Host Modification of Chlamydiae: 
Presence of an Egg Antigen on the Surface of Chlamydiae Grown in the Chick Embryo

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Egg-grown chlamydiae (EGO) have a yolk sac antigen associated with their surface which is absent from cell monolayer-grown organisms (CGO). EGO infectivity was specifically neutralized by rabbit antiserum to normal yolk sac; CGO infectivity, before or after incubation with normal yolk sac material, was not neutralized. Treatment of EGO with Clostridium welchii culture filtrate, containing phospholipase C, abolished spontaneous infectivity for monolayers and neutralization by anti-yolk sac antiserum but did not affect centrifuge-assisted infectivity. The possible significance of host antigen on the chlamydial surface is considered.

INTRODUCTION

Monolayer cultures of established cell lines have now supplanted the chick embryo as the favoured host for isolation of chlamydiae from clinical material. Centrifugation (Gordon & Quan, 1965) is used to enhance the low level of infection which usually results from incubation of organisms with static monolayers (spontaneous infection). With this technique, organisms isolated in eggs can readily be adapted to growth in cell culture without apparent change in antigenic properties (Gordon et al., 1971).

However, studies on infection of cell monolayers by the guinea-pig inclusion conjunctivitis strain (GP-IC) of Chlamydia psittaci have shown that GP-IC grown in the chick embryo (EGO) has a lower spontaneous infectivity for cell cultures than GP-IC grown in cell monolayers (CGO; Griffiths et al., 1976). EGO and CGO behave as two homogeneous populations which differ phenotypically – most probably in surface properties affecting their attachment to monolayers – and are interconvertible by a single passage in the appropriate host (Allan & Pearce, 1979b).

Sim & Stephen (1975), during purification of an egg-grown strain of C. trachomatis, observed that organisms were retained by immunosorbent prepared from antibody to normal yolk sac (NYS) material. Pretreatment of the organism with phospholipase C, however, markedly influenced the pattern of its interaction with both specific and unrelated immunosorbents, suggesting that alteration of the particle surface had occurred. In the light of these observations we have now examined whether EGO differs from CGO in having an associated egg antigen that allows its neutralization by antibody to NYS material and whether this behaviour is affected by enzyme pretreatment. A preliminary account of the work has appeared (Allan et al., 1976).
METHODS

Organism. *Chlamydia psittaci*, strain guinea-pig inclusion conjunctivitis (GP-IC), was grown in eggs (EGO) or irradiated McCoy cells (CGO). Harvesting, purification and enumeration of organisms were as previously described (Allan & Pearce, 1979b).

Cell culture. Preparation of irradiated McCoy cell monolayers and titration of spontaneous and centrifuge-assisted infectivities of organisms were as previously described (Allan & Pearce, 1979b).

Antisera. Antiserum to GP-IC (anti-GP-IC) was produced following the AT2 immunization schedule of Sim (1973), using egg-grown, Renografin-purified (Allan & Pearce, 1979b), live GP-IC as antigen. Rabbits were given subcutaneous injections of 10⁵, 10⁶, 10⁷ and 10⁸ organisms in complete Freund’s adjuvant on days 0, 10, 17 and 25, respectively, and intravenous injections of 10⁷ organisms (without adjuvant) on days 46, 75 and 154. Rabbits were bled on day 164.

A globulin concentrate of rabbit antiserum to normal yolk sac material (anti-NYS) was a gift from Dr J. Stephen, Department of Microbiology, University of Birmingham (Sim & Stephen, 1975). Sheep antiserum to normal rabbit globulin (antiglobulin) was a gift from Dr D. R. Stanworth, Department of Immunology, University of Birmingham.

The sera and concentrated globulin preparation were stored at -20 °C; before use, the globulin preparation was diluted 1:10 in Hanks’ balanced salts solution (HBSS) supplemented with 10% (v/v) foetal bovine serum (HFBS) to give a globulin concentration equivalent to that in serum. The sera and globulin preparation were heat-inactivated at 56 °C for 30 min; further dilutions were made in HFBS.

Enzymic treatment of GP-IC. Suspensions (1 ml) containing 10⁸ organisms were incubated with enzyme solution (1 ml) for 60 min at 37 °C. Enzyme solutions used were: (i) a Clostridium welchii type A freeze-dried culture filtrate containing phospholipase C activity, a gift from Dr J. Stephen (Sim & Stephen, 1975), reconstituted with HFBS to a concentration of 1 mg ml⁻¹ in HBSS; (ii) bacterial protease, type V (Pronase AS, Sigma; 1 mg ml⁻¹ in HBSS); (iii) trypsin, type XII (Sigma; 2 mg ml⁻¹ in phosphate-buffered saline, pH 7.2). After incubation with enzyme solution, organisms were washed twice by centrifugation (12000 g, 30 min, 4 °C) and resuspension in HFBS (10 ml), and titrated for centrifuge-assisted and spontaneous infectivity. Organisms treated with *C. welchii* culture filtrate were rapidly filtered through a column (1 ml) of Sephadex G-15 (Sim & Stephen, 1975) before washing.

Infectivity neutralization. The effect on organism infectivity of treatment with either anti-GP-IC or anti-NYS (stage 1 neutralization) was examined. In some cases this was followed by treatment with antiglobulin (stage 2 neutralization).

For stage 1 neutralization, equal volumes of GP-IC suspension (0-3 to 0-5 ml, containing 10⁷ organisms) and dilutions of anti-GP-IC or anti-NYS (dilution shown in Tables) were incubated at 37 °C for 15 min. As controls, organism suspensions were incubated with appropriate dilutions of normal rabbit serum or HFBS alone. For stage 2 neutralization, samples of the incubated suspensions were then incubated at 37 °C for 15 min with an equal volume of antiglobulin (1 in 3 dilution) or HFBS. As controls, organism suspensions incubated with normal rabbit serum or HFBS in stage 1 neutralization were then incubated with antiglobulin. All samples were diluted in HFBS and titrated for spontaneous and centrifuge-assisted infectivity.

