MATING REACTION IN *HANSENULA WINGEI*

Relation of Cell Surface Properties to Agglutination

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Hansenula wingei is an ideal organism for the study of the physiology and biochemistry of heterothallic mating systems. When suspensions of the two mating types are brought together under appropriate conditions, a mass agglutination of the cells occurs, resulting in a clump which settles out of suspension (Wickerham, 1956). Since the agglutination is a macroscopically visible reaction, it presents the possibility for a quantitative assay of mating, and the opportunity to study readily the effect of a number of factors on mating. Since most other heterothallic yeasts present no such strong attraction between mating types, it appears that the surface components responsible in *H. wingei* are more intensely developed, opening up the possibility for extracting and characterizing the components involved. The agglutination is the first step in the mating sequence, serving only to bring the cells of the two types into intimate contact; fusion follows rapidly. It is hoped that a study of the initial reactions involved in this mating system might begin to clarify physiologically and biochemically the nature of mating systems in heterothallic organisms.

H. wingei was first isolated by Wickerham (1956), who has analyzed the relationship between agglutination and zygote formation. Preliminary work (Brock, 1958*a*) has shown the feasibility of studying the mating reaction of this yeast quantitatively and has presented an assay for the degree of agglutination. The necessity of cations as cofactors in the agglutination has been shown, as well as the effects of pH, dielectric constant, and heat. The presence of a specific protein component on one mating type has also been indicated (Brock, 1958*b*). The present paper will attempt to determine what cell surface property is responsible for agglutination.

METHODS

The yeasts used in this study were *H. wingei* NRRL Y-2340, strains 5 and 21, which are the haploid agglutinative mating types. The strains were supplied by Dr. L. J. Wickerham.

The medium used had the following composition: glucose, 30 g; yeast extract, 7 g; KH_2PO_4, 5 g; with or without agar, 20 g; in 1000 ml of distilled water.

The cells were grown on the surface of agar in large trays for 3 days and harvested by washing with distilled water, filtering through cheesecloth, and washing 2 or 3 times with distilled water. Since heating intensified the reaction, reduced the need for cofactors, and rendered the cells more stable to storage by reducing autolysis, the washed suspensions were heated at 100 C for 5 min. Cells treated in this manner were stable at 4 C for at least 2 months. Cells could also be dried in acetone and stored in a desiccator for long periods of time and still exhibit strong agglutination after being rehydrated. However, certain other surface properties of the cells were altered by this treatment, so that such cells were not used for any of the studies reported here. Occasionally cells were grown in 100-ml amounts of liquid medium in 500-ml Erlenmeyer flasks on a reciprocating shaker for 18 hr, and then treated as above.

In qualitative tests for agglutinability, dense suspensions containing 200 to 300 mg wet cells per ml were used. These were tested by placing small drops of the two strains adjacent on a glass slide and stirring together rapidly for a few seconds. If the cells were agglutinable, it was macroscopically evident almost immediately after stirring. The quantitative assay used was as described earlier (Brock, 1958*a*). The cells of the two types were mixed in 1 per cent $MgSO_4 \cdot 7H_2O$ with appropriate additives, centrifuged to pack, and then resuspended. If agglutination had occurred during centrifuging, the sediment would not resuspend but immediately settled out. By reading the turbidity of this resuspended mixture after 5 or 10 min, the degree of agglutinability of the cells could be estimated. The quantitative

results are presented as percentage reduction in turbidity over unagglutinated controls. This assay must be done in a centrifuge with swinging cups, since in an angle head the cells stick to one side of the tube and make subsequent optical density determinations uncertain.

In a number of experiments to study the nature of the cell surface, the effect of various agents on the suspension stability of the individual strains was studied. In these experiments dense suspensions were used. To distinguish the mixed agglutination of the mating types from the agglutination of separate mating types due to added agents, the latter will be called self-agglutination, although it should be understood that suspensions of the individual strains in distilled water were highly stable.

