plasts some cells plasmolyze into more than one unit, thus releasing small protoplasts; (ii) once released, some protoplasts have been observed to rupture, release cell contents, and then heal to form smaller protoplasts with normal cyclosis. These partial protoplasts, usually few in number, generally disappear within 2 days on agar.

We attempted to determine whether the number of protoplasts released from peeled *Avena* coleoptiles by *Myrothecium* enzyme could be increased by addition of other substances. A pectin esterase preparation (Pectinol 42-E concentrate) and a polygalacturonase preparation (Pectinol 41-P concentrate; Rohm and Haas), at concentrations of 0.5 percent in the cellulase digestion mixture with mannitol, both inhibited protoplast release by more than 50 percent. In the presence of mannitol, $CaCl^2$ or $MgCl^2$ at $0.01M$ and EDTA (ethylenediaminetetraacetate) at concentrations greater than $5 \times 10^{-2}M$ also strongly inhibited release. The reason for this inhibition by calcium, as contrasted with its promotive effect when alone or with KCl (Table 1), is not clear, but the fact is well established. Concentrations of EDTA below $5 \times 10^{-2}M$ had little effect, although $10^{-5}M$ caused a small but significant increase in the number of protoplasts released.

Protoplasts prepared in the standard way were exposed to each of the above additions for 1 hour before determination of the percentage survival. None of the additions caused significant bursting, nor did they, in separate experiments, affect the action of cellulase on carboxymethyl cellulose. These inhibitions, therefore, resulted from effects upon the wall-removal process itself, and not from bursting of the protoplasts after they had been released. The unexpected inhibition by high concentrations of EDTA suggests that EDTA probably does not loosen the middle lamella of coleoptile cells, since loosening would result in exposure of more cells to cellulase attack. EDTA does assist in the release of single cells from some tissues (*4*).

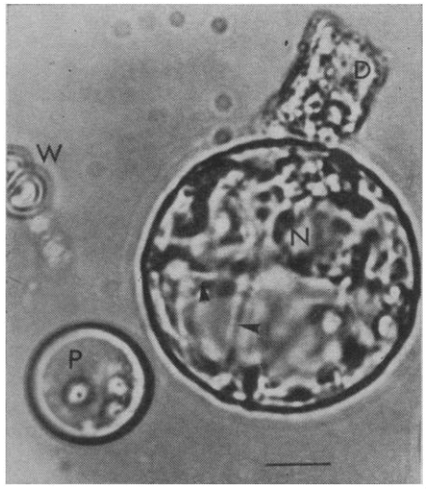

Fig. 1. An *Avena* coleoptile protoplast. After enzymic digestion of cell walls, the protoplasts were placed on 0.6 percent agar for 20 hours before transfer to a microscope slide for photography. *N*, nucleus; *W*, rings of secondary walls of digested vascular elements; *D*, debris from a disintegrated protoplast; *P*, partial protoplast (see text). The darts indicate transvacuolar strands; the bar is 10 μ.

Several tissues of physiological interest have been surveyed for ability to release viable protoplasts when treated with *Myrothecium* cellulase in 0.5 molal mannitol (Table 2). Both leaf and root tissue of oat seedlings release protoplasts in good yield; a region 2 to 4 mm behind the root tip released protoplasts as well as the tip itself, although in neither case was viability (indicated by incidence of cyclosis and general phase-contrast appearance) as good as that of the coleoptile protoplasts. For the standard preparation, 5-mm subapical segments of 20-mm coleoptiles were used; protoplasts were released readily from segments of larger coleoptiles, but only in smaller yield from coleoptiles 10-mm long, largely because the epidermis of the short coleoptiles could not be removed cleanly. The fact that *Convolvulus* callus has exhibited colony formation from single cells is evidence that the individual cells retain the ability to grow and divide (*5*). Cocking (*1*) has found that various tissues of the tomato plant also release protoplasts by similar procedures.

Among the unsuitable tissues, only the *Acer* and potato-tuber cells showed signs of significant wall digestion. The *Acer* cells released no protoplasts when exposed to the cellulase for 2 hours, but, a few minutes after their return to distilled water, a few cells squirted their contents out of presumed holes in the weakened wall, allowing the cell wall to contract with a jerk. When potato tissue was placed in cellulase, digestion of some wall occurred, but the protoplasts apparently ruptured, leaving only starch grains visible.

To prepare protoplasts, the methods and tissues must be carefully matched. Obviously no one method is suitable for all cells. Although the addition, to the basic cellulase digestion mixture, of pectinase or hemicellulase did not enhance release from coleoptile tissue, it may release protoplasts from some of the tissues we have listed as unsuitable; small amounts of these enzymes are, of course, already present in the *Myrothecium* enzyme mixture. In addition to steric factors and recalcitrant wall materials, one is also faced with the presence in many plants of cellulase inhibitors (*6*), which may be a limiting factor in the enzymic removal of cell walls around living plant protoplasts.