RESULTS

Antibody neutralization as a probe of chlamydial surface properties

Although antibody can inhibit chlamydial attachment to cell monolayers in spontaneous infection, measurement poses technical problems for organisms with inherently low spontaneous infectivity (Ainsworth *et al.*, 1979). Centrifugation, which enhances infectivity, cannot be applied as it overcomes attachment inhibition by alteration of the organism–cell interaction (Allan & Pearce, 1979a). However, if antibody-sensitized organisms are treated with antiserum to the antibody globulin (Gerloff & Watson, 1967; Blyth & Taverne, 1974), neutralization of infectivity can be followed by the centrifugation technique.

We tested the procedure using rabbit antiserum to GP-IC (anti-GP-IC) and sheep antiserum to rabbit globulin (antiglobulin; Table 1). Stage 1 neutralization (anti-GP-IC alone) was 91% for spontaneous infection but only 16% for centrifuge-assisted infection. However, addition of antiglobulin (stage 2 neutralization) gave at least 95% neutralization for both infection modes. Non-specific neutralization by normal serum (stage 1), or HFBS or normal serum followed by antiglobulin (stage 2) was low (maximum 20%) throughout. The method thus appeared suitable for analysis of surface differences between EGO and CGO.
Table 1. Differential neutralization of centrifuge-assisted and spontaneous GP-IC infectivities by antiserum to GP-IC

Suspensions of egg-grown organisms (EGO) were incubated (37 °C, 15 min) in stage 1 neutralization with anti-GP-IC or, as controls, with Hanks' balanced salts solution supplemented with 10 % (v/v) foetal bovine serum (HFBS) or normal rabbit serum. In stage 2 neutralization, samples of the incubated suspensions from stage 1 were incubated (37 °C, 15 min) with antiglobulin or, as a control, with HFBS. After both treatments, samples were further diluted in HFBS and titrated for centrifuge-assisted (CA) and spontaneous (S) infectivities (three replicate monolayers per sample). Neutralization is expressed as the percentage reduction in infectivity relative to that for suspensions incubated in HFBS in both stages 1 and 2.

<table>
<thead>
<tr>
<th>Neutralization treatments</th>
<th>Infectivity neutralization (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1</td>
</tr>
<tr>
<td>HFBS</td>
<td>HFBS</td>
</tr>
<tr>
<td>Anti-GP-IC (1:96)*</td>
<td>HFBS</td>
</tr>
<tr>
<td>Normal serum (1:96)</td>
<td>HFBS</td>
</tr>
<tr>
<td>HFBS</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>Anti-GP-IC (1:96)</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>Normal serum (1:96)</td>
<td>Antiglobulin (1:6)</td>
</tr>
</tbody>
</table>

* Fractions in parentheses indicate serum dilutions during neutralization of organisms.

Table 2. Effect of antiserum to normal yolk sac material (NYS) on the centrifuge-assisted and spontaneous infectivities of egg- and monolayer-grown organisms

Experimental design and presentation of results are as in Table 1, except that anti-NYS was used in place of anti-GP-IC.

<table>
<thead>
<tr>
<th>Neutralization treatments</th>
<th>Infectivity neutralization (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1</td>
</tr>
<tr>
<td>HFBS</td>
<td>HFBS</td>
</tr>
<tr>
<td>Anti-NYS (1:6)</td>
<td>HFBS</td>
</tr>
<tr>
<td>Normal serum (1:6)</td>
<td>HFBS</td>
</tr>
<tr>
<td>HFBS</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>Anti-NYS (1:6)</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>Normal serum (1:6)</td>
<td>Antiglobulin (1:6)</td>
</tr>
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</table>

* Negative values indicate enhancement of infectivity.

Presence of yolk sac antigen on the chlamydial surface

The possibility that EGO might differ from CGO in having an egg antigen associated with their surface was tested by measuring the extent of infectivity neutralization of EGO and CGO by anti-NYS and antiglobulin (Table 2). For EGO, there was considerable neutralization of infectivity, following the same pattern as for anti-GP-IC (Table 1). Thus, only spontaneous infection was reduced in stage 1 neutralization but both centrifuge-assisted and spontaneous infection were reduced in stage 2 neutralization (Table 2). In contrast, CGO showed only low-level neutralization (Table 2) implying that neutralization of EGO by anti-NYS was specific. To confirm this point, anti-NYS was incubated with an equal volume of solution containing 500 µg pelleted NYS substance ml⁻¹ at 37 °C for 15 min, and then its neutralization capacity was tested. Neutralization of the spontaneous infectivity of EGO was reduced from 78 to 13 % (stage 1 procedure) and after treatment with antiglobulin (stage 2 procedure) neutralization of centrifuge-assisted infectivity was reduced from 82 to 22 %; similar treatment of normal serum with NYS material had no effect on EGO infectivity.

Thus, EGO differed from CGO in having a surface-associated yolk sac antigen. If this
Table 3. Effect of incubation of monolayer-grown organisms (CGO) with normal yolk sac material (NYS) on neutralization of CGO infectivity with antiserum to NYS

CGO were incubated at 37 °C for 60 min with 1 mg pelleted NYS substance ml⁻¹, and then washed twice with HFBS by centrifugation (12,000 g, 30 min, 4 °C) and resuspension, to remove excess NYS; these organisms are designated CGO/NYS. As controls, suspensions of CGO and EGO were incubated in HFBS and washed in parallel with the test sample. CGO/NYS and EGO control suspensions were then subjected to the two-stage neutralization procedure and titrated for centrifuge-assisted (CA) and spontaneous (S) infectivities (see Methods and Table 1).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Neutralization treatments</th>
<th>Infectivity neutralization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1</td>
<td>Stage 2</td>
</tr>
<tr>
<td>CGO</td>
<td>HFBS</td>
<td>HFBS</td>
</tr>
<tr>
<td>CGO/NYS</td>
<td>HFBS</td>
<td>HFBS</td>
</tr>
<tr>
<td>EGO</td>
<td>HFBS</td>
<td>HFBS</td>
</tr>
<tr>
<td>CGO</td>
<td>Anti-NYS (1:6)</td>
<td>HFBS</td>
</tr>
<tr>
<td>CGO/NYS</td>
<td>Anti-NYS (1:6)</td>
<td>HFBS</td>
</tr>
<tr>
<td>EGO</td>
<td>Anti-NYS (1:6)</td>
<td>HFBS</td>
</tr>
<tr>
<td>CGO</td>
<td>Anti-NYS (1:6)</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>CGO/NYS</td>
<td>Anti-NYS (1:6)</td>
<td>Antiglobulin (1:6)</td>
</tr>
<tr>
<td>EGO</td>
<td>Anti-NYS (1:6)</td>
<td>Antiglobulin (1:6)</td>
</tr>
</tbody>
</table>