RESULTS

The mating components responsible for mixed agglutination reside on the cell walls. This has been shown by the preparation of cell walls by mechanical disintegration with glass beads in a Nossal shaker, followed by extensive centrifugation to free the walls of cytoplasmic constituents. These isolated cell walls agglutinated strongly when mixed. Because of the refractory nature of this yeast, it was difficult to prepare large scale batches of cell walls, so all of the work that follows was carried out with whole cells.

Two possible explanations for the strong attraction between the two mating types come to mind: (a) the two strains may have electrostatic charges that are opposite in sign in the pH range in which they agglutinate; (b) each strain has a specific component, which may be a small part of the total surface, which is complementary to a component of the opposite strain, and the two types agglutinate because of the close fit of these components, in a reaction analogous to an antibody-antigen reaction. The present work explores both of these possibilities.

Electrostatic charge. Three independent procedures were used to determine if the two strains had electrostatic charges that were opposite in sign or of widely differing intensities. These were electrophoresis, dye adsorption, and acid agglutination. Electrophoretic studies of the cells were done in a 10-mm diameter U-tube with arms 10 cm long. Dense suspensions were added at the appropriate pH and ionic strength (kept constant at 0.1 μ). Platinum electrodes were then submerged at the top of each arm. A constant current of 40 ma was run through the apparatus. After 5 min a marked reduction in turbidity occurred in one arm, and a marked increase in the other arm, indicating that the cells had moved towards one of the poles. At this time, 0.5-ml samples were removed from the top of each arm, diluted to 10 ml with distilled water, and the turbidities read on the colorimeter. The degree and direction of mobility could be estimated from the differences in turbidity in the two arms. Since the only information sought was whether there was any difference in electrostatic charge on the two strains, this procedure seemed reasonably valid and accurate.

The cells moved to the anode and were negatively charged at pH 2.5 or above, and moved to the cathode and were positively charged at pH 1.5. There was no noticeable difference between either strain in degree of movement. The isoelectric pH as measured here is somewhere between pH 1.5 and 2.5, which is of the same order as that found by Jansen and Mendlik (1951) for the yeast *Saccharomyces cerevisiae*. Since the mating reaction does not occur strongly below pH 2.5, it is obvious that both strains are negatively charged in the region of active agglutination.

The two strains do not adsorb the dye acid fuchsin at pH values above 5. At lower pH, the dye is adsorbed in varying amounts, and this can be measured by colorimetric determination of residual dye after removing the cells. At pH 2.5 practically 100 per cent of the dye was removed from a solution of 20 μg per ml by dense suspensions. Determinations at different pH values revealed no difference in dye adsorption between the two strains, indicating no difference in electrostatic charge.

An indirect way of determining electrostatic charge is to determine the pH at which suspensions of the individual strains are agglutinated (Lamanna and Mallette, 1953; Mudd, 1933). With the yeasts under study here, no self-agglutination was obtained at any pH value down to pH 0. This would be expected of highly hydrophilic organisms. It was discovered, however, that if the cells were suspended in 40 per cent acetone, they behaved like hydrophobic cells and were readily self-agglutinated by acid or salt, although 40 per cent acetone itself did not cause self-agglutination in the absence of acid or salt. This presented another technique for determin-

ing if the two strains differed in electrostatic charge, since a difference in charge would be reflected in a difference in pH necessary for self-agglutination.

An experiment was set up in which various concentrations of HCl in 40 per cent acetone were used and the highest pH which just caused agglutination for each strain was determined. The pH of the HCl-acetone mixtures was measured with the glass electrode. Strain 5 just agglutinated at pH 2.8, and strain 21 just agglutinated at pH 3.1. These results further show that there is essentially no difference in electrostatic charge between the two strains. That the pH which just causes agglutination is higher than the isoelectric pH as measured by electrophoresis is not surprising, since cells usually self-agglutinate at pH values somewhat above the isoelectric pH (Lamanna and Mallette, 1953).

