CONJUGATION IN *TETRAHYMENA PYRIFORMIS*

The Effect of Polylysine, Concanavalin A, and Bivalent Metals on the Conjugation Process

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ABSTRACT

The polycation polylysine, at different degrees of polymerization, was found to cause a marked inhibition of the conjugation process. Inhibition of conjugation by polylysine was highly dependent on the molecular weight of the polymer. When polylysine of a mol wt of 1,250 (degree of polymerization = 6) was used, a concentration of 1.6×10^{-5} M was required for a complete inhibition of conjugation, while only 2×10^{-7} M of polylysine of a mol wt of 71,000 (degree of polymerization = 340) was needed for the same effect. Polyaspartic acid prevented the inhibition of conjugation by polylysine.

Chelators of bivalent metals such as O-phenanthroline (10^{-3} M), EDTA (10^{-3} M), and EGTA (5×10^{-3} M) strongly inhibit the conjugation process in *Tetrahymena pyriformis*. The inhibition was partially prevented when bivalent metals such as Zn^{++}, Fe^{++}, and Ca^{++} were added together with the chelators.

The lectin concanavalin A (25 $\mu g/ml$) completely prevented the conjugation process, while other lectins, such as phytohemagglutinin (500 $\mu g/ml$), soybean agglutinin (75 $\mu g/ml$) and wheat germ agglutinin (250 $\mu g/ml$) had no effect. Inhibition of conjugation by concanavalin A is completely reversible by 40 mM of α-methyl-D-mannoside.

Conjugation in *Tetrahymena pyriformis* constitutes the sexual reproduction phase of these cells (Elliot and Hayes, 1953). During this process, cells of opposite mating types collide and form pairs, and membranes of adjacent cells subsequently fuse at very specific sites. A starvation period, during which most of the cells divide and reach the G_1 phase, is essential for inducing the conjugation process (Ron, 1973; Wolfe, 1973). Bruns and Brussard (1974) have shown that a series of specific events leads from vegetatively growing cells to conjugating cells. They have divided these events into two stages: initiation and costimulation. The first stage, initiation, occurs during the starvation period in the absence of interaction between the mating types and is dependent upon the cellular environment. In contrast to initiation, costimulation occurs after the two mating types have been mixed (Bruns and Palestine, 1975). This step requires specific cell-cell interaction between initiated cells of the two mating types. A period of active cell contact between starved cells before pairs form was also suggested by McCoy (1972) and Wolfe (1973).

Virtually nothing is known about the molecular events which take place at the surface of the mating type of *T. pyriformis* during the costimulation period. Investigation of these events may help also

to elucidate some of the steps that control specific adhesion reactions in eukaryotic cells. Furthermore, a better understanding of the biochemical reactions which induce the fusion step during the conjugation process will help to clarify the as yet unknown mechanism of cell fusion.

The purpose of the present work was to study the effect of two high molecular weight compounds, the polycation polylysine and the lectin concanavalin A (Con A), on the conjugation system when added during the costimulation period. In addition, the effect of bivalent cation was also studied.

MATERIALS AND METHODS

Cells

T. pyriformis mating type I (Strain WH-6) and mating type III (Strain WH-52) were obtained from the American type culture collection and were grown in 0.5% proteose peptone and 0.5% bacto tryptone under sterile conditions at 26°C.

Medium

The medium used for washing cells, and starvation and conjugation experiments was 20 mM tricine-NaOH pH 7.4.

Induction and Determination of Conjugation

Conjugation was induced essentially as described by Elliot and Hayes (1953). After 48-64 h of growth, cells of each of the mating types were washed four times with 20 mM tricine-NaOH, pH 7.4, and suspended in the above-mentioned buffer to give a turbidity of 100 Klett units at filter 54 (about 10^6/ml cells). The suspended cells were incubated at 30°C for 20-40 h (starvation period). For conjugation, the two mating types were mixed, if not otherwise stated, by introducing 0.5 ml of each type to a 20-ml glass flask, and then incubated at 30°C without shaking. If not otherwise stated, the extent of conjugation was determined after 4 h of incubation.

