CELL AGGLUTINATION IN THE YEAST, *HANSENULA WINGEI*, CORRELATED WITH CHANGES IN ULTRASTRUCTURE

A Dissertation

Presented to

the Faculty of the Department of Biology

College of Arts and Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

F. Glenn Anders, Jr.

December 1971

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CELL AGGLUTINATION IN THE YEAST, *HANSENULA WINGEI*, CORRELATED WITH CHANGES IN ULTRASTRUCTURE

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ABSTRACT

The yeast Hansenula wingei exhibits a cellular agglutination of opposite mating types as an early step in sexual reproduction by conjugation. Freshly harvested and washed cells show a low agglutinability in the absence of cations. Previous investigations have shown that cell agglutinability may be activated by either chemical or physical treatments. In this thesis ultrastructure of the cell walls and external fringe, viewed with electron microscopy, is correlated with certain treatments known to affect the agglutinability of the cells. Activation of agglutinability with cations is freely reversible, strictly dependent upon the presence of dissolved salts, and appears to cause no noticable ultrastructural changes in the cell wall or fringe. Activation of agglutinability with brief heat treatment is not freely reversible, but makes the agglutinable state independent of dissolved salts, and appears to remove the fringe material quantitatively from the cell wall surface. Cell agglutination of both heat activated and magnesium activated cells is freely and repeatedly reversible in 8M urea, and this confirms a previously published conclusion that cell agglutination involves weak chemical bonding. Several methods of measuring the intensity of binding and the rates of

agglutination show that heat activated cells demonstrate a more intense binding than cation activated cells. The results suggest further that the agglutinative component molecules are located in both the external fringe and the outer layer of the cell wall proper. It is also noted that the mucopolysaccharide specific stain, ruthenium red, is localized in both of these layers.

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I. INTRODUCTION

CHAPTER I

INTRODUCTION

The heterothallic yeast Hansenula wingei was isolated as a diploid yeast from bark-beetle frass of coniferous trees by Wickerham (1956). He described the species and was the first to observe massive cell agglutination in this species when cell suspensions of haploid isolates were mixed. He also was able to correlate haploid mating types with this cell agglutination phenomenon, referred to as sexual agglutination, as it involved an early step in sexual reproduction by conjugation.

The conjugation of H. wingei was first studied with the electron microscope by Conti and Brock (1965). Protuberances formed by adjacent cells at the point of contact eventually fused forming a conjugation tube. After the dissolution of the fused cross wall in the conjugation tube, the nuclei moved into the conjugation tube, and the diploid nucleus moved into the newly formed bud. A cross wall then developed, separating the diploid bud from the conjugation tube.

Mating type is commonly determined by a one-locus, two-allele genetic system in heterothallic yeast (Raper, 1966). Wickerham (1956) completed genetic crosses which gave strong

indications that both cell agglutination and mating type were inherited as simple Mendelian traits. Occasional genetic recombinants and a few spontaneous mutants suggested further that the genetic specificities for agglutination and mating type might originate from two separate but closely linked genetic loci. This early impression was strengthened by genetic results of Herman,et. al. (1966), which showed that the agglutinating factor nearly always segregated with the mating type factor. Herman and Griffith (1967) obtained evidence suggesting that non-agglutinative strains still possessed a gene for agglutination, but its expression appeared to be masked by the action of a repressor gene. Crandall and Brock (1968b) also postulated a repressor gene, to explain the non-agglutinative nature of diploid strains.

Wickerham (1956) observed that non-agglutinative strains conjugated but at a much lower frequency than agglutinative strains. From this evidence he concluded that sexual agglutination was a mechanism that increased the probability for conjugation. The two agglutinative isolates of opposite mating type, strain-5 and strain-21, used in this investigation were derived by Gertrude Lindegren in October, 1951, as progeny of one of Wickerham's original diploid isolates (Y-2340) and were used extensively in investigations of the agglutination process (see review by Crandall and Brock, 1968b).

Cell agglutination in H. wingei is a cell-surface phenomenon involving a specific binding between complementary agglutinating factors located on the surface of the cell wall. Strains 5 and 21 demonstrated specificity in that neither would agglutinate with themselves (autoagglutination) nor with the non-agglutinative diploid strains. The agglutinating factors on strains 5 and 21 were complementary and nonidentical, as shown by their specificity of reaction and by the differential sensitivities of whole cells of both strains to both physical and chemical treatments (Table I). Brock (1959a) showed that 8M urea prevented the agglutination of 21 and 5 cells and also deagglutinated agglutinated cell masses. The agglutinating factors were isolated and partially purified with snail juice enzymes, mechanical disruption or proteolytic enzymes and characterized as heterogenous, low molecular weight glycoproteins (Taylor 1964a, 1964b, 1965; Taylor and Orton, 1967, 1968a, 1968b, 1970; Brock 1965; Crandall and Brock 1968a). The molecular weight of the smallest functional unit was not determined.

