable, which is consistent with a receptor-mediated mechanism of attachment. Binding occurred rapidly and was only slowly reversible. Binding was also independent of pH from pH 5.5 to 8, of cation concentrations, and of competition by sugars up to 1.0 M concentrations. Only a portion of CPS was capable of binding, and strains varied in the extent to which their CPS bound. CPS-15-IV (peak IV was the major polysaccharide peak on DEAE-cellulose chromatography of CPS from strain 15) had the highest proportion of binding (40%), followed by CPS from strains 98, 6, 145, 110, and 15-III (peak III was an earlier eluting fraction of CPS from strain 15). The CPSs differed similarly in their ability to competitively inhibit binding. Treatment of CPS, but not yeast cells, with proteinase XIV abolished binding without altering the CPS gross structure. Treatment of yeast cells with proteases, heat, or formaldehyde did not alter binding, and both strain 602 and Cap59 bound CPS similarly. Binding to encapsulated yeast cells was minimal.

The capsule of *Cryptococcus neoformans* affects the pathogenesis of cryptococcosis. Capsule-free mutants produce a more inflammatory and localized disease in humans and experimental animals than encapsulated strains, which produce a more severe and chronic disease (7, 8, 11). The lesions often lack a tissue reaction (1), implying that the capsule acts as a virulence factor by suppressing the host immune response.

Many investigations have addressed this property of the cryptococcal capsule. Encapsulated strains resist phagocytosis in vitro better than capsule-free or hypocapsular strains. For example, uptake by macrophages is inversely proportional to capsule size (25). *C. neoformans* 602, a capsule-free mutant, can bind exogenously supplied cryptococcal capsular polysaccharide (CPS) and resist phagocytosis by mouse macrophages (15, 18). Binding was evaluated by immunofluorescence, and a receptor was postulated. More recently, a radiolabel binding assay for cryptococcal polysaccharide was described (17). Binding of <sup>125</sup>I-labeled CPS (<sup>125</sup>I-CPS) was specific, and CPS from strains of each serotype competed for binding. De-O-acetylated, carboxyl-reduced, periodate-oxidized and -reduced (polyalcohol), and Smith-degraded CPS all competed for binding (17). Other properties of CPS include the induction of tolerance and suppression of lymphocyte responsiveness (3, 21, 26).

To begin to understand mechanisms of capsule activity, CPS was examined from strains of *C. neoformans* serotype A denoted 6L, 15S, 98M, 110M, and 145M. S, M, and L

indicate small, medium, and large in vitro capsule sizes, respectively. These strains vary in capsule size, animal virulence, and resistance to phagocytosis and also display strain-related differences in molecular size and subunit composition of their CPSs (25, 29; J. M. Small and T. G. Mitchell, manuscript in preparation). The purpose of this investigation was to devise a quantitative assay to evaluate the binding of the radiolabeled CPS of each of these five strains to mutant strain 602 and to Cap59, another capsule-free strain (13), and to use this system to characterize the nature of the binding and to document strain variation in CPSs within serotype A.

### MATERIALS AND METHODS

**Organisms and media.** *C. neoformans* 6, 15, 98, 110, and 145 have been characterized as to capsule size, resistance to phagocytosis, and relative molecular sizes and compositions of their CPSs (25, 29). Thomas R. Kozel and Eric S. Jacobson generously donated the capsule-free mutant strains 602 (16) and Cap59 (13), respectively. Other yeasts used were *Candida albicans* 4918 (23) and a laboratory strain of *Saccharomyces cerevisiae*. All yeasts were maintained on slants of yeast morphology agar supplemented with 1% additional agar (YMA; Difco Laboratories, Detroit, Mich.). Strain 602 was cultured on solid media because it formed clumps of 5 to 20 yeast cells in all of the broth media tested, including phosphate-buffered glutamine-glycine-asparagine (GGA-B) broth (29), Wickerham carbon base medium (Difco), glucose-yeast extract, tryptic soy broth, and brain heart infusion broth. Cultures of strain 602 harvested from slants of glucose-yeast extract agar and YMA consisted of single yeast cells and yeast cells with buds; YMA was

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selected for routine use because it is a chemically defined medium.

For routine binding assays, organisms were transferred to YMA and incubated for 2 days at 25°C. Yeast cells were harvested by washing slants with sterile 0.01 M phosphate-buffered normal saline (PBS; pH 7.0), enumerated by hemacytometer count, and were kept at 4°C for not more than 1 week.

Yeast cells designated formaldehyde killed were prepared by suspending growth from YMA slants as described above in 0.33% (vol/vol) formaldehyde and allowing to stand at 4°C overnight. Yeast cells were then washed and suspended in PBS as described above. Yeast cells designated heat killed were prepared by placing yeast cell suspensions in a 65°C water bath, followed by washing as described above. Lack of viability of both preparations was established by negative culture.

**CPS preparation.** CPSs of *C. neoformans* 6, 15, 98, 110, and 145, all serotype A, were prepared by ethanol precipitation from broth culture filtrates followed by DEAE-cellulose chromatography, as described previously (29). CPSs from the various strains used are designated CPS-6, CPS-98, CPS-110, and CPS-145; CPS-15-III and CPS-15-IV are major peaks III and IV from DEAE-cellulose chromatography of CPS-15 (29). Other polysaccharides used for controls were dextran T-2000 and dextran sulfate (Pharmacia Fine Chemicals, Piscataway, N.J.) and yeast mannan (Sigma Chemical Co., St. Louis, Mo.).

**Tyramination of CPS.** CPS was covalently linked to tyramine by a method based on that of Keck (14). Solutions of CPS were dialyzed against PBS and brought to a concentration of 2 mg/ml by the phenol-sulfuric acid assay (6) with a glucose standard. Thirty microliters of 0.01 M sodium metaperiodate (Fisher Scientific Co., Springfield, N.J.) was added to 1 ml of CPS in PBS with stirring, and the solution was kept in the dark for 24 h at 4°C. Then, 0.5 ml of 0.1 M tyramine hydrochloride (Sigma) and 0.5 ml of a 0.5 M borate buffer (pH 9.5) were added, and 10 min later 50 µl of 2 M sodium borohydride (Sigma) was added. After the solution was kept at 4°C overnight, it was passed over a 10-ml desalting column of Sephadex G-25 Fine (Pharmacia), and 1-ml fractions were assayed by determining the  $A_{280}$  for bound tyramine and by the phenol-sulfuric acid assay (6) for carbohydrate.