When large amounts of conjugated cells were required, cells were suspended in a vol of 5 ml in a 100 ml Erlenmeyer flask. For determination of the extent of conjugation, the cells were removed from the reaction medium and were fixed with an equal vol of 4% formaldehyde. The number of cells was counted with a Clay Adams cytometer under a phase microscope.

Percent of conjugation was calculated as follows:

$$\text{Conjugation (\%)} = \frac{\text{No. of conjugants} \times 2}{\substack{\text{Total no. of cells} \\ \text{(single and conjugants)}}} \times 100.$$

All experiments in the present work were performed in duplicate, and each one was counted four times; each time, between 75 and 100 cells were counted. The results given are an average of four counts. The variation between individual counts never exceeded 10%.

Determination of [³H]Con A Binding

Con A was acetylated with [³H]acetic anhydride as described by Miller and Great (1972). Unless otherwise stated, [³H]Con A (usually of about 0.5×10^6 cpm/mg protein) was added to 1 ml of cells (10^6/ml) and incubated for 1 h at 30°C. At the end of the incubation period, the cell suspensions were filtered over glass filters (Type A, Tamer Corp., Israel), and washed once with another 30 ml of Tricine-NaOH buffered at pH 7.4. The filters were then dried, transferred to 10 ml of toluene scintillation liquid, and counted in a scintillation counter (Packard).

In experiments where α-methyl-D-mannoside was used, 10 µl of [³H]Con A (25 µg) were incubated for 10 min with 100 µl of α-methyl-D-mannoside (usually 50 mM), and the mixture was then added to the cell suspension.

Polylysine of various degrees of polymerization, Con A and soybean agglutinin were purchased from Miles-Yeda, Rehovot, Israel, and phytohemagglutinin from Difco Laboratories, Inc., Detroit, Mich. Wheat germ agglutinin was a generous gift of Professor N. Sharon, Biophysics Department, Weizmann Institute of Science, Rehovot, Israel. All other chemicals were of purest grade available.

RESULTS

The Effect of Polyamino Acids on the Conjugation Process

Exposed charged groups may play an important role in the specific interaction between the two mating types. Experiments were therefore designed to study the effects of positively and negatively charged polymers on the conjugation reaction.

As can be seen in Fig. 1, conjugation was strongly inhibited by the basic polymer polylysine. Inhibition was highly dependent on the length of the polymer, the monomer lysine being the less effective. Concentrations of 200 µg/ml (1.7×10^{-4} M) and 15 µg/ml (2×10^{-7} M) of polylysine, of a polymerization degree of 6 and 340, respectively, were required for complete inhibition of conjugation. However, as much as 10 mg (5×10^{-2} M) of the monomer lysine were required for blocking conjugation (Table I, Fig. 1). Polyaspartic acid of a degree of polymerization of 38 did not affect conjugation even at a concentration as high as 400 µg/ml (Table II). Furthermore, the addi-
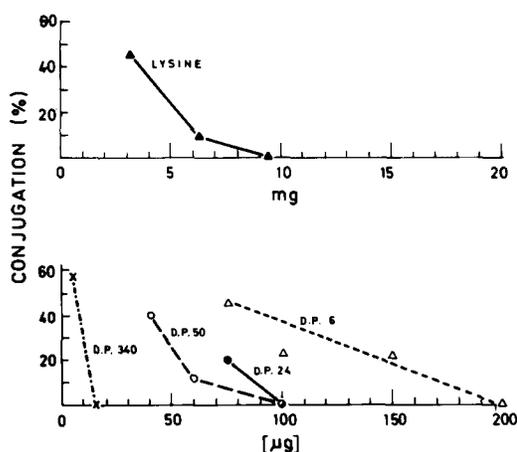
FIGURE 1. The effect of polylysine of different degrees of polymerization on the conjugation process. Lysine (upper panel) or polylysines of different degree of polymerization (lower panel) were added to the conjugation medium at zero time. Conjugation in all samples was determined after 4 h of incubation. The volume of the conjugation medium was 1 ml.