Table 4. Effect of enzymes on centrifuge-assisted and spontaneous infectivities of egg- and monolayer-grown organisms

Suspensions of EGO and CGO were incubated at 37 °C for 60 min with culture filtrate (CF) from Cl. welchii, hyaluronidase, trypsin or protease (see Methods) or, as controls, with HFBS. After treatment, suspensions were washed twice by centrifugation and resuspension. Samples were diluted in HFBS and titrated for centrifuge-assisted (CA) and spontaneous (S) infectivities (three replicate monolayers per sample).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Enzymic treatment</th>
<th>CA</th>
<th>S</th>
<th>S:CA</th>
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<tbody>
<tr>
<td>EGO</td>
<td>HFBS</td>
<td>1050</td>
<td>1:35</td>
<td>1:780</td>
</tr>
<tr>
<td>EGO</td>
<td>CF</td>
<td>700</td>
<td>0*</td>
<td>—</td>
</tr>
<tr>
<td>EGO</td>
<td>Hyaluronidase</td>
<td>890</td>
<td>1:00</td>
<td>1:890</td>
</tr>
<tr>
<td>EGO</td>
<td>Protease</td>
<td>800</td>
<td>1:15</td>
<td>1:695</td>
</tr>
<tr>
<td>EGO</td>
<td>Trypsin</td>
<td>650</td>
<td>0:85</td>
<td>1:765</td>
</tr>
<tr>
<td>CGO</td>
<td>HFBS</td>
<td>95</td>
<td>3:70</td>
<td>1:26</td>
</tr>
<tr>
<td>CGO</td>
<td>CF</td>
<td>160</td>
<td>5:50</td>
<td>1:29</td>
</tr>
<tr>
<td>CGO</td>
<td>Hyaluronidase</td>
<td>70</td>
<td>1:70</td>
<td>1:41</td>
</tr>
<tr>
<td>CGO</td>
<td>Protease</td>
<td>130</td>
<td>4:10</td>
<td>1:31</td>
</tr>
<tr>
<td>CGO</td>
<td>Trypsin</td>
<td>230</td>
<td>8:00</td>
<td>1:29</td>
</tr>
</tbody>
</table>

* No inclusions detected in 100 microscope fields examined per monolayer.

had become adsorbed during isolation of organisms from the yolk sac then it would be reasonable to infer that CGO, established as a phenotypic variant of EGO, could be converted to EGO by incubation with yolk sac material. However, incubation at 37 °C for 60 min with 1 mg pelleted NYS substance ml⁻¹ failed to convert CGO (spontaneous:centrifuge-assisted infectivity ratio, 1:20) to EGO-like infectivity properties (spontaneous:centrifuge-assisted infectivity ratio, 1:700; Allan & Pearce, 1979b). Nor was there any evident adsorption of yolk sac antigen to CGO by the more stringent test of anti-NYS neutralization (Table 3).

Susceptibility of chlamydial surface antigens to enzymic treatment

The failure of CGO to adsorb yolk sac antigen suggested that the egg antigen associated with EGO was not an artefact of organism isolation. To gain further information on the
Host modification of the chlamydial surface

Table 5. Effect of treatment of egg-grown organisms (EGO) with Cl. welchii culture filtrate on the ability of antiserum to normal yolk sac material (NYS) to neutralize EGO infectivity

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Neutralization treatments</th>
<th>(10^{-4}\times) Infectivity titre (inclusion-forming units ml(^{-1}))</th>
<th>Neutralization (% of CA infectivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGO</td>
<td>HFBS HFBS</td>
<td>1200 4.6 0</td>
<td></td>
</tr>
<tr>
<td>EGO</td>
<td>Anti-NYS HFBS</td>
<td>1300 0.81 -8</td>
<td></td>
</tr>
<tr>
<td>EGO</td>
<td>Anti-NYS Antiglobulin</td>
<td>51 NT 96</td>
<td></td>
</tr>
<tr>
<td>EGO/CF</td>
<td>HFBS HFBS</td>
<td>1100 0.23 9</td>
<td></td>
</tr>
<tr>
<td>EGO/CF</td>
<td>Anti-NYS HFBS</td>
<td>910 NT 24</td>
<td></td>
</tr>
<tr>
<td>EGO/CF</td>
<td>Anti-NYS Antiglobulin</td>
<td>1040 NT 13</td>
<td></td>
</tr>
</tbody>
</table>

NT, Not tested: CF abolished spontaneous infectivity of EGO (Table 4).

nature of the association, the effect of various enzymes on infectivity was examined. Somewhat surprisingly, most enzymes caused little change (Table 4). Hyaluronidase, protease and trypsin did not alter EGO infectivity appreciably. However, Cl. welchii culture filtrate (CF) containing phospholipase C activity completely abolished spontaneous infectivity while leaving centrifuge-assisted infectivity unimpaired - retention of the latter indicated that no inactivation had occurred. None of the treatments reduced CGO infectivity (Table 4); indeed, trypsin and CF caused some increase, probably by disaggregation of small clumps of organisms.

The differential action of CF on spontaneous infectivity provided further evidence of surface differences between EGO and CGO. Since anti-NYS had also neutralized the spontaneous infectivity of EGO it seemed possible that the egg antigen present on EGO might have been affected by the CF treatment. This was examined by comparing neutralization, by anti-NYS, of EGO infectivity before and after enzyme treatment, applying antiglobulin (stage 2 neutralization) to test for alteration in centrifuge-assisted infectivity. The latter was largely unimpaired (Table 5), indicating that CF had abolished egg antigen specificity on EGO.

**DISCUSSION**

EGO were previously distinguished from CGO by their lower spontaneous infectivity (Allan & Pearce, 1979b). We have now shown that they also differ in bearing an egg antigen (or cross-reacting antigen) and in their susceptibility to the Cl. welchii culture filtrate. The unilateral inhibition by CF of spontaneous infection, but not centrifuge-assisted infection, is similar to the effect of attachment-inhibiting antibody (Ainsworth et al., 1979) and provides further evidence that the organism–cell interaction is altered in centrifuge-assisted infection (Allan & Pearce, 1979a).