**Radioiodination of CPS.** Tyraminated CPS (TCPS) was iodinated by the chloramine T method of Greenwood et al. (10). All reactions were carried out under a fume hood. In a 1.5-ml polypropylene microfuge tube were mixed 50 µl of 0.5 M sodium phosphate (PB; pH 7.4), 150 µg of TCPS in PBS, and 0.5 to 1 mCi of carrier-free Na<sup>125</sup>I (Amersham Corp., Arlington Heights, Ill., or New England Nuclear Corp., Boston, Mass.). Fifty microliters of 0.4% (wt/vol) chloramine T in PB was added. Five minutes later 200 µl of 2% (wt/vol) sodium metabisulfite in PB and 10 µl of 40% aqueous potassium iodide were added and the mixture (<sup>125</sup>I-CPS) was desalted over Sephadex G-25 Fine which had been equilibrated with PBS.

**Binding studies.** Polypropylene microfuge tubes of 1.5-ml capacity (Eppendorf; Brinkmann Instruments, Inc., Westbury, N.Y.) were precoated by filling them with 10% fetal calf serum in PBS to reduce nonspecific adherence. After aspiration of the coating solution, each tube was filled with 1.0 ml of PBS and a sample of yeast cells (usually 10<sup>7</sup> yeast cells, as determined by hemacytometer counts). The tubes were centrifuged for 3 min in a microfuge (Eppendorf model 5414 or 5412; Brinkmann), and the supernatant fluid was

aspirated. One milliliter of a solution containing radiolabeled CPS, PBS, or other reagent and any test substances, as appropriate, was added. The yeast cells were suspended by vortexing, and the tubes were incubated at room temperature for 2 h. Results of preliminary experiments had determined that binding was near maximum by this time. The tubes were then vortexed and centrifuged as described above, and the supernatant fluid was removed. The yeasts were washed by the addition of 1 ml of PBS, vortexing, and centrifugation. Yeast cell pellets were suspended, transferred to glass tubes, and counted in a gamma counter (model 1185; Searle Analytic Inc.) with a 30% counting efficiency. Samples of test solution(s) were also measured to determine the total counts per minute added.

**Protease studies.** The following enzyme preparations were obtained from Sigma: proteinase XIV from *Streptomyces griseus*, proteinase XI from *Triturachium album* (protease K), pepsin and trypsin. Papain was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. A stock preparation of each enzyme, or the control of bovine serum albumin (Sigma), was prepared at 0.4 mg/ml in PBS (or in 0.05 M citrate buffer [pH 3.5] for pepsin) and mixed with an equal volume of 1-µg/ml solutions of <sup>125</sup>I-CPS. After digestion of various times (overnight in most cases, or from 2 to 24 h with proteinase XIV; see Fig. 7), proteases were inactivated by heating in a boiling water bath for 15 min. In one experiment 1-ml samples of proteinase XIV were (i) mixed with 25 µl of a 40 mM solution of phenylmethylsulfonyl fluoride (PMSF) in ethanol; (ii) mixed with 25 µl of ethanol; or (iii) heated to 65°C for 5 min. These enzyme samples were then added to <sup>125</sup>I-CPS as described above. Digested samples of <sup>125</sup>I-CPS were tested for their ability to bind in the standard binding assay. In other experiments, 10<sup>7</sup> formalinized yeast cells of strain 602 were suspended in 1 ml of trypsin or proteinase XIV solution for 45 min, washed, and assayed by the binding assay. Proteolytic activity of enzyme preparations was confirmed by mixing each enzyme solution with an equal volume of 1% (wt/vol) casein in PBS at room temperature for 1 h, after which the undigested casein was precipitated with 5% trichloroacetic acid and centrifuged, and the absorbance of the supernatant fluid was measured at 280 nm.

## RESULTS

**Tyramination of CPS.** Preliminary experiments showed that mixing CPS-6, periodate, and tyramine at pH 9 for a short time, as recommended by Keck (14), resulted in poor labeling and partially degraded CPS. To improve the labeling procedure, CPS was first oxidized with periodate at pH 7.4, and then tyramine was added with enough borate buffer to bring the pH to 9 to stabilize the Schiff base. The final addition of sodium borohydride converted the Schiff base to a stable amine. The periodate oxidation step required 24 h to adequately derivatize CPS, dextran, dextran sulfate, and yeast mannan. Azide interfered with tyramine coupling and, when present, had to be removed by dialysis. Coupled tyramine, as measured by determining the  $A_{280}$ , was directly proportional to the quantity of periodate added (data not shown). In a typical experiment the absorbance at 280 nm of TCPS was 0.050, which on a standard curve corresponded to  $5 \times 10^{-5}$  M tyramine. The concentration of CPS was 600 µg/ml. Assuming an average sugar residue molecular mass of 180 daltons, there were 3.33 µmol/ml sugar residues (i.e., 3.33 mM), yielding 1.5% substitution (i.e., [100]  $5 \times 10^{-5}$  M Tyr/3.33  $\times 10^{-3}$  M sugars).

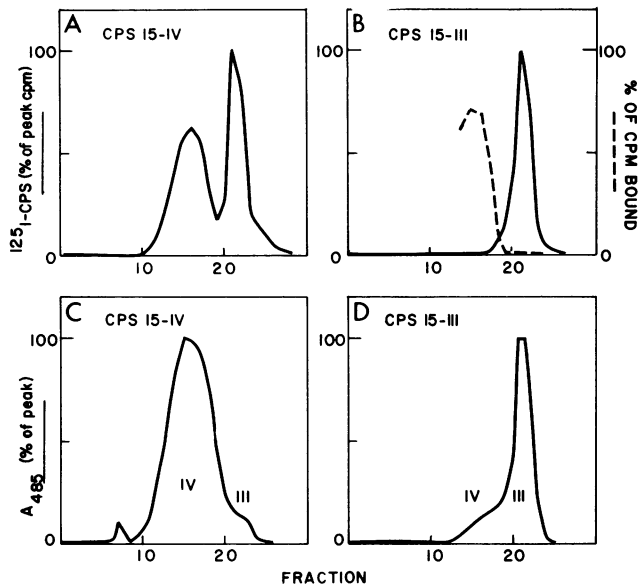


FIG. 1. Relative efficiencies of labeling and binding of CPS-15-III and CPS-15-IV. Samples of each CPS from DEAE-cellulose chromatography (29) were fractionated on a column (1.7 by 45 cm) of Sepharose CL-2B and eluted with 0.01 M PBS, and the fractions were assayed for carbohydrate by the phenol-sulfuric acid method (6). Carbohydrate is expressed as the percentage of peak absorbance at 485 nm (C and D). Samples of each CPS were then tyraminated and radiolabeled as described in the text and fractionated on the same column, and the radioactivity in each fraction was measured.  $^{125}\text{I}$ -CPS is expressed as a percentage of peak counts per minute (A and B). Finally, 1.0-ml samples of fractions of  $^{125}\text{I}$ -CPS-15-III were added to  $10^7$  yeast cells of *C. neoformans* 602 and incubated for 1 h, and the bound radioactivity was determined. The percentage of counts per minute added that bound is shown by the dashed line (B); fractions before fraction 14 were not evaluated because of insufficient activity.