It can be concluded from the above work that the agglutination in the mating reaction is not the result of an attraction between cells of opposite or quantitatively different charge.

Evidence for specific mating components. The second possibility suggested above as an explanation for the mating reaction was examined by a search for specific cell treatments which would alter or eliminate the agglutinability of one mating type without affecting the other. In this way it was felt that by a knowledge of the action of such differential treatments, the chemical nature of the components might be deduced.

(1) Effect of extractive agents:—Since preliminary work with solvents and drying had indicated that the agglutination components were quite stable, a series of experiments were set up to determine what solvents might remove something from the cells which was essential for agglutination. The experimental procedure was to extract the cells of each strain with the solvent for 5 min at room temperature, then wash the cells and test for agglutination with extracted and unextracted cells of the opposite type. The following solvents had no effect on either strain: methanol, ethanol, *n*-butanol, acetone, 1,4-dioxane, diethyl ether, ethanol:ether (1:3), 8 M urea, 1 N HCl, and 0.1 N NaOH. Sodium hydroxide (1 N) completely destroyed the agglutinability of both strains, whereas 80 per cent phenol destroyed the agglutinability of strain 21 but had no effect on strain 5. These results indicate that organic solvents do not affect the surface components responsible for agglutination.

Since 8 M urea, which should break hydrogen bonds, is also ineffective, this indicates that the components are tightly bound to the surface. However, when the cells are tested for mixed agglutination in the presence of 8 M urea, the reaction is inhibited, indicating that hydrogen bonds are necessary to hold the two cell types together.

Since 80 per cent phenol is usually considered to extract proteins, this may indicate that protein is necessary for agglutination of strain 21, but not of strain 5. Morphologically the cells of both strains were considerably affected by this extraction procedure, but strain 5 continues to agglutinate normally. A large amount of precipitate developed during this extraction which was probably denatured protein. No assays for protein were run, because the acid extraction and enzyme treatments outlined below were more favorable for quantitative studies.

(2) Acid extraction of cells:—The clue obtained above, indicating the possible importance of protein for strain 21, was further exploited using other techniques. Treatment with hot dilute acid was found to give similar results to phenol extraction. The cells used had been heated in water and then washed 3 times to remove any loosely bound protein. They were highly agglutinable. Cells, 50 mg wet weight, were then suspended in 1.0 ml of 0.01 N HCl and heated at 100 C for various periods of time. The cells were immediately neutralized by adding a calculated amount of NaOH, then cooled, centrifuged, and washed. The supernatants from the cells were saved and assayed for protein by the method of Lowry *et al.* (1951) and for carbohydrate by the anthrone method (Trevelyan and Harrison, 1952). The washed cells were tested for agglutination quantitatively, using untreated cells of the opposite type as testers.

The results of these tests are given in table 1. The data show clearly that the hot dilute acid treatment removes about the same amount of protein and carbohydrate from each cell type, but only the agglutination of strain 21 is affected. At the end of 15 min heating, strain 21 has completely lost the power to agglutinate, whereas strain 5 agglutinates just as strongly as the control. It is evident from these results that strain 21 possesses some component removable by hot

TABLE 1

Agglutination of 0.01 N HCl extracted cells: protein and carbohydrate assays on the extracts

Treatment	Agglutinability		Protein Assay		Carbohydrate Assay	
	Strain		Strain		Strain	
	5	21	5	21	5	21
	%		μg/ml		μg/ml	
Room temp, 1 min	—	93	—	—	—	—
Room temp, 15 min	84	94	80	120	36	6
100 C, 1 min	78	27	300	300	40	38
100 C, 3 min	90	5	400	360	54	42
100 C, 5 min	85	1	420	410	50	65
100 C, 10 min	76	3	500	440	80	76
100 C, 15 min	87	3	540	500	106	110
Control	81	95	—	—	—	—

Protein assay: crystalline lysozyme standard. Carbohydrate assay: glucose standard.

dilute acid which is essential for agglutination. The results above with phenol extraction had indicated that this component was protein. The acid results confirm this. They also indicate the specificity of protein involved, since a quantitatively similar amount of protein was removed from strain 5 without affecting its agglutination. It is interesting that carbohydrate material is also removed by the extraction. Although there is no indication that the protein and carbohydrate are coming off together, results of Eddy (1958*a*) with Saccharomyces have indicated that agglutinable strains of this organism possess a mannan-protein complex which is removable as a unit.

