Molecular Aspects of Specific Cell Contact

Abstract. *Complementary macromolecules were isolated from yeasts of opposite mating type. These cell-surface molecules neutralize each other as do antibodies and antigens. Both yeast factors are glycoproteins of low molecular weight. Other specific cell associations may be due to the interaction of such complementary macromolecules.*

Tissue formation and sexual union of gametes are two examples of a large class of interactions requiring specific cell contacts. It has been proposed that the specificity involved in these cell associations may be due to an interaction between complementary macromolecules on the cell surfaces (*1*), or to an interaction which involves the adhesiveness of the cell surface as a whole (*2*). The main advantage of the former theory is that it postulates entities which should be capable of being isolated by biochemical methods. Studies of cell aggregation in marine sponges (*3*, *4*) have led to the isolation of an "aggregating factor" of large molecular weight (10^6) which contains glycoprotein subunits. Agglutinins have been isolated from male and female strains of *Chlamydomonas* (*5*); these substances are also glycoproteins but have even larger molecular weight (10^8). We now report studies of the biochemical determinants of specificity of cell contact during mating in yeast; we have isolated complementary macromolecules with relatively low molecular weights. Since there are two different but complementary cell types in this system, the development of an assay for each cell-surface agglutination factor was facilitated. However, it seems reasonable that the procedures which we have used may have wider applicability.

We used *Hansenula wingei*, a sexually agglutinative yeast. When cell suspensions of the two mating types, strains 5 and 21, are mixed, massive agglutination occurs (*6*) due to the strong adhesive forces between cells of opposite mating type. The specificity of agglutination is shown by the fact that neither mating type agglutinates with itself or with the diploid hybrid. Genetic analysis of the agglutination characteristic has shown that agglutination type specificity segregates as a simple allele which is either identical to or closely linked to the mating-type locus (*7*). We present evidence here that this specificity of agglutination between opposite mating types is due to the presence, on their cell surfaces, of complementary macromolecules which are glycoproteins. These glycoproteins

interact in the manner of antibody and antigen.

A cell-surface agglutination factor may be isolated from strain 5 by digestion of whole cells with snail juice enzyme (*8*), or subtilisin (*9*). This agglutination factor, called 5-factor, specifically agglutinates cells of strain 21 and has a large molecular weight (500,000). 5-Factor with lower molecular weight can be isolated from cytoplasmic extracts prepared by alumina grinding or ballistic disintegration (*10*). Material of several molecular sizes was seen in sucrose-gradient centrifugation, and the smallest three particles which had agglutinating activity had sedimentation constants of 3.5, 6.5, and $9.0S_{20,w}$. Since 5-factor is an agglutinin, it is presumably multivalent, in the immunological sense of this term.

The activity of 5-factor is destroyed by mercaptoethanol (*8*, *11*), and by proteolytic enzymes, such as trypsin, chemotrypsin, and pronase (*10*). Mannose is the only sugar found in purified 5-factor preparations of either high or low molecular weight (*8*, *10*).

No agglutinin can be found in strain-21 cells (*8*, *10*, *12*). However, strain-21 cells do yield a specific cell-surface component, called the 21-factor, which can be assayed by its ability to inhibit the agglutination activity of 5-factor. Since 21-factor neutralized 5-factor, cell-free preparations of each were mixed for several minutes to allow for complex formation, and then cells of strain 21 were added to measure any residual 5-factor agglutinating activity. The details of this inhibition assay and the definition of a unit of 21-factor activity are described in (*12*).

An inhibition assay of this type may prove misleading if the assay has not first been shown to be specific. Conceivably many substances could inhibit such an agglutination assay nonspecifically. A rather striking example of the difficulties involved in such an inhibition assay was the discovery of two distinguishable inhibitory activities both

Table 1. Differences between nonspecific inhibitor (NSI) and 21-factor.

NSI	21-Factor
Specificity criteria	
Released from all strains of *H. wingei*	Released only from cells of strain-21 mating type
Eluted from cell surface by heating	Not eluted by heating
Not eluted by trypsin digestion	Eluted in biologically active form by trypsin digestion
Adsorbs nonspecifically to strain-5 and strain-21 cells	Adsorbs only to strain-5 cells
Biological assay	
Add to strain-21 cells, then add 5-factor	Add to 5-factor, then add strain-21 cells
Biochemical properties	
Soluble in saturated ammonium sulfate	Precipitated by saturated ammonium sulfate
Stable to pH 11.5, 1 hour	Unstable to pH 11.5, 1 hour
Heat stable	Heat labile, 50 percent loss in activity after 30 minutes at 62°C
Heterogeneous in molecular size	Homogeneous in molecular size
Large molecular size	Low molecular size, $2.9S_{20,w}$

Table 2. Purification of 21-factor. For complete details, see (*12*).