**Radioiodination of TCPS.** Specific activities of  $1 \times 10^6$  to  $3 \times 10^6$  cpm/ $\mu\text{g}$  of CPS were repeatedly achieved with each of the six preparations of CPS. Nontyraminated CPS-6 took up approximately 2% as much label. The elution profiles from Sepharose CL-2B of freshly iodinated CPS ( $^{125}\text{I}$ -CPS) were similar to those of unlabeled material. However, radiodegradation occurred after a month of storage, as evinced by a change in elution profile and decreased binding.  $^{125}\text{I}$ -CPS, with a lower specific activity produced by labeling with 0.25  $\mu\text{Ci}$  of  $^{125}\text{I}$ , degraded more slowly and had adequate activity for experiments. Gel chromatography profiles of  $^{125}\text{I}$ -CPS-15-III and -15-IV revealed that CPS-15-III incorpo-

TABLE 1. Specificity of the binding of  $^{125}\text{I}$ -CPS-6 to various yeast species

Organism <sup>a</sup>	cpm added	cpm bound
<i>C. neoformans</i> 602	760,000	108,101
<i>C. neoformans</i> Cap59	760,000	114,179
<i>Candida albicans</i> 4918	760,000	91
<i>S. cerevisiae</i>	760,000	116
<i>C. neoformans</i> 15	460,000	1,000

<sup>a</sup> One milliliter of PSB containing 1  $\mu\text{g}$  of  $^{125}\text{I}$ -CPS-6 (DEAE-cellulose purified CPS-6 that had been tyraminated and radioiodinated as described in the text) was added to  $10^7$  live yeast cells of the indicated strain. After 1 h the bound radioactivity was measured.

TABLE 2. Specificity of polysaccharide binding to *C. neoformans* 602

$^{125}\text{I}$ -labeled polysaccharide <sup>a</sup>	cpm added	cpm bound
Dextran T-2000	216,000	85
Dextran sulfate	10,000	148
Yeast mannan	469,810	76
CPS-6	760,000	108,101

<sup>a</sup> The indicated polysaccharides were tyraminated and radioiodinated, and 1  $\mu\text{g}$  in 1 ml PBS was added to  $10^7$  yeast cells of *C. neoformans* 602. Bound radioactivity was determined as described in the text.

rated label more efficiently than CPS-15-IV (Fig. 1). CPS-15-IV eluted with a small shoulder corresponding to that of CPS-15-III (Fig. 1C), while radiolabeled CPS-15-IV showed a larger peak corresponding to that of CPS-15-III (Fig. 1A).

**Binding studies.**  $^{125}\text{I}$ -CPS bound avidly to yeast cells of *C. neoformans* 602 and Cap59, two capsule-free mutants, but did not bind to yeast cells of *Candida albicans* 4918, *S. cerevisiae*, or *C. neoformans* 15, a strain with a small capsule (Table 1). Comparable binding occurred with heat- or formaldehyde-killed strain 602, and subsequent binding experiments were performed with formaldehyde-killed 602.

**Polysaccharide specificity.** The specificity of polysaccharide binding is demonstrated in Table 2. Radioiodinated dextran T-2000, dextran sulfate, and radioiodinated yeast mannan did not bind to strain 602.

**Time course.** Yeasts of strain 602 were incubated for various time periods with 1  $\mu\text{g}$  of  $^{125}\text{I}$ -CPS with or without a

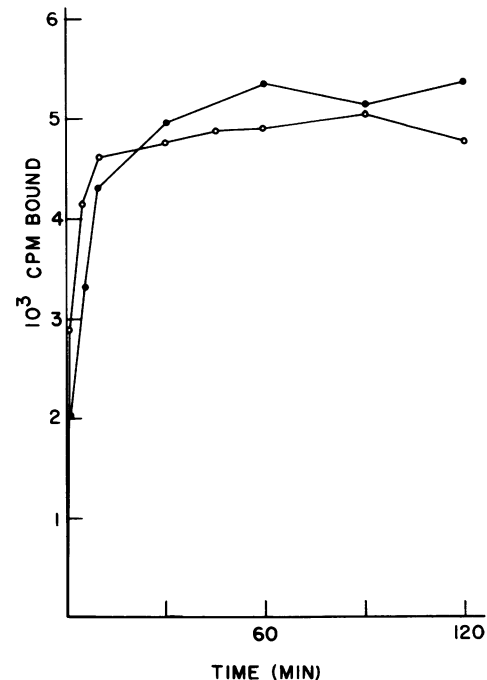


FIG. 2. Time course of binding of  $^{125}\text{I}$ -CPS-6 to the capsule-free isolate *C. neoformans* 602. One milliliter of PBS containing 1  $\mu\text{g}$  of  $^{125}\text{I}$ -CPS-6 per ml with (○) or without (●) 100  $\mu\text{g}$  of unlabeled CPS-6 per ml was added to tubes containing  $10^7$  yeast cells of strain 602, and the tubes were incubated at 25°C. After the indicated times, the tubes were centrifuged, the yeast pellet was removed, and the bound radioactivity was measured. Each point is the mean of duplicate determinations.

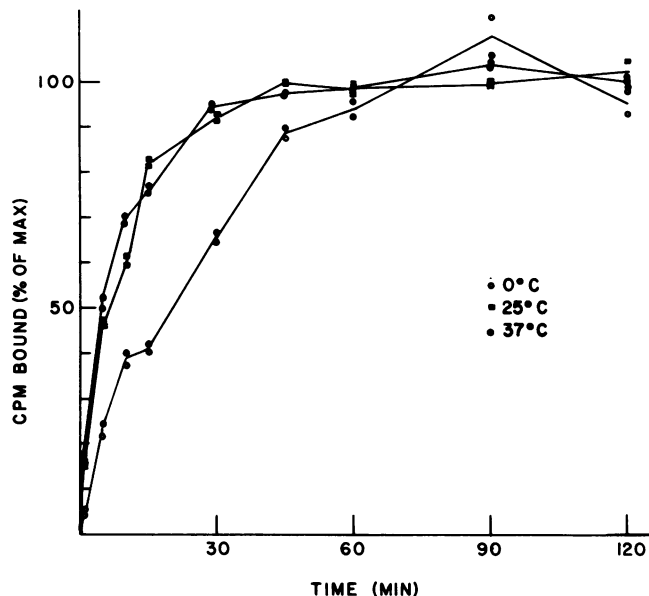


FIG. 3. Effect of time and temperature on binding of  $^{125}\text{I}$ -CPS-15-IV to *C. neoformans* 602. One milliliter of PBS containing  $1\ \mu\text{g}$  of  $^{125}\text{I}$ -CPS-15-IV per ml which had been equilibrated at indicated temperatures was added to tubes containing  $10^7$  yeast cells of strain 602, and the tubes were incubated at 0, 25, or  $37^\circ\text{C}$ . After the indicated times, bound radioactivity was measured. Individual determinations are depicted.