Electron microscope studies on yeast cell walls demonstrated an outermost hairlike fringe of filamentous material oriented at right angles to the layering of the cell wall proper (Forman, 1967). Cytochemical studies of Northcote and Horne (1952) indicated that the yeast cell wall consisted of at least two regions. One of these layers consisted primarily of

TABLE I

DIFFERENCES IN AGGLUTINABILITY BETWEEN CELLS OF STRAINS 5 AND 21

Agent or Treatment	Response Strain 21	Response Strain 5	Reference
Proteolytic enzymes:			
trypsin	destroyed	resistant	Brock 1958b
papain	destroyed	resistant	Brock 1959a
lipase	destroyed	resistant	Brock 1959a
pancreatin	destroyed	resistant	Brock 1959a
80% Phenol	destroyed	resistant	Brock 1959a
Acid (hot dilute)	destroyed	resistant	Brock 1959a
Esterification (Methanol + H^+)	destroyed	resistant	Brock 1959a
Thorium nitrate	destroyed	resistant	Brock 1959a
Na Periodate, 37°C	more resistant	destroyed	Brock 1959a
Na Periodate, 5°C	resistant	destroyed	Snider (unpublished)
Mercaptoethanol	resistant	destroyed	Taylor 1964
0.1% DNFB	resistant	destroyed	Taylor 1964
0.1M Na_2SO_3 + 4M urea	resistant	destroyed	Taylor 1964
Prolonged heat (100°C, 3 hrs)	destroyed	resistant	Henderson 1966

mannan associated with protein and the other layer had a glucan component. Forman (1967) used enzymatic digestion with Helix pomatia gastric juice to show the removal of the inner glucan-containing layer. Attempting to stain preferentially the polysaccharides of the yeast cell wall for an electron microscope examination, Mundkur (1960) obtained evidence that the mannan is located in the outer region of the wall. Better observational definition of the surface fringe in H. wingei was obtained recently by Black (1971) with the mucopolysaccharide-specific stain ruthenium red. The heavy element ruthenium (A.W. 101) greatly increases the electron-density of the hairlike fringe.

Brock (1958a) demonstrated a reversible, non-specific cation requirement for agglutination. Appropriate heat treatment of washed cells prior to agglutination also activated cells but differed from cation activation in several respects. Heat treatment of cells irreversibly activated cells and led to a more intensive agglutination than occurred in the presence of cations (Brock, 1958a). Activation by both cations and heat treatment is discussed further in Chapter 3.

This different in strength of agglutination was observed here in several different lines of experimentation. The studies of Brock and Taylor on the effects of certain treatments upon agglutinability were extended in an attempt to

elucidate further the mechanism of cell agglutination. The new data are consistent with the hypothesis that cell agglutination involves weak chemical bonding between dissimilar, complementary components. The principal aim of this thesis was an attempt to find changes in the microstructure of the cell wall that could be correlated with states of agglutinability induced by various laboratory treatments. The correlation was a success. It also made possible an explanation for the different binding strength of magnesium and heat activated cells. The observation was also noted that agglutinability seemed to be associated with material that stained with ruthenium red.

II. METHODS AND MATERIALS

CHAPTER II

METHODS AND MATERIALS

Organism

The haploid strains, 5 and 21, of Hansenula wingei used in this work were siblings of opposite mating and agglutinating types. These strains were isolated from the parent diploid strain Y-2340 in the laboratory of Dr. L. J. Wickerham and kindly supplied by him. They were maintained on stock slant cultures, and they have continued to possess the cultural and physical characteristics ascribed to them by Wickerham (1956).

Cultural conditions

For most of the work the yeast cells were grown in the broth or solid medium of Brock (1958a). Brock's medium (BM) is a complex medium composed of 5g of KH_2PO_4, 30g of glucose and 7g of yeast extract (Difco) in 1 liter of distilled water (pH about 5.3). For the electron microscopic work a second medium was used at the suggestion of Dr. S.H. Black. This medium (Wickerham and Stodola, 1960) is referred to here as Yeast-Malt (YM) medium and consists of 3g malt extract (Difco), 3g yeast extract (Difco), 5g peptone (Difco), and 10g glucose in