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Gamma-A Cold Agglutinin: Importance of Disulfide Bonds in Activity and Structure

Abstract. *Cleavage of disulfide bonds caused by mild reduction with mercaptoethanol produces a reversible loss of cold agglutinin activity and antigen-binding ability in a gamma-A polymer cold agglutinin. Return of agglutination and antigen-binding ability occur only in those molecules that have not been depolymerized by reduction. Loss of activity becomes irreversible if reduction is followed immediately by alkylation.*

The difficulty of isolating γA antibodies has limited the opportunities to examine the role of disulfide bonds in both biological activity and structure of these antibodies. Cold agglutination of red blood cells, observed in the serum of a patient with reticulum cell sarcoma (*1*), was found to be caused by a γA antibody with a

sedimentation coefficient of 10.5*S* (9.5 to 11.5*S*), which indicates a γA polymer. Cold agglutinin was detectable in the serum at high dilutions and could readily be isolated by elution from the red cells at 37°C. In my experiment reduction and alkylation of this γA cold agglutinin caused a marked and irreversible decrease in the titer of the agglutinin. Reduction and removal of the reducing agent permitted a partial return of agglutinin activity (25 to 50 percent), the maximum return requiring approximately 24 hours (Table 1).

Reduction was carried out with the use of 0.1*M* 2-mercaptoethanol in phosphate-buffered saline (PBS), *p*H 7.0, for 2 hours at room temperature with a 1 : 8 dilution of serum containing the γA cold agglutinin. Alkylation was accomplished by adding recrystallized iodoacetamide in sufficient quantity to make the final concentration 0.2*M*. Iodoacetamide and mercaptoethanol were removed by dialysis against PBS, with bath changes every 15 minutes for 2 hours (*2*). Cold agglutinin titers were determined as described previously (*1*).

Recovery of activity after removal of mercaptoethanol was not improved by increasing the temperature to 37°C or by changing the *p*H of the solutions to 5.5 or 8.0. Dialysis of reduced serum against the following oxidizing agents did not improve recovery: 0.0016*M* oxidized glutathione, 0.00046*M* cystine, and 0.001*M* dehydroascorbic acid. Dialysis against 0.0001*M* mercaptoethanol after removal of the 0.1*M* mercaptoethanol and finally the removal of all mercaptoethanol by dialysis against PBS did not affect the return of cold agglutinin activity.