100-fold excess of unlabeled CPS. The yeast cells were then washed, and the radioactivity was measured (Fig. 2). Binding was complete between 30 and 60 min. Similar experiments were performed at 0, 37, and  $25^\circ\text{C}$ . Incubation at  $0^\circ\text{C}$  reduced the rate but not the maximum level of binding (Fig. 3).

**Nature of binding.** Once attached,  $^{125}\text{I}$ -CPS bound tenaciously to the yeast cells. Treatment of yeast cells of strain 602 with bound  $^{125}\text{I}$ -CPS at room temperature overnight with 8 M urea, 6 M guanidine, 0.4 mg of proteinase XIV per ml,

TABLE 3. Effect of monosaccharides on binding of  $^{125}\text{I}$ -CPS of strains 6, 15, 98, and 145 to *C. neoformans* 602

Sugar <sup>a</sup>	% of control $^{125}\text{I}$ -CPS of strain <sup>b</sup> :			
	6	15-IV	98	145
Galactose	91	95	102	101
Glucuronic acid	87	108	98	95
Mannose	68	86	83	73
<i>N</i> -Acetyl glucosamine	88	111	97	99
Xylose	89	96	91	105
$\alpha$ -Methyl mannoside	85	100	100	95

<sup>a</sup> A total of  $10^7$  yeast cells of strain 602 were suspended for 2 h in a 1.0 M solution of the indicated sugar and  $1\ \mu\text{g}$  of the indicated radiolabeled CPS per ml, and bound radioactivity was determined.

<sup>b</sup> Percentage of control (PBS without sugar). Values are means of duplicate determinations.

which is similar to pronase, 1% sodium dodecyl sulfate, 1 M NaCl, 0.05 M EDTA, or  $100\ \mu\text{g}$  of unlabeled CPS-6 per ml released less than 10% of the radioactivity. Binding was comparable in PBS, Hanks balanced salt solution with 10% fetal calf serum, and 0.01 M EDTA. Binding curves were similar from pH 5.5 to 8. To evaluate the possibility of a lectinlike receptor, binding experiments were performed in the presence of 1 M galactose, mannose, xylose, glucuronic acid,  $\alpha$ -methyl mannoside, and *N*-acetyl glucosamine (Table 3). Only mannose was weakly inhibitory.

**Extent of binding.** To examine the homogeneity of  $^{125}\text{I}$ -CPS in binding,  $1\ \mu\text{g}$  of  $^{125}\text{I}$ -CPS-6 per ml was added to  $10^7$  yeast cells of strain 602, the mixture was incubated for 2 h, and the supernatant fluid was transferred to another tube containing  $10^7$  fresh 602 cells for a second binding. A third transfer and binding was then carried out. The first pellet contained  $1.9 \times 10^5$  cpm of the radioactivity added ( $1.25 \times 10^6$  cpm); the second contained  $0.2 \times 10^5$  cpm, and the third contained  $0.03 \times 10^5$  cpm. Thus, only 15% of the available radioactivity in  $^{125}\text{I}$ -CPS-6 was capable of binding. To confirm that this percentage was a property of the CPS and not an artifact of the labeling procedure, four samples of CPS-6 were tyraminated and radiolabeled separately; each preparation bound to the same extent. Similar experiments deter-

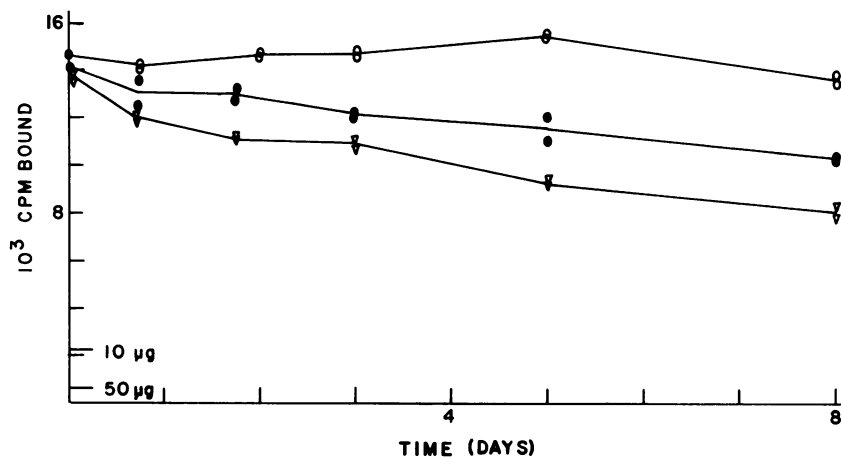


FIG. 4. Dissociation of bound  $^{125}\text{I}$ -CPS-15-IV in the presence of excess unlabeled CPS-15-IV. One milliliter of PBS containing  $1\ \mu\text{g}$  of  $^{125}\text{I}$ -CPS-15-IV per ml was added to tubes containing  $10^7$  yeast cells of *C. neoformans* 602, and the tubes were incubated at  $25^\circ\text{C}$ . After 1 h the tubes were centrifuged, the supernatant fluid was removed, and the yeasts were suspended in 1 ml of PBS containing 0 ( $\circ$ ), 10 ( $\bullet$ ), or 50 ( $\nabla$ )  $\mu\text{g}$  of unlabeled CPS-15-IV. Bound radioactivity was measured at the indicated times. Individual determinations are depicted. Controls consisted of strain 602 incubated in a mixture of  $1\ \mu\text{g}$  of  $^{125}\text{I}$ -CPS-15-IV per ml and 10 or 50  $\mu\text{g}$  of unlabeled CPS-15-IV per ml for 2 h, followed by determination of bound radioactivity. Control binding is indicated near the origin.