Mannan is characterized by the fact that it is precipitated by Fehling's solution, due to the complexing of the alkaline copper with the adjacent hydroxyl groups of the mannose sugar. No other polysaccharide has been reported to precipitate with Fehling's solution (Eddy, 1958*b*). The presence of mannan in these acid extracts could be shown by this procedure. Thus the acid is removing both a protein and a mannan component.

A neutralized acid extract was tested to see whether it would inhibit agglutination. A 0.1-ml amount of extract equivalent to 5 mg wet weight of cells was mixed with a very dilute suspension of cells of the opposite type and allowed to stand for 15 min at 37 C for adsorption. Then 1 ml of 1 per cent $MgSO_4$ and 1 drop of tester cells of the same type as the extract were added and agglutination tested by centrifuging. The extracts had no effect on agglutination under these conditions.

Tests were also made to determine the ability of cells to adsorb protein from the extracts of opposite type, thus indicating a possible specificity. One ml of extract equivalent to 100 mg wet weight of cells was mixed with 1 ml of cell suspension containing 100 mg wet weight. The cells had been previously treated with $MgSO_4$, since this substance is stimulatory to agglutination. The cells were allowed to stand in contact with the extract for 15 min at 37 C, then centrifuged and the supernatant assayed for protein. This experiment was done in all four possible combinations, strain 5 extract with strain 5 and 21 cells, and strain 21 extract with strain 5 and 21 cells.

When strain 5 was the source of the extract, cells of both strains 5 and 21 adsorbed about 55 per cent of the extract protein. When strain 21 was the source of the extract, cells of both strains 5 and 21 adsorbed about 70 per cent of the extract protein. These values are corrected for dilution by intercellular water. These results indicate that there was a lack of specificity in the adsorptions, although a considerable amount of the extracted protein was adsorbed by both cell types. The fact that the extracts from the two cell types were adsorbed in different amounts may indicate that the two extracts do differ. It is possible that the lack of specificity observed between strain 5 and 21 cells is because the protein component was denatured during the hot dilute acid extraction. Such an extraction might be considered to be somewhat rigorous. An indication of such denaturation can be seen from comparing the rate of protein removal with the rate of decrease in agglutinability of strain 21 in the data of table 1. Agglutinability decreases faster than protein is extracted. This may indicate denaturation of the protein while it is still attached to the cells.

(3) Treatment with enzymes:—Proteolytic enzymes such as trypsin are known to remove M protein from streptococci. Such enzymes have also been used by Eddy (1958*a*) to remove a protein-mannan complex from brewer's yeast. Consequently the effect of proteolytic enzymes

was tested on the removal of protein and on the agglutination of strains 5 and 21. In table 2 are listed the actions of various enzymes on the agglutinability of the mating types (all of the enzymes tried), which included several nonproteolytic enzymes as well. It can be seen that trypsin and other enzymes with proteolytic activity, such as papain, lipase (impure), pancreatin, but not pepsin, were able to cause a complete loss in agglutinating power in strain 21, but were without effect in strain 5. This observation is in complete agreement with the results of phenol and acid extraction.

Detailed experiments were then run with trypsin. In these experiments, 0.5 ml of cells washed 3 times (500 mg wet weight per ml) were mixed with 0.5 ml of various trypsin solutions dissolved in pH 8.0 tris(hydroxymethyl)aminomethane (Tris) (0.02 M) buffer. After enzyme action the cells were removed by centrifugation, washed, and tested for agglutination. The supernatants were assayed for protein and corrected for the amount of trypsin present.