1 liter of distilled water (pH 6.0-6.5). Two percent agar (Difco) was added to either formula when solid medium was required. Both media were autoclaved for 20 minutes and incubated overnight for a sterility check before using. Liquid cultures were aerated by growing in Erlenmeyer flasks containing medium to no more than two-fifths of capacity and incubated on a rotary shaker at 200-250 rev/min. Flasks containing 50 or 100 ml of medium were inoculated correspondingly with 5 or 10 ml of inoculum from a stationary phase culture and incubated on the rotary shaker at 27-30°C. Under these conditions, log phase growth was obtainable within 10 hours incubation. Changes in pH during the growth period showed an initial drop followed by a gradual climb, with a final pH not exceeding 6.8 after 90 days on Brock's medium and a pH of 8.5 after 30 days on YM media. Cells were typically harvested after 3 to 6 days of incubation, although experiments indicated no difference in the agglutinability of cultures older than 6 days. Cells were harvested by centrifugation at 10,000 RPM (12,000 x g) for 5 minutes and washed three times with demineralized water. The washed pellet was then suspended in either demineralized water or 1% $MgSO_4$ solution (depending upon method of activation) and standardized by adjusting the concentration to a turbidity of 500 Klett units (#42 blue filter). This turbidity was equivalent to a concentration of about 10^8 cells/ml for either strain.

Activation of Cells

Cells suspensions were heat activated in volumes no greater than 10 ml per test tube by immersing the tubes in a water bath already at 100° C and holding the tubes at 100° C for 10 minutes before removing them to cool to room temperature. Although the heat treatment killed the cells, controls showed heating did not release active agglutinating factors into the supernate and, therefore, there was usually no reason to wash and restandardize the cells after heat treatment. The supernate of heat activated cells of strains 5 and 21 was analyzed by flame photometry for the presence of several cations that might be expected to be extracted by the heating. This was a reasonable expectation, since we knew from quantitative results obtained by Henderson (1966) that the thermal death point for H. wingei was between 60° - 70° C. Significant amounts of potassium ion were detected in the supernate, but there were no detectable amounts of magnesium ion, sodium ion or calcium ion. The threshold of detectability for magnesium ion was 3.6×10^{-5}M and for sodium ion, potassium ion and calcium ion, it was about 10^{-7}M. Nevertheless, heat activated suspensions were washed several times before use in any experiment in which there was a concern for possible activating effects of cations released by the heat-activating treatment. Bulk amounts of cell suspensions were heat activated

by placing a 500 ml Erlenmeyer flask with 250 ml of standardized cell suspension in loose steam (100° C) for 15 minutes.

Magnesium activation was accomplished by using a 1% $MgSO_4$ (W/V) solution for the final wash, then resuspending the washed cells in a 1% $MgSO_4$ (0.04M) solution and standardizing the solution to 500 Klett units turbidity. Earlier work in our laboratory indicated a direct correlation of the time of exposure to magnesium cations with the degree of activation. In the present studies, no difference in level of activation by magnesium cations was observed when the total exposure time was 20 minutes, overnight (12 hours) or longer. A two hour exposure period was adopted as standard procedure.

Standard Agglutinating Assay

The standard agglutinating assay used was a modification of the rapid quantitative method according to Brock (1958a). In this assay 2.5 ml each of standardized and activated cells of strain 21 and strain 5 were mixed (total volume 5 ml) in optically matched Klett tubes. The mixed cells were stirred with a stainless steel spatula for about 30 seconds or until massive agglutination and precipitation became apparent. The agglutinated cells were then centrifuged in the Klett tubes at 6,000 RPM (4300 x g) for three minutes. The pellet was resuspended by stirring with the spatula and then allowed to

settle for ten minutes. The final turbidity in the supernatant was read on a Klett-Sumerson colorimeter (filter number 42).

The difference between the initial (T_i) and final (T_f) turbidity was a measure of the degree of agglutination. The change in turbidity (T) or final turbidity (T_f) could be easily converted into expressions of the percent of cells agglutinated or left in suspension, respectively. Results are presented here in terms of final turbidity (T_f); thus, it is important to keep in mind that low T_f values correspond to a high intensity of agglutination. Non-activated cells gave essentially no reduction in turbidity, whereas heat activated cells yielded a mean final turbidity of 16± 7 Klett units and magnesium activated cells produced a mean final turbidity of 32± 10 Klett units. These values of final turbidity corresponded to ninety-seven or ninety-eight percent removal of the cells initially in suspension. In some experiments other methods than pouring and centrifuging were explored for mixing the cells and packing them to facilitate agglutination (see Chapter 4).

Deagglutination by Urea

Deagglutination by 8M urea (refer to Chapter 4, urea deagglutination) was accomplished by pelleting the agglutinated cell mass, decanting the supernate and resuspending the cells in freshly prepared 8M urea. Deagglutination kinetics were followed by reading the final turbidity of resuspended cells

that remain in suspension after a ten minute settling period. After a reading, the tube was stirred again and allowed to settle for another ten minute period before reading again. The T_f of deagglutination, in contrast to the agglutination assay, is thus directly proportional to the amount of deagglutination.