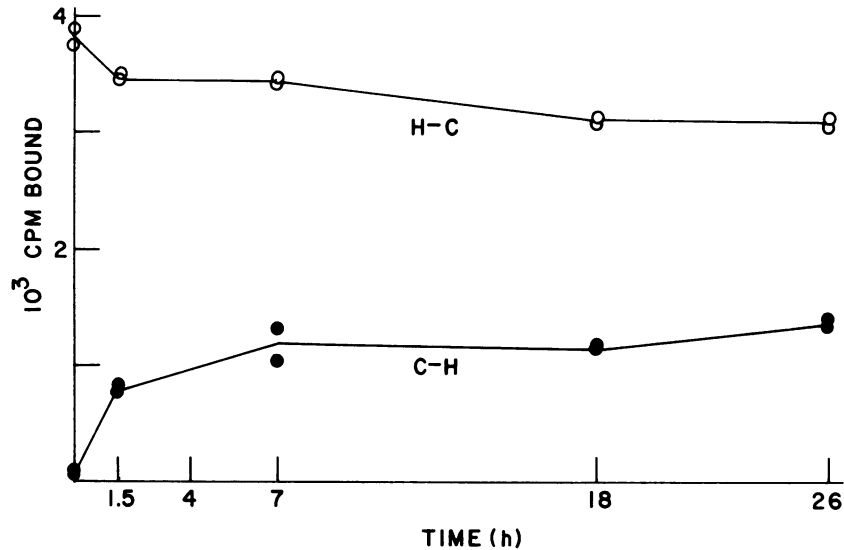


FIG. 5. Slow approach to equilibrium of CPS binding. To test whether final binding was independent of starting conditions, as required for equilibrium binding,  $10^7$  yeast cells of *C. neoformans* 602, were preincubated for 1 h either in solution H (10  $\mu$ g of CPS-15-IV plus 1  $\mu$ g of  $^{125}$ I-CPS-15-IV per ml) or in solution C (10  $\mu$ g of CPS-15-IV per ml). Yeast cells were then pelleted and suspended in the other solution. At the indicated times bound radioactivity was determined. C  $\rightarrow$  H represents yeasts preincubated in solution C (no radiolabel), while H  $\rightarrow$  C represents yeasts preincubated with solution H. Individual determinations are depicted.

mined that <2% of CPS 15-III, 40% of CPS-15-IV, 23% of CPS-98, 8% of CPS-110, and 14% of CPS-145 were able to bind to strain 602. When fractions from gel chromatography of  $^{125}$ I-CPS from strain 15 (Fig. 1) were analyzed in the binding assay, 70% of the radioactivity eluting at the volume corresponding to CPS-15-IV bound, while less than 2% of the radioactivity eluting at CPS-15-III bound.

**Reversibility.** Analysis by standard binding equations requires readily reversible binding at equilibrium; it is not sufficient to simply demonstrate that binding has reached a steady state (31). To test these conditions, 1  $\mu$ g of  $^{125}$ I-CPS-15-IV per ml was bound to strain 602 for 1.5 h, the yeast cells

were pelleted, and the supernatant fluid was removed. Then, 1.0 ml of PBS containing 0, 10, or 50  $\mu$ g of unlabeled CPS-15-IV was added, and the tubes were incubated for up to 8 days. Controls consisted of yeast cells incubated with a mixture of 1  $\mu$ g of  $^{125}$ I-CPS-15-IV and 0, 10, or 50  $\mu$ g of unlabeled CPS-15-IV per ml. Adherent counts did not reach control levels even after 8 days (Fig. 4). In another experiment, yeast cells were preincubated with 10.0  $\mu$ g of CPS-15-IV per ml (solution C) for 1.0 h and then they were suspended in a solution of 1.0  $\mu$ g of  $^{125}$ I-CPS-15-IV per ml and 10.0  $\mu$ g of CPS-15-IV per ml (solution H) (C  $\rightarrow$  H); another sample of yeast cells was suspended in the latter

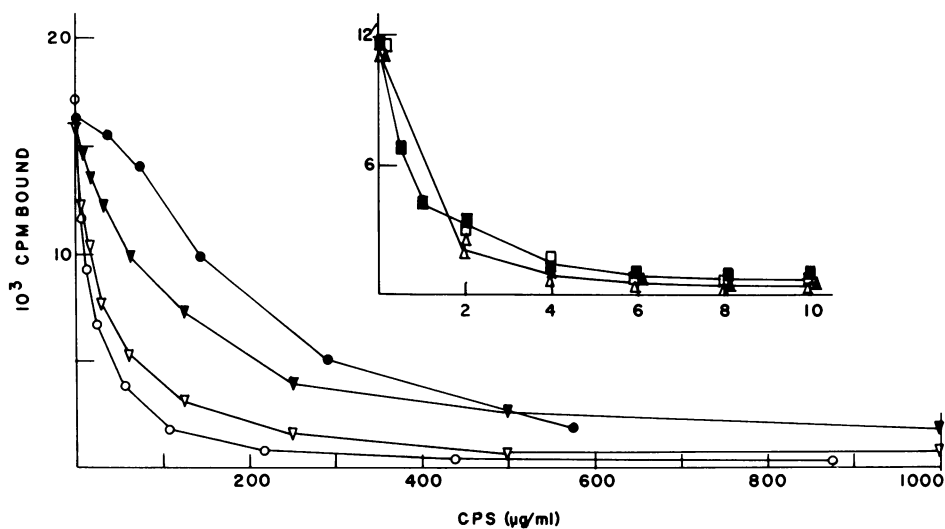


FIG. 6. Saturation of binding of CPS. One milliliter of PBS containing the indicated concentrations of unlabeled CPS-6 ( $\circ$ ), 15-III ( $\bullet$ ), 15-IV ( $\blacksquare$ ), 110 ( $\blacktriangledown$ ), or 145 ( $\nabla$ ) was added to tubes containing  $10^7$  formaldehyde-killed yeast cells of *C. neoformans* 602 and incubated at 25°C. After 1.5 h, 1  $\mu$ g of  $^{125}$ I-CPS-15-IV was added to each tube as a probe. After 1 additional h, bound radioactivity was measured. Each point is the mean of duplicate determinations. The inset depicts a similar experiment with CPS-15-IV and live (solid) and formaldehyde-killed (open) *C. neoformans* 602 ( $\square$ ) and Cap59 ( $\triangle$ ).

TABLE 4. Competition for binding of <sup>125</sup>I-CPS to *C. neoformans* 602 by unlabeled CPS

Competing CPS <sup>a</sup>	% Radioactivity bound, <sup>125</sup> I-CPS of strain <sup>b</sup> :			
	6	15-IV	98	145
6	20	29	8.9	4.5
15-IV	7.0	3.8	4.1	2.1
98	15	17	9.9	3.7
110	40	81	41	15
145	32	55	24	6.0
Mannan	100	105	100	100

<sup>a</sup> CPS at 50 µg/ml and 1.0 µg of indicated <sup>125</sup>I-CPS per ml was added to 10<sup>7</sup> yeast cells of strain 602, and bound radioactivity was determined.  
<sup>b</sup> Percentage of radioactivity that bound in the absence of competing CPS.

solution for 1.0 h and then washed and suspended in 10.0 µg of CPS-15-IV per ml (H → C). If equilibrium conditions pertain, binding under conditions of C → H and H → C should converge over time; they did not, even after 26 h (Fig. 5).