In the first experiment, the effect of various concentrations of trypsin during 1-hr incubation at 37 C was examined. The results in table 3 show

TABLE 2

Action of enzymes on agglutinability of cells; 1 hr at 37 C

Enzyme Treatment	Agglutination*	
	Strain	
	5	21
Pepsin, 25 mg/ml; pH 2.3; KCl buffer	+	+
Trypsin, 25 mg/ml; pH 7.5; phosphate buffer	+	−
Trypsin, 500 μg/ml; pH 8.0; Tris buffer	+	−
Pancreatin, 25 mg/ml; pH 7.5; phosphate buffer	+	−
Lipase, 25 mg/ml; pH 7.5; phosphate buffer	+	−
Papain, 500 μg/ml; cystein, 1.2 mg/ml; pH 4.5; phosphate buffer	+	−
Ribonuclease, 500 μg/ml; pH 7.8; Tris buffer	+	+
Malt diastase, 500 μg/ml; pH 4.5; acetate buffer	+	+

* + = Good agglutination, − = no agglutination.

TABLE 3

Action of trypsin on agglutinability of cells and on the removal of protein from cells; 1 hr at 37 C

Treatment	Agglutinability		Protein	
	Strain		Strain	
	5	21	5	21
	%		μg/ml	
Water control	85	80	50	50
Tris buffer control	80	75	55	70
Trypsin:				
1 μg/ml	80	80	120	140
10 μg/ml	85	75	220	220
50 μg/ml	85	60	375	370
100 μg/ml	85	40	670	500
500 μg/ml	85	5	1120	820
1000 μg/ml	90	5	1250	1100

Protein assay: crystalline lysozyme standard, corrected for trypsin present.

that approximately the same amount of protein is removed from both strain 5 and 21 cells but that only the agglutination of strain 21 cells is affected. This is in close agreement with the results above for acid extraction. In table 4 are presented the results of a time study using 100 μg trypsin per ml. Again it can be seen that the agglutination of strain 5 cells is completely unaffected, even though an amount of protein comparable to that of strain 21 was removed. It is interesting to compare the rate of removal of protein and rate of reduction of agglutinating power here with the results for acid extraction. In the latter case, the agglutinating power dropped off faster than the protein extraction. In the case of trypsin, protein extraction proceeds at a faster rate than is agglutinating power destroyed. This seems to indicate that with the enzyme, considerable amounts of protein are removed that are not necessary for agglutination, so that it may be concluded that only a part of the protein removable by trypsin is actually the mating component, whereas much of the protein is nonspecific material. Anthrone assays of the supernatants from trypsin action are also presented in table 4. It can be seen that there are significant amounts of carbohydrate removed by the trypsin action, and the results are similar to those for the acid extraction. Again there seems

TABLE 4

Action of trypsin on agglutinability of cells and on the removal of protein and carbohydrate from cells: trypsin concentration 100 μg/ml, 37 C

Treatment	Agglutinability		Protein		Carbohydrate	
	Strain		Strain		Strain	
	5	21	5	21	5	21
	%		μg/ml		μg/ml	
Trypsin:						
15 min.....	95	90	200	240	170	170
30 min.....	95	85	370	320	190	170
1 hr.....	95	70	530	490	200	200
2 hr.....	95	45	970	710	230	260
4 hr.....	95	35	1410	1080	370	390
24 hr.....	85	10	1330	1250	180	350
Tris buffer control:						
4 hr.....	95	90	100	150	120	190
24 hr.....	90	90	100	100	110	140

Protein assay: crystalline lysozyme standard, corrected for trypsin present. Carbohydrate assay: glucose standard.

to be little difference between the two strains in amounts released.

It was of great interest to determine whether the trypsin extracts would be capable of inhibiting agglutination, even though the acid extracts had not done so. Unfortunately, the trypsin in the extracts had a marked inhibiting effect on the agglutination, thus preventing either a quantitative or qualitative assay of agglutination inhibition. Until methods can be devised for removing the enzyme extracted protein from the trypsin (itself a protein), further work along these lines will not be possible. This same difficulty prevents a study of possible differences in protein adsorption between the two strains.