Unlike Blyth & Taverne (1974) we observed little non-specific neutralization after treatment of organisms with normal serum and antiglobulin. In view of their findings it is probably always desirable to demonstrate the specificity of the antibody interaction by appropriate absorption, as was done with NYS absorption of anti-NYS.

Our results do not resolve the question of how the differences between EGO and CGO arise. EGO may differ from CGO only in respect of their egg antigen. They may carry other distinctive antigens, not so far detected, which together determine EGO affinity for cell monolayers (which probably governs spontaneous infectivity) and their behaviour towards CF. The simplest possibility is that EGO are CGO with adventitiously adsorbed egg antigen. 
However, incubation of NYS with CGO did not affect spontaneous infectivity or anti-NYS neutralization. Moreover, CF, although apparently removing egg antigen, did not restore the spontaneous infectivity shown by CGO. It seems unlikely that CF treatment left adsorbed egg antigen residues on the EGO surface which, although no longer serologically functional, were capable of completely inhibiting spontaneous infection.

Egg antigen may be present on all chlamydiae grown in the chick embryo. In early work Moulder & Weiss (1951) used anti-egg antiserum to purify egg-grown C. psittaci (strain feline pneumonia) and recorded partial losses. For serological purposes, antisera raised against egg-grown chlamydiae are frequently absorbed with yolk sac material because of the yolk sac antigen invariably found in chlamydial preparations. The presence of egg antigen on the chlamydial surface would thus go unrecognized unless organisms were reacted with anti-egg antibody.

Our observations raise the question whether animal or tissue cells endow chlamydiae with a host antigen. In the preceding paper we mentioned instances in which the host appears to exert an effect on chlamydial properties (Allan & Pearce, 1979b). If host antigens are present on the particle surface in close juxtaposition to chlamydial antigens, then autoimmune reactions could be induced which would contribute to the immunopathological damage seen in chronic trachoma. Further, if organisms grown in one host cell infect that cell more readily than others (CGO have a higher spontaneous infectivity for cell monolayers than EGO) then modification by the host could be viewed as moderating the host range of chlamydial infections.

We thank Dr J. Stephen, and other colleagues, for helpful discussion. I.A. was in receipt of an MRC research studentship.

REFERENCES


The Binding of Enzymes to Fungal β-Glucans

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Purified preparations of the extracellular glucan of Sphacelia sorghi and the walls of Claviceps purpurea were shown to contain enzymically active protein. The glucans and walls were treated in various ways to liberate the protein and determine the nature and strength of its binding, if any, to each matrix. Binding of protein to the glucan appeared to be non-covalent and was specific only in that the native protein seemed to bind more firmly than protein derived from other fungal genera. Binding of protein to walls appeared to be stronger than to the glucan but in each case protein was found to be less stable when freed from its carbohydrate matrix. Protein liberated from glucan showed enhanced activity although its $K_m$ remained the same; thus, this matrix acted in the same manner as a non-competitive inhibitor. Protein liberated from walls was less active than when attached. The differences between the two matrices, with respect to protein binding, are discussed in the light of possible differences in their tertiary structure.

INTRODUCTION

The presence of β-linked glucans in fungi is now well established; they are found both intra- and extracellularly and in the walls (Clarke & Stone, 1963; Perlin & Taber, 1963; Buck et al., 1968; Bartnicki-Garcia, 1970; Dickerson et al., 1970; Wessels et al., 1972; Bartnicki-Garcia, 1973). Wall glucan and extracellular glucans have been proposed as ‘elicitors’ of host response for fungi that are pathogenic on plants (Keen, 1975; Keen et al., 1975; Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976a, b, c; Ebel et al., 1976; Anderson, 1978; Stekoll & West, 1978). Glucans of the same type are also known to have antitumour activity (Whistler et al., 1976). The commonest of the glucans is a homopolymer of glucose residues with a main chain of (1→3)-β-D linkages. Occasionally, other residues are present (Clarke & Stone, 1963) and ‘elicitors’ have been reported to contain 4% mannosyl residues (Ayers et al., 1976c). The main chain of these polysaccharides is frequently branched; the commonest type of branch is a single (1→6)-β-linked glucose residue, but longer branches are known to exist (Fleet & Manners, 1976, 1977; Sietsma & Wessels, 1977). The molecular weights reported for these polymers range widely, from about $3 \times 10^3$ to $5 \times 10^6$ (Clarke & Stone, 1963; Anderson-Prouty & Albersheim, 1975; Ayers et al., 1976c). Extracellular glucans, in addition to wall glucans, of the above type are a common feature of several fungi including the plant pathogens Claviceps and Sphacelia (Perlin & Taber, 1963; Buck et al., 1968). During the course of structural studies on purified extracellular glucans from these fungi grown in culture, it was noted that some glucan samples underwent slow hydrolysis (about 1% h$^{-1}$) when incubated alone in buffers of pH 4.5 to 7.5. Hydrolysis was subsequently found to be due to an associated exo-(1→3)-β-D-glucanase (Dickerson et al., 1970). Similar phenomena have been reported elsewhere, e.g. from studies on walls of Schizosaccharomyces (Fleet & Phaff, 1974) and Saprolegnia (Fèvre, 1977). The autohydrolysis of the branched extracellular glucans of Claviceps purpurea (Perlin & Taber, * Present address: Abbott Laboratories Ltd, Queenborough, Kent ME11 5EL.

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1963) and Claviceps fusiformis (Buck et al., 1968) yielded only glucose; the presence of other enzymes capable of hydrolysing (1→6)-ß linkages was therefore suspected.

We report here studies on the nature of the association between enzymes and fungal glucans with special reference to a ß-fructofuranosidase (‘invertase’) from Sphacelia sorghi and an extracellular glucan produced by the same fungus.

METHODS

Organisms. The Claviceps purpurea strain (29/4) was the same as that used by Dickerson et al. (1976). Sphacelia sorghi strain R18B is a derivative of a strain originally isolated from sclerotia on Sorghum vulgare in Nigeria (Mantle & Waight, 1968; Mantle, 1968). Basidiomycete QM806 (CMI 155, 771) was supplied by the Commonwealth Mycological Institute, Kew, Surrey.