Step	Volume (ml)	Biological activity (units)	Protein (mg)	Unit/mg protein	Fold purification	Recovery (%)
Trypsin digest of strain-21 cells	200	16,400	670	24	1	100
Adsorption to strain-5 cells and elution with $8M$ urea	60	11,900	12.6	945	40	73
$CHCl_3$, $MnCl_2$ extraction	68	15,400	3.4	4,540	190	94
Sephadex G-200	7	7,350	0.45	16,300	680	45
Starch-block electrophoresis	2	1,780	0.06	28,000	1,200	11

Property	5-Factor	21-Factor
Valency	Multivalent	Univalent
Molecular heterogeneity	Heterogeneous	Homogeneous
Molecular size	3.5, 6.5, 9.0, $31S_{20,w}$ plus larger fractions	$2.9S_{20,w}$
Heat sensitivity	Resistant	Sensitive
Alkali sensitivity	Resistant	Sensitive
Composition	Mannan-protein	Mannan-protein
Specificity	Found only in strain-5 cells and adsorbs only to strain-21 cells	Found only in strain-21 cells and adsorbs only to strain-5 cells

present on strain-21 cells. It was possible to demonstrate that one inhibitor was the specific 21-factor and that the other inhibitor was nonspecific by testing each inhibitor preparation for ability to adsorb to each mating type. Since two different cell types are available, specificity is defined in terms of adsorption of a factor to the opposite cell type. The specific inhibitor, that is, the 21-factor, was completely and specifically adsorbed to strain-5 cells, but not to strain-21 cells or to cells of the nonagglutinative diploid hybrid. However, the nonspecific inhibitor (NSI) was adsorbed to a small extent to both haploid cell types. Further studies showed that the NSI and the 21-factor also differed in their physical and chemical properties as well as in their mode of preparation and their manner of inhibiting the 5-factor activity (Table 1) (12).

Although 21-factor is present in cell-free cytoplasmic extracts, it can be isolated in higher yields by trypsin digestion, since trypsin causes the release of 21-factor from the cell surface in biologically active form. It had been reported (13) that trypsin destroyed the ability of strain-21 cells to agglutinate. It is apparent now that this inactivation was not due to a destruction of the agglutination factor, but to its release from the cells (12). This should provide a precautionary note on the interpretation of the molecular characteristics of substances involved in specific cell adhesion from the sensitivities of whole cells to trypsin (1, 15). Although 21-factor is a protein, this fact could not be inferred from the sensitivity of strain-21 cells to trypsin.

The procedure used for the purification of 21-factor is shown in Table 2. Use was made of the fact that 21-factor is adsorbed specifically to strain-5 cells, from which it can be eluted with $8M$ urea. This reagent was chosen for elution of adsorbed 21-factor since it brings about deagglutination (14). The first elution released very little 21-factor; increasing amounts of 21-factor were released by successive elutions. Most of the extraneous protein bound to the cells was released in the initial elution, so that the first eluate was discarded and the subsequent eluates were pooled. Since the 21-factor was not precipitated or inactivated by extraction with a mixture of chloroform, isoamyl alcohol, and $MnCl_2$, this treatment was used to remove large amounts of extraneous protein and nucleic acid from the eluates. Final purification was achieved by Sephadex-gel filtration and starch-block electrophoresis. Purified 21-factor contains only protein and carbohydrate in a ratio of 65:35; the sugar is probably mannose. Thus, the 21-factor, like the 5-factor (8, 10) and other cell-surface proteins of yeast (16), is a glycoprotein. It appears that cell-surface aggregating molecules as a rule are glycoproteins.

Both crude and purified 21-factor were homogeneous as judged by Sephadex G-200 gel filtration, starch-block electrophoresis (Fig. 1), and sucrose-gradient centrifugation. From sucrose-gradient analysis, the sedimentation constant was calculated to be $2.9S_{20,w}$.

That the 21-factor requires protein for activity is shown by the fact that it is inactivated by alkaline pH, by heat, and by high ionic strength. Since it is an inhibitor rather than an agglutinin, it probably has only one combining site. The differences between the 21-factor and the 5-factor are summarized in Table 3.

We have developed cell-free assays for two specific cell-surface components involved in yeast mating and have used these assays in their purification. Since 5-factor is an agglutinin, its assay was relatively easy to set up, although the assay of 5-factor components of low molecular weight requires rather special conditions. However, the assay of the 21-factor was more difficult, since it required the availability of cell-free 5-factor, and because it was necessary to demonstrate that the inhibitory activity was in fact specific and attributable to 21-factor activity.