(4) Search for a specific component on strain 5:—All of the above work seems to indicate that a specific protein or protein-carbohydrate complex is necessary for agglutination of strain 21. The agglutination of strain 5 was remarkably resistant to these protein extraction methods. It was hypothesized that perhaps strain 5 possesses a specific polysaccharide component, which is complementary to a protein component of strain 21. The work of Eddy (1958*a*) on flocculation (self-agglutination) of brewer's yeasts indicates strongly the function of a mannan-protein complex in this system. In particular, the marked inhibition of flocculation by mannose seems to verify this idea.

Attempts were made to inhibit the agglutination reaction in *H. wingei* with various sugars. Concentrations of sugars up to saturation were used, and none of the following had any effect on the reaction: mannose, glucose, maltose, fructose, lactose, galactose, sucrose, xylose, mannitol, dextrin, and starch. These negative results do not eliminate the possibility of a polysaccharide, since the mating agglutination is much stronger than brewer's yeast flocculation, and it might be expected that low molecular weight sugars would not be able to compete with structurally more complementary cell wall polysaccharides.

Boric acid is known to specifically react with carbohydrates containing *cis*-hydroxyl groups, and mannose and mannans are strong reactants (Deuel *et al.*, 1948). However, in the present work agglutination occurred strongly, whether the agglutination reaction was carried out in 0.5 M boric acid, or whether the individual mating types were treated separately and then washed before testing for agglutination. Boric acid did react with the cells, however, as could be shown by the marked reduction in suspension stability of boric acid treated cells. These results seem to argue against a mannan as a specific mating component.

Periodic acid oxidation revealed the polysaccharide nature of the strain 5 component. This substance oxidizes carbon-to-carbon bonds containing either hydroxyl or hydroxyl and amino groups attached to them, and it has been shown by Hotchkiss (1948) that polysaccharides are the most common biological materials reacting with it. The use of periodic acid oxidation was used by Hirst (1948) to reveal the carbohydrate nature of the receptor on red cells for influenza virus.

By proper selection of periodate concentrations, it was possible to destroy the agglutinability of strain 5, while affecting strain 21 much less (table 5). The reaction appears to be stoichiometric, since extended incubation results in no further decrease in agglutinability, probably because the periodate is used up. At higher periodate concentrations, strain 21 is more affected, but never as completely as strain 5. Glucose completely

TABLE 5

Effect of periodate oxidation on agglutinability of cells

Treatment	Agglutinability	
	Strain	
	5	21
	%	
Control	90	90
Sodium periodate, 37 C:		
0.001 M, 30 min	28	73
0.001 M, 60 min	24	70
0.001 M, 2 hr	19	57
0.001 M, 4 hr	20	67
0.001 M, 8 hr	10	50
0.01 M, 1 hr	3	15
0.01 M + glucose, 0.33 M, 1 hr	90	90

prevents the periodate action. This finding is all the more interesting when it is recalled that strain 5 is quite resistant to many rigorous treatments, such as phenol and hot dilute acid. Since glucose completely prevents the action of periodate, probably by reacting with the periodate and converting it to iodate, it has been possible to study the kinetics of the action of periodate on the cells by stopping the reaction quickly and completely with 0.33 M glucose. Interestingly enough, at 0.01 M periodate, strain 21 is more rapidly affected than strain 5, but the effect levels off sooner, whereas with strain 5, agglutination continues to decrease until it is completely abolished.

These results seem to indicate that a polysaccharide structure may be involved on both strains, possibly in different ways. A tentative hypothesis can be formulated: strain 5 has a specific polysaccharide which is complementary to a specific protein of strain 21. However, the strain 21 protein is attached to the cell in a polysaccharide (mannan?) complex. During periodate oxidation, the structure of this complex is so altered that agglutination is reduced, although never completely eliminated. But in strain 5 the polysaccharide is essential for agglutination, and once oxidized, no further agglutination is possible. Only further work resulting in the extraction of these components and the demonstration of a specific precipitin reaction *in vitro* will make it possible to verify this hypothesis.