TABLE I

The Relationship Between the Length of Polylysine and its Ability to Inhibit Conjugations

Polylysine degree of polymeriza-tion	Mol wt	Minimum concn. required for complete inhibition of conjugation
		(M)
1	183	5×10^{-2}
6	1,250	1.6×10^{-4}
24	5,010	2×10^{-5}
50	10,500	9.5×10^{-6}
340	71,000	2×10^{-7}

The numbers presented in this table were calculated from the data of Fig. 1.

tion of polyaspartic acid together with polylysine prevented the inhibitory effect of polylysine, and a high percentage of conjugation was reached in the presence of a polylysine-polyaspartic acid complex.

The Effect of Chelators of Bivalent Metals on the Conjugation Process

After the observation that the polycation polylysine inhibits conjugation, it was of interest to check whether bivalent cations play any role in the conjugation process. As can be seen in Table III,

the chelators O-phenanthroline, EGTA, and EDTA either reduced or completely blocked the conjugation reaction. Among the chelators tested, O-phenanthroline was found to have the most potent inhibitory effect. At 0.1 mM it significantly reduced conjugation, and at 0.5-1.0 mM it blocked it completely (Table III, Exp. 1 and Fig. 2). EDTA at 1 mM and EGTA at 5 mM also had a pronounced inhibitory effect on the conjugation process (Table III, Exp. 2).

The inhibitory effect of 0.1 mM O-phenanthro-

TABLE II

Conjugation in the Presence of Polylysine-polyaspar-tic-acid Complex

Addition	(μg)	Conjugation
		%
None	–	72
Polylysine	150	0*
Polyaspartic acid	100	68
Polyaspartic acid	400	85
Polylysine and polyas-partic acid	150 + 400	88

Polylysine and polyaspartic acid were added right after mixing of the mating types. Polylysine of degree of polymerization 50 (mol wt = 10,500) and polyaspartic acid of degree of polymerization 38 (mol wt = 4,460) were used.

* After 2 h of incubation, many dead cells were seen in the microscope field.

TABLE III

The Effect of Chelators of Bivalent Metals on the Conjugation Reaction

Chelator	(mM)	(Me++)	(mM)	Conju-gation
				%
Experiment 1				
O-phenan-throline	0			75
	0.1			18
	1			0
	0.1	Zn	0.2	24
	0.1	Zn	0.4	57
	0.1	Fe	0.2	4
	0.1	Fe	0.4	52
Experiment 2				
EDTA	0			63
	1			6
	1	Ca	2	60
EGTA	1			35
	5			16
	1	Ca	2	66

Conjugation was performed by incubating the two mating types at 30°C for 4 h, as described under Materials and Methods. Chelators alone or with the bivalent metals were added to the incubation medium right after mixing the two mating types.
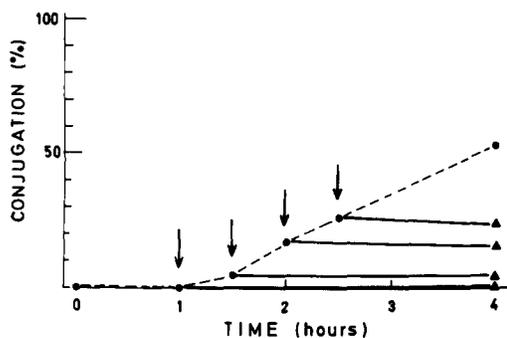
FIGURE 2. Inhibition of conjugation by O-phenanthroline. Conjugation was performed in two parallel cultures, each one consisting of 1 ml of starved cells incubated in glass flasks. At different times (arrows), cells from two parallel cultures were removed and mixed with formaldehyde and counted for percentage of conjugation (control). To one culture, 10 μl of O-phenanthroline were added (arrows) to give a final concentration of 0.5 mM. After 4 h of incubation, cells were removed again, fixed, and counted. (●—●), Conjugation without O-phenanthroline (control system, average of two systems); (●—▲), Conjugation after addition of O-phenanthroline.

line could be largely counteracted through the inclusion of 0.4 mM Zn^{++} or Fe^{++}; the inhibitory effects of 1 mM EDTA or EGTA could be completely relieved in the presence of 2 mM Ca^{++}. Mg^{++} ions could be substituted for Ca^{++} in stimulating conjugation in the presence of EDTA. The various bivalent metals, by themselves, had no effect on the process at the concentrations shown in Table III.