Our work indicates certain precautions which must be considered in any attempts to isolate components involved in specific cell contact from other organisms, since the problem of distinguishing a specific from a nonspecific activity is not necessarily straightforward. If cell association in tissue formation is also due to the interaction of complementary macromolecules, it is likely that since the cells of the tissue are genetically identical, both complementary substances are present on the surface of each cell, spatially separated. If so, the isolation and purification of such substances may be more difficult, since the two eluted substances may neutralize each other in solution, as do our isolated mating factors. Alternatively, both combining
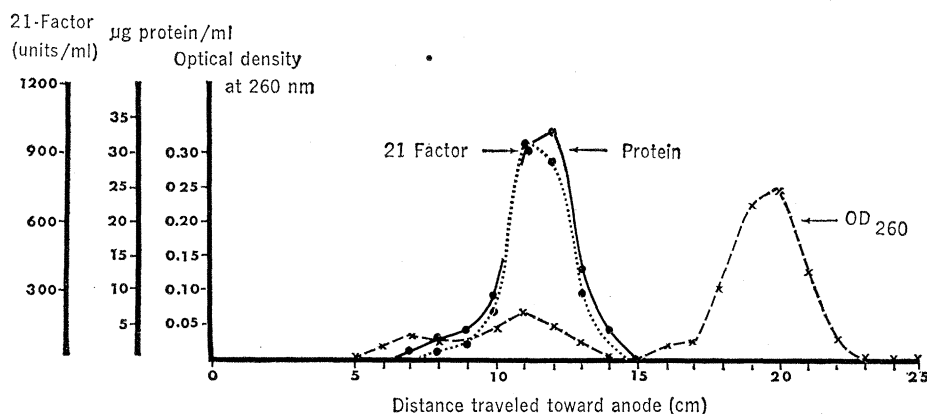

Fig. 1. Starch-block electrophoresis of the purified 21-factor. The combined fractions containing purified 21-factor activity from Sephadex G-200 were concentrated to 0.5 ml and applied to the origin. The electrophoresis was run at 500 volts and 2 ma for 14 hours, at pH 5.5 in 0.01M phosphate buffer. The block was then sectioned, and each section was eluted with 1.5 ml of 0.01M phosphate buffer. Each eluate was assayed for 21-factor activity, protein, and absorbance at 260 nm.

sites might be part of the same macromolecule. If this were so, then the solubilized molecule should be an agglutinin and therefore relatively easy to assay. However, if an agglutinin is obtained which causes aggregation of like cell types, the specificity of this agglutination reaction must be demonstrated because substances like DNA (*17*) or basic proteins (*18*) cause cells to aggregate nonphysiologically. In the yeast mating system, we could define specificity in terms of adsorption of an activity (be it inhibitory or agglutinating) to the opposite cell type from which it was prepared. Perhaps a similar criterion for specificity could be established if it were possible to chemically treat cells from one tissue with a reagent which destroys only one of the two complementary combining sites.

Now that the specific cell-surface components have been isolated and purified from both mating types, the original hypothesis (*14*), that agglutination was due to the association of complementary macromolecules, seems well established. The two substances interact with each other, or interact with cells of opposite type, in a manner precisely analogous to antibodies and antigens. Indeed, the agglutination factors of *H. wingei* provide one of the clearest cases for the hypothesis that cell-to-cell and virus-cell interactions resemble immunological reactions. Since the substances from this yeast system are of relatively low molecular weight, a molecular analysis of their complementary association may be possible. The genetics of the two substances can be analyzed (*7*), and a procedure is available for isolating nonagglutinating mutants (*12*). This system is, thus, an attractive one for further analysis.