(5) Specific chemical groups necessary for agglutination:—The above work indicates strongly the presence of complementary structures which fit together in some stereospecific way and bring about the agglutination of the cells. This would make the reaction analogous to an antibody-antigen reaction. Consequently, studies were performed to determine what chemical groupings on the two cell types might be reacting.

The fact that 8 M urea completely inhibits agglutination without affecting the mating components (see above) indicates that reaction between the two cell types is due to formation of hydrogen bonds. If chemical reagents which combine or react specifically with various chemical groupings are employed, it might be possible indirectly to indicate what structures are necessary for agglutination (Landsteiner, 1945). However, since no reagent is completely specific, results of such experiments must be interpreted carefully.

Uranyl ions react fairly specifically with phosphoric acid groups. However, uranyl nitrate has no effect on the mating reaction, although it reacts quite markedly with both cell types. Use of formaldehyde, nitrous acid, and dinitrofluorobenzene, all fairly specific reactants with amino groups, has shown that free amino groups are unessential for the reaction.

However, it appears that free carboxyl groups may be necessary on strain 21, but not on strain 5. Esterification with methyl alcohol in the presence of acid destroys the agglutinability of strain 21 without affecting strain 5, and this can be partially reversed by hydrolysis of the ester linkages (table 6). Another reagent which complexes the carboxyl group fairly specifically is the thorium ion (Bungenberg de Jong, 1949). When cells of each strain were treated for 5 min with 0.0016 M thorium nitrate in distilled water at room temperature, then washed twice and tested for agglutination, the cells of strain 21 had completely lost the ability to agglutinate, whereas strain 5 cells were unaffected. Thorium nitrate solutions are acid, but cells treated with acid alone were unaffected, indicating the specific effect of the thorium ion. These two sets of independent results seem to indicate that carboxyl groups are necessary for agglutination on strain 21, but not on strain 5.

Unfortunately there is no specific reagent that reacts with the hydroxyl group, so it is not pos-

TABLE 6

Agglutination of cells after esterification with CH_3OH

Treatment	Agglutination*
Normal 5 + normal 21	+
Normal 5 + esterified 21	−
Esterified 5 + normal 21	+
Esterified 5 + esterified 21	−
Normal 5 + normal 21 (CH_3OH control)	+
Normal 5 + normal 21 (0.1 N HCl control)	+
Normal 5 + esterified/hydrolyzed 21†	±

Cells esterified for 1 hr in 80 per cent CH_3OH + 0.1 N HCl at 37 C. Normal cells were untreated suspensions in water. Agglutination tested in presence of Mg^{++}.

* + = Good agglutination; − = no agglutination; ± = slight agglutination.

† After esterification, cells hydrolyzed at 30 C in 0.1 N HCl for 1 hr.

sible to confirm in this way the polysaccharide character of the strain 5 mating component. When strain 5 cells were acetylated with acetic anhydride under conditions in which the hydroxyl groups should be acetylated, they lost the ability to agglutinate. Furthermore, the suspension stability of these acetylated cells was so reduced that it could not be determined if the lack of mating agglutinability was due to a specific effect on hydroxyl groups, or to a general alteration in cell surface characteristics. This treatment also lacks specificity, since the conditions necessary for its performance (100 C for 2 hr), are sufficient to destroy the agglutinability of strain 21, even if strain 21 hydroxyl groups were not necessary for activity.