Conjugation was inhibited completely when 0.5 mM O-phenanthroline was added immediately after the two mating types were mixed (zero time, not shown) or an hour later (Fig. 2). O-phenanthroline, when added to the reaction mixture after 1.5, 2 and 2.5 h of incubation, prevented the formation of further conjugating pairs in the population. An identical picture was obtained with 1 mM EDTA (not shown).

Washing of the cells to remove the chelators from them resulted in a complete recovery of conjugation. Addition of bivalent metals was not required to induce the process, but the achievement of maximal percent pair formation required 7-10 h instead of 3-4 h.

Inhibition of Conjugation by Con A

Fig. 3 A demonstrates the effect of an increasing concentration of Con A on the conjugation

process. The addition of 25 μg of Con A to 1 ml of the conjugation medium at zero time completely prevented the onset of the process. Moreover, conjugation was reduced to 50%, or by 29%, in the presence of 20 μg/ml of Con A (Fig. 3 A). When Con A was added to the conjugation medium at different times, it either blocked the process altogether or else it significantly reduced the percent of pairs obtained before its addition (Fig. 3 B). Con A is a metalo protein, the activity of which is highly dependent on its association with bivalent metals (Yariv et al., 1968); indeed a solution of Con A dialyzed against EDTA lost its inhibitory effect (Table IV).

α-Methyl-D-mannoside either prevented or reversed the inhibitory effect of Con A. The data in Table V show that while conjugation is blocked by Con A, the addition of 40 mM α-methyl-D-mannoside, even after 3 h of treatment with Con A, restores the ability to form pairs virtually com-
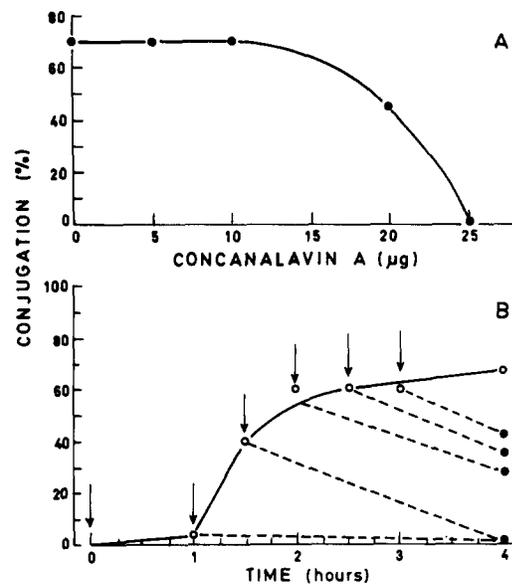

FIGURE 3. Inhibition of conjugation by Con A. (A) The effect of increasing concentration of Con A. Con A was added to the incubation medium at zero time. (B) Addition of Con A at different times of incubation. 25 μg of Con A were added at different times (indicated by arrows). Conjugation was performed in a final vol of 1.5 ml in several parallel glass flasks. To determine conjugation, samples of 0.5 ml were removed at different times (arrows), each time from two flasks, and either Con A (25 μg) or conjugation medium, in a vol of 0.5 ml, were added. Conjugation was determined again after 4 h of incubation.

TABLE IV

The Effect of Dialyzed Con A on the Conjugation Process

Addition	(µg)	Conjugation
		%
None		85
Con A	10	9
Con A	25	0
EDTA-dialyzed Con A	10	70
EDTA-dialyzed Con A	25	65
Ca^{++}-dialyzed Con A	25	0

A solution of Con A (1 mg/ml) was dialyzed for 48 h against either 5 mM tricine-NaOH pH 7.4 + 2 mM EDTA (EDTA-dialyzed-Con A) or 5 mM tricine-NaOH pH 7.4 which contained 0.1 mM Ca^{++} (Ca^{++}-dialyzed Con A). Either 10 µl or 25 µl of the Con A solutions were added to the conjugation system. All other experimental conditions are as described under Materials and Methods.

pletely. The effect of α-methyl-D-mannoside in avoiding or reversing the inhibitory effect of Con A is rather specific. Glucosamine and high concentrations of dextran failed to reverse the effect of Con A, and they did not have any effect on the conjugation process. Glucose and mannose could be used as a substitute for α-methyl-D-mannoside, but higher concentrations are required (80–100 mM, not shown).