Medium and growth conditions. These were the same as those described by Dickerson et al. (1970).

Analytical methods. Total nitrogen was determined, after digestion of samples in concentrated sulphuric acid and selenium followed by distillation in a micro-Kjeldahl apparatus, using the alkaline phenol reagent (Garza & Weissler, 1967). Total carbohydrate was estimated by the anthrone method (Morris, 1948) and by use of the phenol-sulphuric acid reagent (Dubois et al., 1951, 1956). Protein and nucleic acids in solution were determined from absorbance measurements at 260 and 280 nm; protein was also determined by the method of Lowry et al. (1951) and from total nitrogen measurements on the assumption that all the nitrogen was derived from protein. Phosphorus was determined by the method of Fennell et al. (1957) using quinoline hydrochloride. Chromatographic procedures for analysis of sugars obtained by acid or enzymic hydrolysis of the glucan were similar to those described by Buck et al. (1968). Values are the mean of at least three replicates with a variation of less than 10% overall for both analyses and assay procedures (see below). Unless otherwise indicated, other results given are the mean of values obtained from at least three different preparations (see below) with a variation not exceeding 15% overall.

Production of exo-(1→3)-ß-d-glucanase. This enzyme was isolated and purified from culture filtrates of Basidiomycete QM806 following the methods of Reese & Mandels (1966).

Enzyme assays. The activity of ß-glucosidase was determined as described by Dickerson et al. (1970) and ‘invertase’ (ß-fructofuranosidase) was assayed by methods similar to those described by Dickerson et al. (1970) and Dickerson (1972). Phosphatase activity was estimated by the method of Brandenberger & Henson (1953) using O-carboxyphenylphosphate. Incubations were normally carried out with 1 mg protein (or equivalent weight of glucan with the protein) per ml incubate using 1-5% (w/v) substrate. Incubations with exo-(1→3)-ß-d-glucanase were carried out as described by Buck et al. (1968). Results obtained as µmol substrate utilized, or product formed, per ml incubate per mg protein were subjected to first-order plots from which the rate constant (k) was determined.

Isolation, purification and analysis of glucan. Glucan was separated from fungal tissue by successive filtrations through filter paper (Whatman no. 54) until no further cell debris could be detected on microscopic examination of the filtrates. Polysaccharide was precipitated by mixing the filtrate with 1 to 2 vol. absolute ethanol or acetone. The precipitated polysaccharide was collected on a glass rod, drained and resuspended in water using the same volume as that of the original filtrate. The polysaccharide was then reprecipitated and collected as before. The procedure was repeated until the supernatant from ethanol and washed in a Mickle disintegrator in the presence of 8 M-urea (Buck et al., 1968). Results obtained as µmol substrate utilized, or product formed, per ml incubate per mg protein were subjected to first-order plots from which the rate constant (k) was determined.

Isolation and purification of walls. Fungal wall preparations were obtained using methods essentially similar to those of Fleet & Manners (1976). The cells were crushed using an X-press (Dickerson et al., 1970) and washed in a Mickle disintegrator in the presence of 8 M-urea (Buck et al., 1968). All preparations were checked microscopically to ensure that the walls had no adhering cytoplasm.

Separation of glucan from bound protein. The following methods were used:

(i) A low viscosity solution of glucan (0-4%, w/v) in 8 M-urea, at approximately neutral pH, was stirred for 1 h at room temperature; the polysaccharide was then selectively precipitated by the slow addition of ice-cold absolute ethanol or acetone until a concentration of 45 to 50% (v/v) was reached. With this procedure the protein remained in solution. After removing the polysaccharide with a glass rod, the protein was precipitated by increasing the concentration of solvent to 70% (v/v) The whole process was repeated three more times using the same polysaccharide sample. Protein was recovered from the combined alcoholic mixtures by centrifugation at 15 000 g for 20 min; it was then washed in 70% (v/v) ethanol and water, dissolved in 5 mM-acetate buffer, pH 5-2, (1 mg ml⁻¹) and dialysed overnight at 4°C against the same buffer.
Enzyme binding to fungal glucans

The dialysed product was freeze-dried. The whole procedure was repeated replacing the urea by either 6 M-guanidine hydrochloride or 2% (w/v) sodium dodecyl sulphate.

(ii) Glucan solutions, as above, were stirred for 1 h at 25 °C with 0-4% (w/v) DEAE-cellulose (Whatman DE11) in 0-1 M-acetate buffer, pH 4-0, 5-1 or 5-6. The cellulose was recovered by centrifugation at 15000 g for 10 min, washed with the same buffer as used initially, recentrifuged and the residue was brought back to its original w/v by adding 0-2 M-NaCl in 10 mm-acetate buffer at the corresponding pH. The cellulose was removed as before and the supernatant was dialysed overnight at 4 °C against 5 mm-acetate buffer, pH 5-2, containing 0-5 mm-EDTA. The dialysed product was freeze-dried.

(iii) Glucan (4%, w/v) in 50 mm-acetate buffer, pH 5-2, was hydrolysed for 3 h at 30 °C using the exo-(1→3)-β-d-glucanase from Basidiomycete QMS06. Protein, initially bound to the glucan, was liberated from the products of hydrolysis (glucose only in unboiled, enzymically active samples; glucose and gentiobiose, in a molar ratio of approximately 2:1, in previously boiled samples) by dialysis as in (ii). The invertase in the dialysis residue was partially purified and freed from exo-(1→3)-β-d-glucanase by passage through a column (46 × 3 cm) of Sephadex G-100 at 4 °C using 10 mm-sodium phosphate buffer, pH 6-0, as eluant. Walls were hydrolysed in a similar manner for 16 h; solid residue following hydrolysis was removed by centrifugation at 15000 g for 10 min before column chromatography.