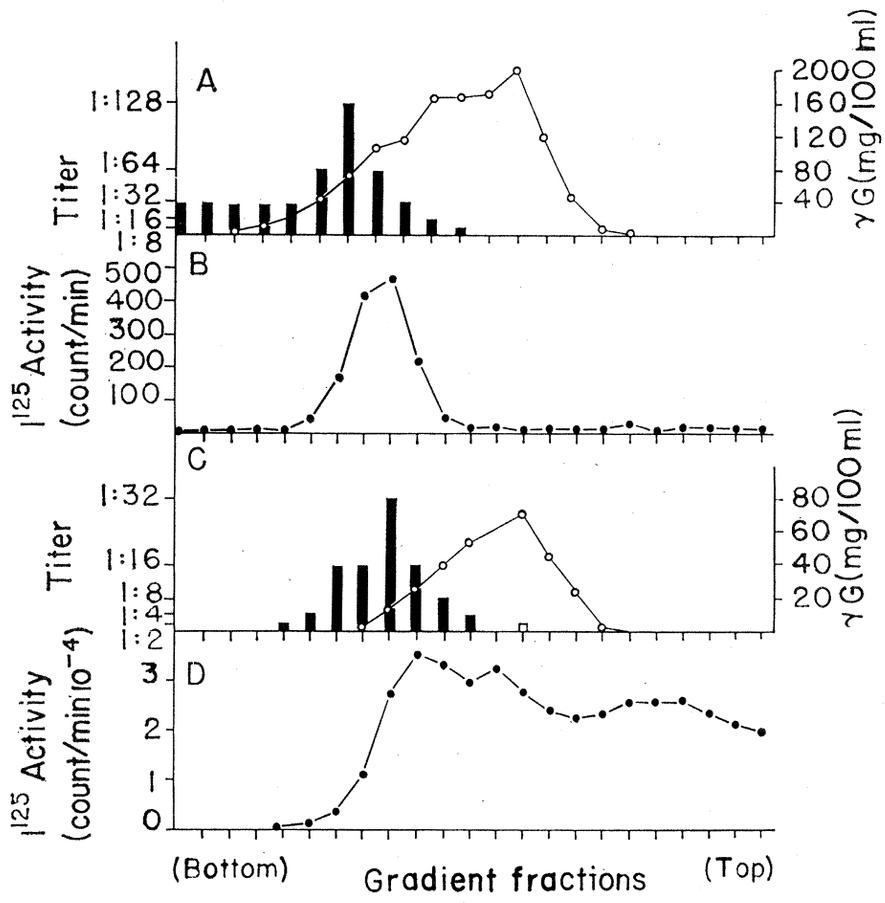

Fig. 1. Titer and radioactivity of γA cold agglutinin in four experiments. Centrifugation was performed in a Beckman-Spinco model L ultracentrifuge with an SW 39 rotor. Samples were centrifuged at 35,000 rev/min (134,211*g*) for 17 hours at 4°C; gradient volume was 4.8 ml and sample volume, 0.2 ml. Solid bars indicate titers of cold agglutinin; open bar, increase in titer of cold agglutinin with antiserum to γA; open circles, γG globulin (mg/100 ml); and solid circles, I^{125} activity. (A) Whole serum containing a γA cold agglutinin was centrifuged through a 5 to 20 percent sucrose gradient. Titer of cold agglutinin and level of γG globulin were determined in each fraction. (B) Isolated γA cold agglutinin was labeled with I^{125} and centrifuged as described in (A); radioactivity of the fractions was then determined. (C) Serum containing γA cold agglutinin was reduced for 2 hours with 0.1*M* mercaptoethanol and promptly layered on a sucrose gradient containing the same concentration of mercaptoethanol. After centrifugation the fractions were individually dialyzed and allowed to regain maximum activity before titration of cold agglutinin and determination of γG globulin. (D) Isolated I^{125}-labeled γA cold agglutinin was reduced for 2 hours with 0.1*M* mercaptoethanol and centrifuged through a sucrose gradient containing 0.1*M* mercaptoethanol, and radioactivity of the fractions was determined.

Haber (*3*) has pointed out that the correct re-formation of intramolecular disulfide bonds is favored in dilute solutions while, in more concentrated solutions, intermolecular bond formation is favored. In my study undiluted serum containing the cold agglutinating antibody was treated at room temperature with 0.1*M* 2-mercaptoethanol for 2 hours, and portions were then diluted 1 : 2, 1 : 16, 1 : 64, 1 : 256, or 1 : 1024. Mercaptoethanol was removed by dialysis as previously described, and the samples were allowed to remain at room temperature for 24 hours. An untreated control had a cold agglutinin titer of 1 : 1024, while the titers of the reduced and diluted samples were all 1 : 512 regardless of their original dilutions during the period of disulfide bond re-formation. Therefore, it seemed possible that intramolecular disulfide bonds essential to the cold agglutinin activity were reduced but, because of their location, they were not readily susceptible to incorrect disulfide bond formation and thus were not adversely affected when the experiment was performed in more concentrated solutions.

Further evidence suggesting the intramolecular location of the disulfide bonds that are responsible for recovery of cold agglutinin activity was provided by sucrose-density-gradient ultracentrifugation. A reduced but unalkylated serum sample was layered on a 5 to 20 percent sucrose gradient that contained 0.1*M* mercaptoethanol. Recovery of activity could not have occurred as a result of re-formation of disulfide bonds during the period of centrifugation because of the presence of 0.1*M* mercaptoethanol. The gradients were then divided into 23 fractions, and mercaptoethanol was removed by dialysis against PBS for 24 hours before performing cold agglutinin titrations (Fig. 1C). Gamma-G globulin as measured by an immunodiffusion method (*4*) served as an internal marker with which to calculate the sedimentation rate (*5*). The reduced cold agglutinin had a sedimentation coefficient of

10.5*S*, approximately the same as that of the untreated antibody (Fig. 1A), which finding demonstrated that the molecules that regained activity had not been depolymerized during reduction.

Deutsch (*6*) reported complete depolymerization of a γA polymer after 10 to 16 hours of reduction with 0.1*M* mercaptoethanol, but he failed to find repolymerization at neutral *p*H. In my experiment a cold agglutinating antibody was isolated by elution from red blood cells (*1*), followed by fractionation with Sephadex G-200. The isolated antibody was labeled with I^{125} (*7*) by the method of Bale *et al.* (*8*), except that the use of catalase was omitted because of the low level of radioactivity. The sedimentation coefficient of I^{125}-labeled antibody (Fig. 1B) was similar to that of cold agglutinin from untreated serum (Fig. 1A). After 2 hours of reduction with 0.1*M* mercaptoethanol the I^{125}-labeled antibody was centrifuged through a sucrose gradient containing 0.1*M* mercaptoethanol to prevent repolymerization. Radioactivity was determined in a well-type scintillation counter, and the results (Fig. 1D) indicate that partial depolymerization does occur.