Experiments with labeled Con A revealed that 24–35% of the added lectin precipitated with the conjugated cells on the glass filter. Addition of α-methyl-D-mannoside prevented about 80–90% of the binding of Con A (Table VI).

The effect of Con A on the conjugation process was found to be specific. Other lectins such as phytohemagglutinin, 500 µg/ml, soybean agglutinin, 75 µg/ml, and wheat germ agglutinin, 250 µg/ml, failed to inhibit conjugation even at very high concentrations (not shown).

DISCUSSION

The quantitative measurements of conjugation presented in this work were obtained by the use of a light microscope. This technique is able to distinguish only between conjugating and nonconjugated cells, and not the ultrastructure changes which take place in the membranes of the conjugated cells. Therefore, the results presented in this investigation deal mainly with the first step of the conjugation process, namely the pairing step at which the two mating types collide and adhere to each other. Whether the present results can also

TABLE V

Restoration of Con A Inhibited Conjugation by α-Methyl-D-mannoside

Addition	Monosaccharide	Time of additions* of α-methyl-D-mannoside	Conjugation
	mM		%
Experiment 1			
None	–	–	72
Con A	0	–	0
Con A and α-methyl-D-mannoside	9.4	–	10
Con A and α-methyl-D-mannoside	37.5	–	64
Con A and mannose	37.5	–	15
Con A and glucose	37.5	–	0
Experiment 2			
None	–	–	66
Con A	0	–	0‡
Con A and α-methyl-D-mannoside	40	0	45
Con A and α-methyl-D-mannoside	40	60	45
Con A and α-methyl-D-mannoside	40	180	60

Experiment 1: 25 µg of Con A were incubated at room temperature for 1 h with gentle stirring with α-methyl-D-mannoside and mannose or glucose in a final vol of 0.1 ml. At the end of the incubation period the mixture was added right after mixing of the two mating types of *T. pyriformis*. Conjugation was determined after 4 h of incubation.
Experiment 2: In the experiment where α-methyl-D-mannoside was added, conjugation was continued 4 h after its addition. Con A at 25 µg/ml was added right after mixing of the two mating types (zero time).
* Minutes after addition of Con A and mixing of the two mating types.
‡ Samples were counted after 4 h and after 7 h of incubation.

TABLE VI

Binding of [³H]Con A to the Mating Types of Tetrahymena pyriformis

Mating type	Con A bound/system
	µg
I	7
II	4
I + III	6
I + α-methyl-D-mannoside	1.1
III + α-methyl-D-mannoside	0.4
I + III + α-methyl-D-mannoside	0.78

[³H]Con A, 25 µg, was added alone or together with 40 mM of α-methyl-D-mannoside to 1 ml containing 10^6 starved cells of either of each of the mating types or a mixture of the mating types. After 1 h of incubation at 30°C, binding of [³H]Con A was determined as described under Materials and Methods.

be applied to the membrane fusion step is still unclear.

From the data presented in this work, it appears that the pairing reaction in *T. pyriformis* requires bivalent metals. Chelators such as *O*-phenanthroline, EDTA, or EGTA cause a marked inhibition of the process. However, experiments to reveal a direct effect of cations on the process failed; therefore, the possibility that the chelators inhibit pairing by a direct effect on the cell membranes cannot be excluded. All three reagents, *O*-phenanthroline, EDTA and EGTA, prevented conjugation, and their effect was overcome by the addition of bivalent cations. Nevertheless, a high degree of conjugation was reached after removing the above reagents by washing without any addition of cations.