**Saturation.** To determine the saturability of binding of CPS to strain 602, various quantities of unlabeled CPS were added to the yeast cells and the tubes were incubated for 1.5 h, and then <sup>125</sup>I-CPS-15-IV was added and incubation was continued for 1.0 h, after which time the bound radioactivity was determined. The binding was saturable, as increasing concentrations of CPS progressively inhibited the binding of <sup>125</sup>I-CPS (Fig. 6). Each CPS was able to compete with any of the other CPSs for binding (Table 4).

CPSs from different strains varied considerably in their ability to inhibit binding. The CPSs with the highest inhibition potencies in Fig. 6 were the ones with the most efficient binding, viz., 15-IV, 6, and 145. Since most experiments were performed with formaldehyde-killed yeast cells of strain 602, the binding to viable and killed cells of both 602 and Cap59 were compared. All bound comparable amounts of CPS-15-IV.

**Binding to encapsulated yeasts.** To investigate the binding of CPS to encapsulated strains, *C. neoformans* 6, 15, 98, 110, and 145 were cultured for 4 days in GGA-B (29) and binding of <sup>125</sup>I-CPS was evaluated (Table 5). Only negligible amounts (<1%) of radioactivity adhered to encapsulated yeasts, and the addition of 50 µg of CPS-15-IV per ml did not decrease binding; in contrast, the same amount of CPS decreased binding to 602 by more than 90%.

**Proteolytic enzyme treatments.** To determine if the putative receptor on yeast cells could be removed by proteolytic digestion, yeast cells of strain 602 were treated with trypsin or proteinase XIV for 45 min; no change in binding of <sup>125</sup>I-CPS occurred. However, treatment of <sup>125</sup>I-CPS with 0.2 mg of proteinase XIV per ml dramatically decreased <sup>125</sup>I-CPS binding (Fig. 7). Equal concentrations of trypsin, pepsin, papain, or proteinase K had no effect on binding. Activities of all enzymes were confirmed by casein hydrolysis. Treatment of proteinase XIV with PMSF did not change the binding inhibition activity, but heating the enzyme preparation to 65°C for 5 min destroyed the inhibition of binding, while most of the proteolytic activity for casein remained (Table 6). Treatment of <sup>125</sup>I-CPS-6, -15, or -98 with proteinase XIV had no effect on the elution profile from Sepharose CL-2B. Therefore, enzymatic digestion inhibited binding but had no discernible effect on the size or structural integrity of the CPS molecule and was not simply releasing radiolabel. To confirm further that proteinase XIV acted on native CPS as well as on <sup>125</sup>I-CPS, 50 µg of CPS-15-IV per ml was digested overnight at 37°C with 0.2 mg of proteinase XIV per

TABLE 5. Binding of <sup>125</sup>I-CPS to yeast cells of encapsulated strains of *C. neoformans*

<sup>125</sup> I-CPS <sup>a</sup>	<i>C. neoformans</i> strain													
	6		15		98		110		145		602			
cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	cpm added <sup>b</sup>	cpm bound	
6	1.8 × 10 <sup>5</sup>	77	1.8 × 10 <sup>5</sup>	134	2.4 × 10 <sup>5</sup>	210	160	1.8 × 10 <sup>5</sup>	158	2.4 × 10 <sup>5</sup>	259	2.2 × 10 <sup>5</sup>	33,787	2,398
15	2.5 × 10 <sup>5</sup>	288	2.5 × 10 <sup>5</sup>	440	3.2 × 10 <sup>5</sup>	442	345	2.5 × 10 <sup>5</sup>	500	3.2 × 10 <sup>5</sup>	940	3.0 × 10 <sup>5</sup>	98,298	9,158
98	2.0 × 10 <sup>5</sup>	282	2.0 × 10 <sup>5</sup>	484	2.8 × 10 <sup>5</sup>	441	392	2.1 × 10 <sup>5</sup>	444	2.8 × 10 <sup>5</sup>	740	2.2 × 10 <sup>5</sup>	57,476	4,950
110	1.4 × 10 <sup>5</sup>	40	1.4 × 10 <sup>5</sup>	56	1.9 × 10 <sup>5</sup>	72	69	1.5 × 10 <sup>5</sup>	168	1.9 × 10 <sup>5</sup>	93	52	14,018	592
145	9.8 × 10 <sup>4</sup>	48	9.8 × 10 <sup>4</sup>	67	1.2 × 10 <sup>5</sup>	98	72	9.6 × 10 <sup>4</sup>	64	1.2 × 10 <sup>5</sup>	106	72	15,403	519
Mannan	4.1 × 10 <sup>5</sup>	281	4.1 × 10 <sup>5</sup>	142	5.4 × 10 <sup>5</sup>	72	66	4.3 × 10 <sup>5</sup>	487	5.4 × 10 <sup>5</sup>	61	134	76	98

<sup>a</sup> Between 0.25 and 0.5 µg of the indicated <sup>125</sup>I-CPS was added to 10<sup>7</sup> live yeast cells of the indicated strain, and bound radioactivity was determined.  
<sup>b</sup> Total counts per minute added.  
<sup>c</sup> In addition, 50 µg of unlabeled CPS-15-IV was added.