Gel electrophoresis. Glucan (1-0 mg) was suspended in 100 ml of 50% (w/v) sorbitol in distilled water. Samples (10 ml) were applied to 8% (w/v) gels prepared by the method of Weber et al. (1972) for undenatured protein and run at 4 °C for 16 h at 7 mA per gel. Gels were stained for protein with Coomassie blue (Chrambach et al., 1967), for carboxylate with periodic acid–Schiff reagent (Fairbanks et al., 1971) and for invertase specifically with 2,3,5-triphenyltetrazolium chloride after pre-incubation of the gel with 1-5% (w/v) sucrose (Gabriel & Wang, 1969). Stained gels were scanned using a Gilford 240 spectrophotometer with linear transport attachment.

Assay of glucan on gels. Transverse sections of gels were macerated and then incubated overnight with exo-(1→3)-β-d-glucanase in the usual manner (see above). The mixture was then filtered through glass wool to remove gel and the filtrate plus washings were analysed for products.

Assay of β-fructofuranosidase (invertase) on gels. Invertase was specifically identified by the method described above. In addition, transverse sections of gels were macerated and the protein was eluted at 4 °C by immersion overnight in 10 mm-sodium phosphate buffer, pH 6-0, (2 ml per section). Gel was then removed, as above, and filtrates and washings were incubated with 1-5% (w/v) sucrose to assay for invertase activity.

Reconstitution of protein–glucan complex. Protein-free glucan, prepared by the urea treatment, was solubilized in 5 mm-acetate buffer, pH 5-2, and stirred for 1 h at 25 °C with glucan-free protein prepared by the action of exo-(1→3)-β-d-glucanase on glucan with bound protein. The ratio of glucan to protein in the stirred solutions was 10:1 and the glucan concentration was normally 0-4% (w/v). The reconstituted product was purified as above by alcohol precipitation.

Continuous flow system for testing the stability of glucan-bound enzyme. The apparatus comprised a sterilized 100 ml Erlenmeyer flask, containing 60 ml of a sterile solution of sucrose (1-5%, w/v) in 1 mm-sodium phosphate buffer, pH 6-0. The flask, which had a narrow side-arm inserted near the base, was mounted on a magnetic stirrer to permit mixing of the contents by a 10 mm PTFE-coated magnetic flea. A drawn-glass jet was sealed through a rubber stopper used to close the flask. The jet was connected to a 1 l substrate reservoir which was fitted with an air filter. The reservoir contained 600 ml of a sterile solution of sucrose (1-5%, w/v) in buffer, as above. The flow rate was governed by a peristaltic 'Varioperpex' pump (LKB Instruments) connected between the reactor side-arm and a fraction collector. A glucan suspension (1-0 ml; 4%, w/v) was enclosed, using aseptic conditions, in each of three sachets of effective length 4 cm, made of previously boiled, 0-635 cm diameter Visking cellophane tubing. Alternatively, one sachet only, containing 1% (w/v) glucan-free protein, was used. Sachets were introduced aseptically into the reactor. Internal pressure ensured that the reaction volume remained constant. The linear flow rate, even at the lowest rate used (1-4 ml h⁻¹), was sufficient to prevent adventitious contamination by bacteria [the fastest moving bacterium, Proteus mirabilis, can achieve velocities of up to 1·0 × 10⁻⁴ cm s⁻¹ (Burrows, 1963); the lowest linear flow rate, using 3 mm diameter tubing, was 0·5 × 10⁻⁴ cm s⁻¹]. Incubations were carried out at 25 °C and the glucose content of the effluent was normally monitored at intervals of 15 min, and not greater than 1 h.

RESULTS

Samples of purified glucan from S. sorghi dried to constant weight at 70 °C had a water content of approximately 10% (w/w). Allowing for the degree of hydration, protein estimations on aqueous solutions of the glucan indicated the presence of between 4 and 14% (w/w) protein for 20 samples tested; the mean was 7·59 (σn−1 = 2·49). The lower protein
Table 1. Isolation of protein from fungal β-glucans

‘Purified’ glucan (100 mg) was subjected to various treatments to separate protein and carbohydrate as described in Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg)</th>
<th>Carbohydrate [mg (mg protein)⁻¹]</th>
<th>Residual glucan [mg (mg protein)⁻¹]</th>
<th>Invertase activity [10⁴ × k (s⁻¹)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.2</td>
<td>12.9*</td>
<td>ND</td>
<td>1.9</td>
</tr>
<tr>
<td>Urea (8 M)</td>
<td>6.3</td>
<td>&lt; 0.1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Guanidine hydrochloride (6 M)</td>
<td>6.7</td>
<td>&lt; 0.1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (2%, w/v)</td>
<td>5.9</td>
<td>&lt; 0.1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>DEAE-cellulose (pH 4.0)</td>
<td>0.3</td>
<td>1.3</td>
<td>1.2</td>
<td>2.6</td>
</tr>
<tr>
<td>DEAE-cellulose (pH 5.1)</td>
<td>0.8</td>
<td>1.4</td>
<td>1.2</td>
<td>3.0</td>
</tr>
<tr>
<td>DEAE-cellulose (pH 5.6)</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>3.2</td>
</tr>
<tr>
<td>DEAE-cellulose (pH 5.6)†</td>
<td>1.0</td>
<td>0.8</td>
<td>0.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Exo-(1→3)-β-D-glucanase</td>
<td>7.0</td>
<td>&lt; 0.1</td>
<td>0</td>
<td>5.8</td>
</tr>
</tbody>
</table>

ND, Not determined.
* Obtained by difference, starting with 100 mg glucan.
† Retreatment of first pH 5.6 treatment.

concentrations were normally found in cultures harvested after 4 to 7 d and the higher concentrations in those harvested after 14 d. The protein concentrations were confirmed by total nitrogen estimations and by analysis of protein and glucose and gentiobiose liberated after treatment of the glucan with the exo-(1→3)-β-D-glucanase. Carbohydrate (i.e. β-glucan) and protein accounted for not less than 98% of the weight of the dehydrated glucan. Absorbance measurements on solutions suggested the presence of nucleic acid to an extent not exceeding 0.5% (w/w); phosphorus estimations showed a concentration of less than 0.1% (w/w), consistent with the presence of a small amount of nucleic acid. Chromatography failed to detect any low molecular weight sugars or amino compounds.