Studies of the mating process in the yeast *Hansenula wingei* have shown that the cell wall factors responsible for agglutination of the opposite mating types are glycoproteins (factor 5 and factor 21) (Crandall and Brock, 1968). Recently, Crandall and Caulton (1973) reported that the synthesis of the 5 and 21 factors is controlled by metal ions. EDTA inhibits the synthesis of factor 5 and hence suppresses agglutination. Sodium metavanadate and sodium molybdate induce the synthesis of this factor. On the other hand, synthesis of factor 21 is induced by EDTA. In addition, Ca^{++} has been reported to be essential for the agglutination of cells of sea sponges by glycoproteins (Gasic and Galanti, 1966), while EDTA prevents this agglutination (Humphreys, 1965). It might well be that exposed membrane glycoproteins are responsible for the adhering step, namely the tight contact between the mating types, in the conjugation process of *T. pyriformis*. Bivalent chelators might exert their effect by specifically inhibiting the synthesis or the exposure of these membrane glycoproteins. Alternatively, the bivalent metals may play a direct role in the pairing process by bridging between two mating types.

Adhesion in many systems has been shown to be due to physical attraction of exposed glycoproteins (Kemp et al., 1973). Indeed, Con A was found in the present work to inhibit the conjugation process in *T. pyriformis*. The inhibition seems to be rather specific to Con A, since other lectins such as phytohemagglutinin, soybean agglutinin, and wheat germ agglutinin failed to affect conjugation. Con A, which possesses at least two binding sites (Yariv et al., 1968), usually causes agglutination of cells, probably by bridging between

two adjacent cells (Kemp et al., 1973). However, in the present case, it inhibits the contact between the two mating types, indicating that the Con A molecules are probably attached only to the surface of one cell, and it is thus unable to bridge between cells. Thus, Con A might inhibit conjugation by steric hindrance of the mating sites. Recently, it has been reported that sperm of the sea urchin *Anthocidaris crassispina* lose their fertilization capacity without losing their mobility, on prior exposure to univalent Con A. Experiments with fluorescein-conjugated Con A revealed that it is bound to the apex of the sperm head and to the midpiece (Aketa, 1975). It should be added that conjugation between the opposite gametes of *Chlamydomonas* is also entirely prevented when androgamone was preincubated with Con A (Wiese and Shoemaker, 1970). However, in the present case, it might be speculated that Con A might inhibit conjugation by precipitating with a glycoprotein secreted by the cell into the medium. The method used in the present work could not differentiate between the binding of Con A to cells and its precipitation with soluble glycoprotein. Glycoproteins have been shown to induce mating in diploid cells of *Hansenula wingei* (Crandall et al., 1974). In addition, sexual differentiation in *Volvox* has been shown to be induced by macromolecules the characteristics of which resemble those of glycoproteins (Pall, 1974).

Inhibition of conjugation by polylysine and its prevention by polyaspartate might indicate that a negatively charged protein, or, more likely, a negatively exposed glycoprotein, is the agglutination site of the mating types of *T. pyriformis*. Like Con A, polylysine usually induced the agglutination of cells (Marikovsky et al., 1966) and may even support cell fusion (De Boer and Loyter, 1971; Sabban and Loyter, 1974), while in the present case it acts in an opposite fashion. Inhibition by polylysine was proportional to its molecular weight and not to the molar concentration of the lysine moieties, indicating that the higher the density of positive charges the greater the inhibition. It was recently reported that the mitogenic effect of soybean agglutinin is markedly stimulated after increasing its molecular weight by cross linking with glutaraldehyde (Lotan et al., 1973). However, in the present case, polylysine might exert its effect by nonspecific steric hindrance of the pairing area.

The fact that α-methyl-D-mannoside reverses the inhibition of conjugation by Con A concomi-

tantly with its removal might indicate that this polymer is not digested by the cells during conjugation. It either interacts with the cells' surface or else binds itself to a soluble factor which is secreted into the medium and is essential for conjugation. Recent experiments in our laboratory using [³H]Con A and fluorescein-conjugated Con A have revealed that Con A can be found both in the medium as an insoluble Con A glycoprotein complex and on specific areas at the cell surface (Frisch, Levkovitz and Loyter, manuscript in preparation).

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