M. A. CRANDALL
T. D. BROCK
Department of Microbiology,
Indiana University, Bloomington 47401

References and Notes

1. A. A. Moscona, *Proc. Nat. Acad. Sci. U.S.* **49**, 742 (1963).
2. M. S. Steinberg, *Science* **141**, 401 (1963).
3. T. Humphreys, in *The Specificity of Cell Surfaces*, B. D. Davis and L. Warren, Eds. (Prentice-Hall, Englewood Cliffs, N.J., 1967), pp. 195–210.
4. E. Margoliash J. K. Schlenck, M. P. Hargie, S. Burokas, W. R. Richter, G. H. Barlow, A. A. Moscona, *Biochem. Biophys. Res. Commun.* **20**, 383 (1965).
5. L. Wiese, *J. Phycology* **1**, 46 (1965).
6. L. J. Wickerham, *Compt. Rend. Trav. Lab. Carlsberg Ser. Physiol.* **26**, 423 (1956).
7. A. Herman, L. J. Wickerham, P. Griffin, *Genetics* **54**, 339 Abstr. (1966).
8. N. W. Taylor, *J. Bacteriol.* **87**, 863 (1964).
9. —— and W. L. Orton, *Arch. Biochem. Biophys.* **120**, 602 (1967).
10. T. D. Brock, *Proc. Nat. Acad. Sci. U.S.* **54**, 1104 (1965).
11. N. W. Taylor, W. L. Orton, G. E. Babcock, *Arch. Biochem. Biophys.* **123**, 265 (1968); N. W. Taylor and W. L. Orton, unpublished.
12. M. A. Crandall, thesis, Indiana University (1968).
13. T. D. Brock, *J. Bacteriol.* **76**, 334 (1958).
14. ——, *ibid.* **78**, 59 (1959).
15. C. W. Metz, in *Sex in Microorganisms*, D. H. Wenrich, Ed. (AAAS, Washington, D.C., 1954), pp. 284–334.
16. W. J. Nickerson G. Falcone, G. Kessler, in *Macromolecular Complexes*, M. V. Edds, Jr., Ed. (Ronald Press, New York, 1961), pp. 205–228; A. A. Eddy, *J. Inst. Brewing* **64**, 19 (1958); N. P. Neumann and J. O. Lampen, *Biochemistry* **6**, 468 (1967) .
17. M. S. Steinberg, *Exp. Cell Res.* **30**, 257 (1963).
18. A. Katchalsky, D. Danon, A. Nevo, A. de Vries, *Biochim. Biophys. Acta* **33**, 120 (1959).
19. We thank Pat Holleman for assistance. Supported by a PHS research career development award and by NSF research grants GB-1964 and GB-6001 to T.D.B. M.A.C. held a PHS predoctoral fellowship.

31 May 1968   ∎

Desmosine Biosynthesis: Nature of Inhibition by D-Penicillamine

Abstract. *Administration of D-penicillamine and lathyrogens such as β-aminopropionitrile to animals markedly alters connective tissue by preventing the normal cross-linkage of elastin and collagen. It had been shown that β-aminopropionitrile blocks the cross-linkage of elastin and collagen by preventing the initial step in cross-linkage: the conversion of lysine in peptide linkage to α-amino adipic-δ-semialdehyde. We show that penicillamine acts after the initial step, causing the accumulation of an elastin rich in α-amino adipic-δ-semialdehyde.*

The desmosine cross-links in elastin arise from condensation of the side chains of four lysine residues in peptide linkage to form a pyridinium ring (*1*). After the synthesis of the protein, the initial step in biosynthesis of desmosine appears to be deamination of a lysine ε-amino group to form the δ-semialdehyde of α-amino adipic acid (*2, 3*). After performic acid oxidation of elastin, α-amino adipic acid, the acid-stable derivative of α-amino adipic-δ-semialdehyde, can be readily detected in elastin hydrolyzates (*3*).

The lathyrogen β-aminopropionitrile blocks biosynthesis of desmosine by interfering with the initial deamination of elastin lysyl residues to α-amino adipic-δ-semialdehyde (*3*). Penicillamine also has been shown to interfere with biosynthesis of desmosine (*4*), but its mode of action has remained unknown. We now present evidence that the inhibition follows the deamination of elastin lysyl residues to α-amino adipic-δ-semialdehyde, perhaps by chemical blockage of the reactive aldehyde group.

D-Penicillamine, β-aminopropionitrile fumarate (BAPN), or saline was injected into the yolk sacs of 10- or 12-day-old fertile eggs. Elastin was isolated as the insoluble residue from minced aortas by these successive extractions in nonhydrolytic solvents: 24 hours in 3 percent Na_2HPO_4 at 5°C; 72 hours in a mixture of $0.5M$ penicillamine, $0.5M$ NaCl, and $0.025M$ tris at pH 7.4 and 5°C; and 24 hours in a mixture of 1-percent sodium lauryl sulfate and $0.05M$ tris at pH 7.4 and 25°C. After these extractions the residues were washed repeatedly with H_2O and lyophilized. Penicillamine has been included in the solvent-extraction preparation of elastin because of its efficacy as a solvent of collagen (*5*).

After overnight oxidation with performic acid (*6*) the insoluble elastin residues were hydrolyzed in $6N$ HCl for 72 hours and analyzed with an automatic amino acid analyzer equipped for accelerated analysis (*7*). Amino acid analysis of unoxidized elastin residues revealed less than 0.5 residue per 1000 total residues of α-amino adipic acid.

Elastin was prepared by solvent extraction rather than by the more usual hot-alkali extraction (*8*) because amino acid analyses of the oxidized alkali extract revealed that appreciable amounts of α-amino adipic acid had been solubilized. Elastin prepared by this method of extraction with nonhydrolytic solvent is similar in amino acid composition to alkali-extracted elastin except for increases in contents of threonine, serine, and the polar amino acids. Amino acid analysis after oxidation revealed less than 0.5 residue per 1000 total residues of α-amino adipic acid in each of the solvent extracts.

Figure 1 (left) shows α-amino adipic acid, desmosine, and lysine contents of insoluble elastin isolated from aortas of