The molar ratio of glucose to gentiobiose liberated by the exo-(1→3)-β-D-glucanase treatment gave the average degree of branching of all the glucan samples tested as approximately one branch on every third residue, as has been found for glucans from Claviceps spp. (Buck et al., 1968; Dickerson et al., 1970); this might be expected as S. sorghi is a closely related organism (Mower et al., 1973). Associated protein exhibited the following hydrolytic enzyme activities when the glucan was incubated with the appropriate substrate (activities, ± 15% for six samples tested, are expressed relative to β-fructofuranosidase activity – see k value, Table 1): β-fructofuranosidase (invertase; 1.00), β-glucosidase (0.65), acid phosphatase (2.02), exo-(1→3)-β-D-glucanase (0.04). In the subsequent studies, only invertase activity was routinely monitored. Table 1 shows typical results for the liberation of protein from a purified glucan sample following various treatments. Fractional precipitation with ethanol in the presence of 8 M-urea, 6 M-guanidine hydrochloride or 2% (w/v) sodium dodecyl sulphate removed 80 to 90% of the protein from the glucan. On recovery, the freed protein was enzymically inactive in each case. Addition of DEAE-cellulose to the glucan resulted in some of the protein being transferred to the cellulose. The best results were obtained at pH 5.6 with an equal weight of cellulose, when approximately 15% of the protein was transferred; increasing the ratio (w/w) of cellulose to glucan did not significantly enhance this value. Protein isolated from the cellulose usually contained an approximately equal weight of carbohydrate; as most of this was accounted for by glucose, following hydrolysis of samples using the exo-(1→3)-β-D-glucanase, it was considered to be residual glucan. Approximately 25% of the residual glucan could be removed after further treatment with DEAE-cellulose at pH 5.6 where it was found that all protein recovered from the first treatment re-bound (Table 1). Liberation of protein by hydrolysis of the glucan matrix with exo-(1→3)-β-D-glucanase resulted in preparations that were completely free from contaminating glucan and had qualitatively the same invertase action (Dickerson,
Table 2. Partial separation of protein and carbohydrate in 'purified' glucan by gel electrophoresis

'Purified' glucan (100 μg) in 10 μl of 50 % (w/v) sorbitol in distilled water was placed on the top of 8 % (w/v) gels. Electrophoresis was carried out at 4 °C for 16 h at 7 mA per gel. Gels were assayed for carbohydrate, protein and β-fructofuranosidase activity as described in Methods.

<table>
<thead>
<tr>
<th>Distance from top of gel (mm)</th>
<th>Percentage of original glucan carbohydrate [10^4 x k (s⁻¹)]</th>
<th>Invertase activity [10^4 x k (s⁻¹)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6</td>
<td>40-0</td>
<td>3-0</td>
</tr>
<tr>
<td>6-26</td>
<td>28.8</td>
<td>0</td>
</tr>
<tr>
<td>26-46</td>
<td>9.2</td>
<td>0</td>
</tr>
<tr>
<td>46-61</td>
<td>&lt; 1.0</td>
<td>0</td>
</tr>
<tr>
<td>61-76</td>
<td>0</td>
<td>5-1</td>
</tr>
<tr>
<td>76-101</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Reconstitution of protein–glucan complex

Protein (10 mg) and protein-free glucan (100 mg; 0.4 %, w/v) were stirred for 1 h at 25 °C in 5 mM-acetate buffer, pH 5.2. The polysaccharide was then precipitated with ethanol and taken through the routine purification procedure (see Methods). Associated protein was measured at each stage after resuspension of the precipitated glucan in water (0.4 %, w/v).

<table>
<thead>
<tr>
<th>No. of ethanol</th>
<th>Associated S. sorghi protein [mg (100 mg glucan)⁻¹]</th>
<th>10^4 x k (s⁻¹)</th>
<th>Associated yeast invertase [mg (100 mg glucan)⁻¹]</th>
<th>10^4 x k (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9-0</td>
<td>ND</td>
<td>6.7</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>ND</td>
<td>4.9</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>8.3</td>
<td>ND</td>
<td>3.7</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>8.2</td>
<td>0.18</td>
<td>3.0</td>
<td>11.4†</td>
</tr>
</tbody>
</table>

ND, Not determined.
* For invertase.
† For the original free enzyme, k = 18.6 x 10⁻³ s⁻¹.

1972) but more than double the specific activity of the associated enzyme. As the \( K_m \) of the associated enzyme (4.0 x 10⁻² M) did not alter on liberation, the presence of glucan must modify the action of the enzyme in a manner analogous to that of a non-competitive inhibitor.

Partial separation of protein and glucan could be effected by gel electrophoresis (Table 2); over 70 % of the glucan was retained within the top 3 cm of the gel, and small amounts diffused down to about 5 cm. The protein was separated into 15 detectable bands; four of these, comprising 27 % of the total protein, were in the same area of the gel as the glucan. The other 11 bands accounted for the rest of the protein. Invertase activity was found in two bands only: one occupied the top 2 mm of the gel, i.e. the topmost of the protein bands in the area of the gel occupied by the glucan, and comprised about 15 % of the total protein; the other was a single band, comprising about 4 % of the total protein, between 6 and 7.5 cm from the top. The activity of the enzyme in the active areas was similar to that observed for the total protein; the electrophoretic procedure used appears, therefore, to inactivate the enzyme significantly. However, as noted before, the enzyme was less active in the presence of the glucan.

Reconstitution of the protein–glucan complex from protein-free glucan and protein isolated from exo-(1→3)-β-D-glucanase digests (Table 3; same sample as that referred to in Table 1) gave preparations which had approximately the same enzyme activity as the original material. A commercial preparation of invertase (baker's yeast; Sigma, grade VI) could replace the S. sorghi protein but the amount remaining firmly associated with the glucan
Fig. 1. Effect of temperature on the enzyme activity of free protein (●) and glucan-associated protein (○). Sucrose (1.5%, w/v) was incubated in 10 mM-sodium phosphate buffer, pH 6.0, with either glucan-associated or free protein (1 mg ml⁻¹) at various temperatures. First-order reaction rates were determined in each case and the results are expressed as pmol substrate utilized (ml incubate)⁻¹ h⁻¹.