DISCUSSION

The results of this paper serve to indicate the areas of most fruitful research in determining the specific biochemical components necessary for the mating reaction. The best hypothesis at the moment is that strain 21 possesses a specific protein which is necessary for agglutination, and the configuration of the surface of this protein is complementary with a structure on the surface of strain 5 which is probably a polysaccharide. The agglutination when the two strains are brought together would then be due to specific groups on the protein, probably carboxyl, forming hydrogen bonds with hydroxyl groups on the polysaccharide, resulting in a very tight fit, and thus an agglutination. The fact that urea inhibits agglutination is in line with the combination being a hydrogen bonding. The pH activity curve, the inhibition of agglutination by nonpolar solvents, and a number of other miscellaneous observations are all in agreement with this hypothesis. The requirement for ions can be viewed as a neutralization of negative charges on the cell surfaces, so that the repulsion of the mating cells can be overcome. The above hypothesis is in agreement with the highly specific nature of the mating reaction, since closely related strains and species show no cross reaction (Wickerham, 1956). Even the diploid hybrid of strains 5 and 21 does not react with either strain (Brock, 1958*a*). Since mating type is gene controlled (Winge and Roberts, 1958), the present system offers the possibility of examining in detail one of the molecular bases of gene action.

The analogy of this agglutinating system with an antibody-antigen system has been pointed out above. Earlier (Brock, 1958*a*) the similarities between this system and the phage-bacterial adsorption system were also noted. Other systems in which antibody-antigen analogies can be advanced are the mating type agglutination in Paramecium (Metz and Butterfield, 1951), flocculation in brewer's yeast (Eddy, 1958*a*), mating compatibility and incompatibility in higher fungi and in green plants (Lewis, 1954), fertilization in animals (Cushing and Campbell, 1957), and conjugation in algae (Hartmann, 1956). It is interesing that in both algae and sea urchins, substances are produced by one member of the mating pair which cause agglutination of the opposite member (algal cells and sperm, respectively) and that in both cases, these substances have been characterized chemically as glycoproteins (Hartmann, 1956). In all of these systems, one important element is that of specificity. The possibility seems good that specificities of quite widely differing biological properties might be controlled by the configuration of macromolecules. In *H. wingei*, a system is available for studying this phenomenon in considerable detail, with the possibility of characterizing it at the biochemical level.

Although simple charge differences have been advanced to explain mating types in *Escherichia*

coli (Maccacaro and Comolli, 1956), these differences are in reality quite small. Since *E. coli* cells seem to come together only at localized areas on their surfaces, the possibility should not be overlooked that conjugation in this organism is also due to complementary structures on the two mating types which come together via hydrogen bonding.

SUMMARY

When cells of the two mating types of the heterothallic yeast *Hansenula wingei* are brought together, they exhibit a strong attraction for each other which results in a mass agglutination of the cells. Most other heterothallic yeasts do not exhibit this phenomenon. This macroscopically visible agglutination has been used to develop a quantitative assay for mating ability which makes it possible to study the effect of different factors and attempt to determine what surface components are responsible. Two hypotheses have been explored in this paper: (a) Agglutination is due to difference in electrostatic charge on the two cell types, (b) it is due to the presence of specific components or groups on the two types which are complementary and combine in a reaction analogous to an antibody-antigen reaction. The former hypothesis is rejected. It has been shown that the component on one strain responsible for agglutination is probably a protein, whereas the component on the other strain is probably a polysaccharide, and the two cell types are probably held together by hydrogen bonds.

ADDENDUM

As a test of the hypothesis that mating agglutination was analogous to an antibody-antigen reaction, experiments were performed to see if guinea pig complement would be fixed during agglutination. There was no evidence for such fixation after 18 hr at 0 C. However, it was noted that mating agglutination was inhibited by the complement. Further tests revealed that gamma globulin (bovine Fraction II, Nutritional Biochemicals Corporation) alone inhibited mating agglutination of dilute suspensions. This seems to confirm the hypothesis that strain 21 has a protein necessary for agglutination. It was also found that human serum inhibits the agglutination of dilute suspensions. Experiments in Dr. Arthur Steinberg's laboratory have shown that this inhibitor is not present in all human sera, but only in certain ones, and the ability to produce it is inherited. This inhibitor of mating agglutination is now being used by him as a genetic marker for studies in human heredity.

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