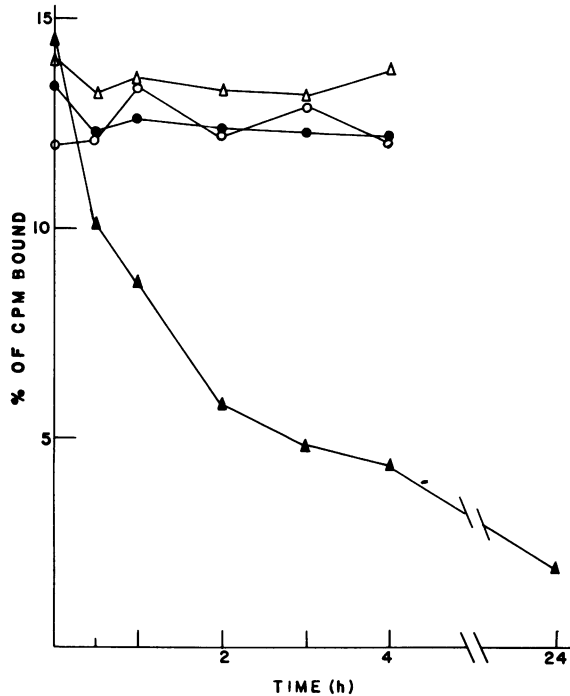


FIG. 7. Abrogation of binding of  $^{125}\text{I}$ -CPS-6 to *C. neoformans* 602 by treatment of  $^{125}\text{I}$ -CPS with proteinase XIV.  $^{125}\text{I}$ -CPS-6 (1  $\mu\text{g}/10 \mu\text{l}$ ) was incubated for the indicated times at  $37^\circ\text{C}$  with an equal volume of 0.4 mg of proteinase XIV per ml (▲), bovine serum albumin (○), PBS (●), or proteinase K (△). After incubation, enzymes were inactivated by boiling, the solutions were diluted to 1  $\mu\text{g}$  of  $^{125}\text{I}$ -CPS per ml, and 1 ml was added to tubes containing  $10^7$  yeast cells of strain 602 for a 2-h incubation. Bound radioactivity was then measured. Points represent means of duplicate determinations. Bound radioactivity is represented as percentage of counts per minute added that bound (approximately 100,000 cpm).

ml and then diluted and used in a saturation assay similar to that depicted in Fig. 6. Digested CPS competed less efficiently than the saline-treated control (Fig. 8).

## DISCUSSION

The major goal of this investigation was to evaluate the binding of CPS to capsule-free mutants of *C. neoformans*. An essential first step was to prepare radiolabeled CPS, and several techniques were considered (14, 20, 22, 28). A method inspired by Keck (14) was developed for radioiodinating CPS that required a moderate pH of 9.5, resulted in high specific activities, caused minimal modification of CPS, allowed the convenience of gamma counting for assay, and did not require the addition of another macromolecule.

CPS required 24 h for adequate derivatization. As CPS consists of an  $\alpha(1\rightarrow3)$  mannan and side chains of glucuronic acid and xylose (2, 5, 24, 29), no *cis*-vicinal diols are present. Periodate oxidation requires vicinal diols, and *cis*-diols are oxidized more rapidly than *trans*-diols (27), probably explaining the slowness of the reaction. CPS-15-III contains galactose with a *cis*-diol pair which may explain its greater efficiency of labeling as compared with peak IV (Fig. 1).

$^{125}\text{I}$ -CPS bound to two capsule-free strains, 602 (15, 16) and Cap59 (13). Results of initial experiments showed that binding of CPS to nonencapsulated *C. neoformans* was receptor mediated, as has been postulated previously (15). The criteria for receptor-mediated binding were specificity,

saturability, and affinity. CPS binding was specific, as radio-labeled dextran, mannan, or dextran sulfate did not bind to strain 602 (Table 2). These data are consistent with previous observations that noncryptococcal polysaccharides do not inhibit the phagocytosis of capsule-free *C. neoformans* (4). Furthermore, CPS did not bind to yeast cells of other species (Table 1), which is in agreement with reports that CPS has no effect on phagocytosis of other microorganisms (4, 15). The prebinding of unlabeled polysaccharide revealed that yeasts did become saturated (Fig. 6). The inhibition of binding with unlabeled CPS also implied saturability (Table 4). Strain 602 had a high affinity for CPS, as most of the available CPS was bound even at concentrations below 1  $\mu\text{g}/\text{ml}$ . Since binding was specific, saturable, and of high affinity, it can be concluded to be mediated by a receptor. This conclusion is, of course, a functional definition and implies nothing about the biochemical interactions involved.

These results are similar to those of Koziel and Hermerath (17), who used different CPS preparations, radiolabeling methods, and binding assays. Both radiolabeling techniques are gentle and easy to perform, especially when compared with the cyanogen bromide reaction (20). The periodate reaction used here cannot be applied to polysaccharides that lack vicinal hydroxyl groups, and the pH approaches 10 during the reduction step. The benzoquinone reaction (17) only requires a single hydroxyl group but, therefore, may not be as specific for carbohydrates. The benzoquinone reaction also adds a bulkier side group of two instead of one aromatic rings. However, the low degree of substitution necessary to achieve high specific radioactivity makes this a minor concern.

The assays used here and by Koziel and Hermerath (17) for evaluation of binding differed mainly in the methods of washing tubes and handling washed counts. Koziel and Hermerath (17) used an exacting method involving extrapolation of bound counts back to zero washes; this technique is undoubtedly superior for rigorous quantitative studies such as the determination of affinities. For the semiquantitative and qualitative questions addressed by this study a more rapid assay was quite satisfactory. Replicate counts yielded coefficients of variation of less than 5%. Furthermore, results of time course and reversibility studies were in excellent agreement. Neither method is appropriate for the measurement of rapidly equilibrating binding. If such binding does occur, a better approach might be a filtration assay.

TABLE 6. Effects of proteases and modified proteases on  $^{125}\text{I}$ -CPS binding to *C. neoformans* 602

Enzyme <sup>a</sup>	$^{125}\text{I}$ -CPS bound (%)	Protease activity ( $A_{280}$ ) <sup>b</sup>
PBS	11.0	0.2
Proteinase XIV <sup>c</sup>	1.4	1.5
Proteinase XIV-PMSF <sup>d</sup>	1.1	1.5
Proteinase XIV (65°C) <sup>e</sup>	11.0	1.1
Papain <sup>c</sup>	11.7	0.55
Trypsin <sup>c</sup>	10.3	1.4
Pepsin citrate (pH 3.5)	11.7	1.3
Citrate buffer (pH 3.5)	11.5	0.16
PMSF	11.5	0.17

<sup>a</sup> One microgram of  $^{125}\text{I}$ -CPS-6 was incubated overnight with the indicated enzyme and the percentage of bound counts per minute was measured.

<sup>b</sup> One milliliter of the enzyme preparation was incubated for 1 h with 1.0 ml of 5% casein. Protease activity is expressed as optical density at 280 nm of a trichloroacetic acid supernatant.

<sup>c</sup> At a concentration of 0.4 mg/ml.

<sup>d</sup> Proteinase XIV was treated with PMSF before incubation with CPS.

<sup>e</sup> Proteinase XIV was heated to  $65^\circ\text{C}$  for 5 min before incubation with  $^{125}\text{I}$ -CPS.