An experiment was performed to determine whether nonagglutinating but antigen-binding antibodies with lower sedimentation rates are produced by mercaptoethanol reduction. After titration of cold agglutinin in fractions obtained from the sucrose-density-gradient experiment in which reduced cold agglutinin was used (Fig. 1C), the red blood cells from that titration were washed at 4°C, mixed with sheep antiserum to γA, and allowed to settle for 1 hour at 4°C. A one-tube increase in agglutination was seen in only one fraction that corresponded to the peak of 7*S* activity, which finding indicated that very little nonagglutinating antigen-binding antibody was present (Fig. 1C).

The combining ability of this γA cold agglutinin was reversibly inactivated by mercaptoethanol. Reduction and alkylation of the antibody abolished not only its agglutinating activity but also its ability to bind to red blood cells at low temperatures, as judged by failure of sheep antiserum to γA to agglutinate the red cells that had been mixed with the reduced-alkylated cold agglutinin. Failure of antiserum to γA to cause agglutination is probably not due to loss of antigenicity of the reduced-alkylated γA cold agglutinin. Preliminary testing of γA polymer from a patient with myeloma showed that the antiserum to γA was able to form agar precipitin bands at the same dilutions of both untreated and reduced-alkylated γA polymers.

Table 1. Return of cold agglutinin activity after reduction with mercaptoethanol. Serums treated with both mercaptoethanol and iodoacetamide were treated first with 0.1*M* mercaptoethanol for 2 hours, then dialyzed for an additional 2 hours against frequent changes of phosphate-buffered saline, *p*H 7.0. Dialysis was discontinued and iodoacetamide was added to the samples at the times indicated. Samples were in contact with iodoacetamide for 2 hours. They were then dialyzed against buffer, and, after 24 hours at room temperature, they were tested for cold agglutinin activity. In testing serums that were treated with mercaptoethanol alone, the mercaptoethanol was removed after 2 hours by dialysis, and the samples were allowed to remain at room temperature for 24 hours before titration. Serums treated with iodoacetamide alone were in contact with this compound for 2 hours; no mercaptoethanol was added. MCE, mercaptoethanol; IA, iodoacetamide.

Treatment of serum	Time between removal of MCE and addition of IA	Titer of cold agglutinin
None		1 : 1024
MCE-IA	0 hour	< 1 : 8
	2 hours	1 : 8
	10 hours	1 : 64
	24 hours	1 : 256
	7 days	1 : 256
MCE		1 : 256
IA		1 : 1024

Red blood cells that initially had been mixed with reduced-alkylated cold agglutinin at 4°C gave the same titer with untreated serum as normal red blood cells did—a further indication that the combining ability of reduced-alkylated cold agglutinin was lost. Nonagglutinating antibody with the combining site intact might have been expected to compete with agglutinating antibody for red blood cell antigen.

Finally, an attempt was made to elute γA antibody from red blood cells that had been mixed at 4°C with reduced-alkylated cold agglutinin. No precipitin band was detected with the eluate and antiserum to γA when the Ouchterlony or Preer methods were used, while a similar eluate from red blood cells combined with untreated cold agglutinating serum demonstrated a precipitin band as far as a dilution of 1 : 5. This indicates that the reduced-alkylated cold agglutinin was unable to adhere to the cell at 4°C.

Ishizaka *et al.* (*9*), using I^{131}-labeled antibody, have demonstrated a similar effect of reduction and alkylation on the combining ability of a γA isoagglutinin. There is no way at present to determine whether disulfide bonds responsible for loss of antigen-binding are present within the combining site itself or at a more remote location that indirectly affects the combining site. The structure of the combining site may be unaltered and the loss of antigen-binding may be the result of steric or charge alterations that prevent access of antigen to the binding site.

Antibodies of the γG class generally have antibody-combining sites in which disulfide bonds are either resistant to these mild conditions of reduction and alkylation or not dependent on the disulfide bonds for their antigen-binding ability. Some γM antibodies (*9–12*) also have combining sites unaffected by similar conditions of reduction and alkylation.

My studies show that this γA antibody is quite sensitive to disulfide bond reduction, and that at least one effect is on antibody-combining ability. This observation and the observations of Ishizaka *et al.* (*9*) and of Rawson and Abelson (*13*) demonstrate that γA agglutinating antibodies have a very wide range of sensitivity to mercaptoethanol. The reason for this is not yet known.

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