Fig. 2. Effect of prolonged incubation on the enzyme activity of free protein (-----) and glucan-associated protein (——). Sucrose (1.5%, w/v) was incubated at 25 °C in 1 mM-sodium phosphate buffer, pH 6.0, with either glucan-associated or free protein, each enclosed in dialysis tubing, in the continuous flow apparatus described in Methods. Results are expressed as the concentration of glucose in the effluent from the apparatus.

was much less than that of the ‘native’ protein. The activity of this enzyme was depressed by the presence of glucan in a similar manner to that of the S. sorghi enzyme.

Wall preparations from C. purpurea (used instead of S. sorghi as little or no glucan is produced in culture), when treated with exo-(1→3)-β-D-glucanase in the same manner as the S. sorghi extracellular glucan, showed a dry weight loss following hydrolysis of between 60 and 70%. Glucose liberated during the treatment accounted for 34 to 36% (w/w) of wall material and liberated protein for 4 to 5%. The original wall preparations were enzymically active (mean \( k = 5.63 \times 10^{-4} \) s⁻¹) but residual wall material after hydrolysis showed no activity. As with the glucan, protein liberated by the action of the exo-(1→3)-β-D-glucanase on the walls was also active but, unlike the glucan protein, less so than the associated form (mean \( k = 0.72 \times 10^{-4} \) s⁻¹).

The stability of the enzymes associated with extracellular glucans and walls was investigated as they were not inactivated by the purification procedures for either matrix and resisted inactivation for periods up to 15 min in boiling water. Figure 1 shows the effect of temperature on invertase associated with the extracellular glucan of S. sorghi and on the freed enzyme, and demonstrates the increased stability of the former. With walls, the stability of the associated protein at elevated temperatures was not so marked; nevertheless, at 50 °C it retained at least 20% of the enzyme activity that it had at 25 °C. Figure 2 shows that the glucan-associated enzyme, enclosed in dialysis tubing in a continuous flow system, stabilized in activity at 5 d and remained at this level for the duration of each experiment (4 to 5 weeks); its functional half-life at several flow rates was at least 30 times that of freed enzyme under the same conditions.
DISCUSSION

Preparations of the extracellular glucan and walls of S. sorghi contained protein that could not be removed by the procedures used to purify the glucan. The amount of protein associated with the extracellular glucan showed some variation; part of this can be accounted for by the tendency of the protein levels associated with the glucan to increase with the age of the culture, but some of the difference might also be attributable to variations in the conformation of both protein and glucan (see below).

The comparatively rigorous methods required for complete separation of the carbohydrate and protein fractions of the mixture, the relative ineffectiveness of DEAE-cellulose in removing protein even with dilute glucan solutions, the ease of reconstitution of the mixture to give a product closely resembling the starting mixture and the thermal stability of the protein associated with the glucan relative to the free protein are all consistent with the protein being non-covalently bound to the glucan. The two polymers, especially if tertiary structure is exhibited and as they are native to the same organism, might be expected to associate by, for example, hydrogen-bonding. The energy derived from this type of association could account for the thermal stability of the bound protein. The native protein, exemplified by invertase, appeared to associate more readily with the glucan than invertase from other fungal sources (e.g. yeast); allowing for the fact that the yeast invertase probably contained inert material in the form of highly denatured protein, this partial specificity might also be explained if the conformation of the native protein molecules corresponded more closely with those of the glucan and thus facilitated binding. Such an affinity might be mediated or enhanced if a specific substrate link between native enzyme and glucan existed. In the continuous flow studies, the initial decline from maximal invertase activity, which was arrested after 2 d, could be attributed to two different invertases associated with the glucan. Alternatively, a single protein only may be represented, the major portion of which is firmly bound and the residue either loosely bound or unbound and hence rapidly inactivated. The electrophoretic studies with glucan-associated protein also demonstrate separable invertase activities; again this may represent two invertases or a single protein with the major portion remaining in the same area of the gel as occupied by the glucan and a minor, more mobile, portion that could represent the loosely bound or unbound enzyme.

The ability of β-glucans, particularly the insoluble variety which normally have a very low degree of branching (Villanueva et al., 1976), to bind proteins has been used to purify, by selective adsorption, hydrolases for which the glucans themselves are the natural substrate (Clarke & Stone, 1965; Villa et al., 1976). Branching increases the solubility of the glucans in aqueous media and from the present work this appears to enhance the degree to which proteins, in addition to those which catalyse glucan hydrolysis and are implicated in wall extension (Villanueva et al., 1976; Kritzman et al., 1978), may bind. From conformational studies a distinct tertiary structure for the unbranched (1→3)-β-linked glucan has been proposed, consisting of a triple helix stabilized by hydrogen bonds between substituents on C-2 of each residue (Atkins et al., 1969; Rees, 1973). As the turns of the helix result in the substituents for branch formation on C-6 being directed towards the outside of the helix and away from its axis, substitution to give branches should not interfere with the basic tertiary structure. An increased facility for the non-covalent binding and stabilization of proteins may, therefore, be due to the addition of branches to the secondary structure of the polymer or by addition to an existing tertiary structure. The conformational entropy of polysaccharides suggests that a distinct tertiary structure is less likely to occur when the polymer is in solution (Rees, 1973); therefore the triple helix might be the predominant form of the glucan in the fungal wall, but not necessarily in the extracellular glucans elaborated in liquid culture. If this structural difference does exist, it could account for our observation that residual protein remaining in the wall was, after washings in the presence of 8 M-urea to remove cytoplasm, still firmly associated, enzymically active and could only
be removed following partial hydrolysis of the wall with exo-(1→3)-β-D-glucanase. The last procedure resulted in loss of enzyme activity which again may be contrasted with the extracellular glucan where an enhancement was observed. Thus, with respect to P-glucans, both secondary and tertiary structure would seem to be important in any binding and stabilizing process for proteins.

Allowing for other elements in the fungal wall which may play a role in protein binding, the great stability of glucan-associated proteins suggests that the glucan could well be the site in the fungal wall where enzymes involved in assimilation are normally bound in vivo; further studies will establish whether other enzymes, concerned with transport and secretion, might also be sited here and whether any binding is more specific than at present suggested. With respect to elicitor activity, wall glucans may owe their role as agents for recognition to their ability to bind to proteins in addition to their ability to bind to other polysaccharides (Moorhouse et al., 1977; Morris et al., 1977). However, if glycoproteins are involved, such binding may merely be another manifestation of carbohydrate interaction.

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