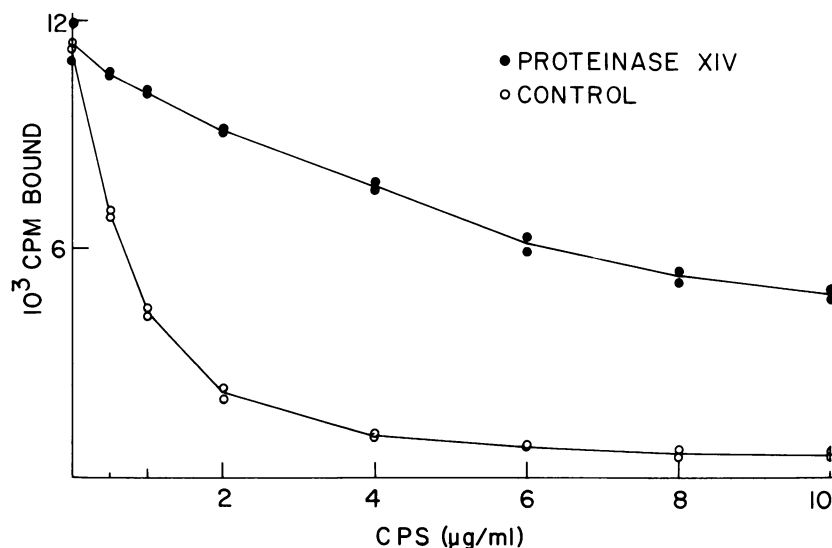


FIG. 8. Reduction of the binding of unlabeled CPS-15-IV to *C. neoformans* 602 by pretreatment of CPS with proteinase XIV. Solutions containing 50 µg of CPS-15-IV per ml were incubated overnight at 37°C with 0.2 mg of proteinase XIV per ml (●) or PBS as a control (○). Protease and control solutions were boiled to inactivate enzymes, diluted to the indicated CPS concentrations, and added to 10<sup>7</sup> strain 602 cells in microfuge tubes. After 1 h, 1 µg of <sup>125</sup>I-CPS-15-IV in 10 µl was added. After a further 1 h of incubation, bound radioactivity was measured. Points represent individual determinations.

The radiolabel binding assay allowed quantitative evaluation of several other aspects of CPS binding. Binding was strong and independent of various assay conditions, including pH, salt or cation concentration, and temperature. Maximum binding occurred within 2 h (Fig. 2).

Classical saturability studies and Scatchard-type analysis could not be applied because the binding curves (data not shown) did not level off at high concentrations, probably because of an inability to measure nonspecific binding and because binding was not freely reversible (Fig. 4 and 5). Scatchard plots (data not shown) were concave upward and did not intersect the *x*-axis.

Methods to evaluate nonspecific binding in a system include withholding some factor, such as a cation, which is essential for binding (12), or adding a large excess (100- or 1,000-fold) of unlabeled ligand and assuming that any resultant binding of label is nonspecific. However, CPS binding seemed to occur equally in all media, and at concentrations higher than 0.5 to 1 mg/ml CPS solutions were too viscous to perform the binding assay. Nevertheless, binding of 1 µg/ml was decreased by more than 90% in the presence of 100-fold excess unlabeled CPS, which indicated that the majority of binding was specific. More accurate determination of nonspecific binding, especially at higher concentrations of <sup>125</sup>I-CPS, was not possible.

The inability to evaluate nonspecific binding may explain the problems with the early saturability studies, but a more serious obstacle to Scatchard analysis arose—binding was not freely reversible. To demonstrate true equilibrium, which is required to validate Scatchard plots, it is necessary to demonstrate that following the addition of excess bound or free ligand, comparable final concentrations of free and bound ligand occur at comparable final total ligand concentrations. Steady-state binding, in which the level of binding does not change with time, is not necessarily equilibrium (31). Binding of CPS to strain 602 reached steady state after 2 h in the routine binding assay, but in two different experiments designed to test the criteria described above equilibrium was not achieved, even after up to 8 days of

incubation (Fig. 4 and 5). Therefore, Scatchard analysis was inappropriate for binding of CPS.

Several experiments were designed to investigate the chemical component of CPS that is recognized by its receptor. Binding was unaffected by 1.0 M xylose, glucuronic acid, and α-methyl mannoside and was only slightly reduced by 1 M mannose (Table 3). A simple lectinlike receptor recognizing a monosaccharide is thus unlikely, but specificity for a disaccharide or other moiety cannot be excluded. Treatment of yeast cells with trypsin, proteinase XIV, or formaldehyde or heating to 70°C did not alter CPS binding—if the receptor is a protein it is remarkably stable, protected, or both. However, despite the very low protein content determined by amino acid analysis (17, 29) treatment of radiolabeled and unlabeled CPSs with protease XIV, a preparation similar to pronase, decreased binding significantly (Fig. 7 and Table 6). Other proteases did not have this effect. Digested and undigested <sup>125</sup>I-CPS had the same elution profiles from Sepharose CL-2B, and unlabeled CPS lost its ability to compete for binding after digestion, eliminating the possibility that Proteinase XIV acted only on labeled material or released label from <sup>125</sup>I-CPS (Fig. 8).

Only a fraction of <sup>125</sup>I-CPS would bind to yeasts. This indicates that some heterogeneity exists in CPS preparations after ion-exchange purification. The nature of the heterogeneity is not known. CPS binding was largely irreversible, even with vigorous chemical treatments. A quantitative nondestructive technique for removing bound CPS would facilitate analysis of the specificity involved in binding.

The binding of solubilized CPS to capsulefree strains may explain the failure to select nonencapsulated mutants on the basis of surface charge by a two-phase separation procedure (13). The two-phase system might be used to select for receptor mutants instead (13, 19).

The periodate-tyramine reaction may have other uses in addition to binding studies. Addition of a UV chromophore like tyramine to a polysaccharide would permit the testing of more dilute solutions to evaluate the dependence of sedimentation behavior on concentration (30). Radiolabeled



polysaccharides would be useful in several other areas, including radioimmunoassays, other types of binding studies, and animal clearance studies.

A multivalent binding model for CPS would predict and explain very slow reversibility of binding and greater binding at higher concentrations, with each bound CPS molecule occupying fewer receptors. Visible capsules might not form even under saturation conditions because CPS molecules could be oriented flush against the yeast cell surface.

However, a multivalent binding model requires that CPS have several sites for binding. The very low protein content in CPS (less than 0.17% [29]) implies that peptide receptors, a strong possibility in light of the protease data, could not be numerous or very large. Monosaccharide residues on CPSs are unlikely candidates as ligands, as none of the sugars examined competed for CPS binding even at 1 M concentrations (Table 3), and the enzyme inhibition curve (Fig. 7) did not show an early plateau. Furthermore, the Sepharose elution profile should change if a contaminating glycosidase in proteinase XIV were removing all of the monosaccharide side chain residues from a CPS molecule. Receptors may recognize a large oligosaccharide unit in the CPS molecule, which is present in only a few copies, or a monosaccharide present in quantities too small to detect. Finally, a lipid moiety such as that described for capsular polysaccharides of gram-negative bacteria must be considered (9). Confirmation or rejection of these possibilities must await finer chemical resolution.

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