Electron Microscopy

Cells were grown in either broth or on solid YM (Yeast-Malt) medium for periods from one to three days. Cultures were stained and prefixed directly in the culture medium and harvested with a minimal amount of washing. Fixation and staining were accomplished simultaneously with ruthenium red (Tetraaminoruthenium hydroxychlorochloride), Alpha Inorganics, New Jersey, and either sodium permanganate according to Black (personal communication) or a modification of a double fixation method (Pate and Ordal, 1967) with sodium permanganate and osmic acid. In the single fixation method, cultures were prefixed with 0.5% sodium permanganate solution for ten minutes. The culture medium was removed by decanting the supernate after short and light centrifugation. The pelleted cells were resuspended for 30 minutes in a fixation mixture consisting of equal proportions of 2% sodium permanganate and 0.1% ruthenium

red solution. In the double fixation method the permanganate fixative was removed by washing with 0.1% ruthenium red solution. These cells were then post fixed with a 5% osmium tetroxide: ruthenium red solution (1:1 by volume) for one hour. All fixations were done in an ice bath. The fixed and stained cells were then embedded in 2% agar blocks according to the method of Conti and Brock (1965). After hardening, the agar blocks were cut into 1 mm cubes and dehydrated in a graded series of ethanol solutions from 30% to 100%, followed by transitional infiltration with propylene oxide. Cells were then embedded in BEEM capsules with Epon 812, as described by Luft (1961), or by a 1:1 mixture of Epon 812 and Araldite according to the method of Mollenhauer (1964). After curing for four days at 60° C, the blocks were trimmed and sectioned on a Sorvall MT-2 Ultramicrotome with either glass knives or a diamond knife. Gray to gold sections (500 - 1000 A) were collected on formvar coated grids and post stained with lead citrate alone or were stained for 15 minutes with 2% uranyl acetate followed by a 10 minute treatment with lead citrate (Reynolds, 1963). Electron micrographs were made at a magnification of 20,000 X or 30,000 X with an A.E.I. EM-6B electron microscope.

III. ACTIVATION OF AGGLUTINABILITY

CHAPTER III

ACTIVATION OF AGGLUTINABILITY

Harvested and washed cells of opposite mating type would normally demonstrate only slight agglutination (1 - 2% of the cells). As demonstrated by Brock in earlier work, cells of H. wingei could be activated to yield a high degree of agglutination (98% of the cells) by treatment with either various cations or by heat treatment. The cells retained full viability during and after activation by cations and agglutination; heat activated cells were killed by the heat treatment. Heat activation, however, not only made the cells highly agglutinable but most signficantly did not alter the specificity of agglutinability. Washed cells of opposite mating type suspended in distilled water failed to demonstrate massive agglutination (Brock, 1958), while resuspending these cells in solutions of various cations resulted in nearly complete agglutination of the cells. The cation requirement was relatively nonspecific. As shown in Table 2, a variety of cations, both monovalent and divalent, were effective as agglutinability activators. Brock (1958) pointed out that the relative effects of various cations did not follow the Hofmeister series. At low concentrations divalent cations seemed more active than the monovalent ions. (Brock, 1958a). In the present study, a single concentration

TABLE 2. LIST OF EFFECTIVE CATION ACTIVATORS

Initial turbidity 500 KU, low T_f indicates high degree of agglutination, all salts were 1% solutions. (gms/vol)

Cation	Salt Used	T_f (Final turbidity)
Li	LiCl	26
Na	NaCl	46
NH_4	NH_4Cl	42
Na	Na Acetate	76
K	K_2SO_4	25
Ca	$CaCl_2 \cdot H_2O$	33
Mn	$MnCl_2$	48
Mg	$MgSO_4 \cdot H_2O$	41
Fe++	$FeSO_4$	24
Zn	$ZnSO_4$	56

of 1% showed essentially no differences in activation by the ions tested. As shown in Table 2, all cationic solutions tested gave uniformly good agglutination, and activation of the cells seemed not to be influenced by the ionic strength of the cationic solutions. Thus, a concentration of 1% appeared to be above the optimal concentration required to stimulate maximal agglutinability with most of the commonly available monovalent and divalent cations. Brock also reported the ability of certain proteins such as gelatin, peptone and protamines to activate washed cells as the inorganic salts did, and, since protamines and gelatin are cationic, such results strongly suggested that cations, rather than anions, are responsible for activating agglutinability.

Magnesium Activation

Magnesium ions were selected as a convenient agent for kinetic studies of the reversibility of cation activation. No difference was observed in the final amount of agglutination for concentrations of magnesium above 10^{-2}M; therefore, the 1% solution (4×10^{-2}M) used by Brock in his earlier work was adopted for the standard cation activation procedures reported here. The extent to which activation by magnesium cation was a freely and quantitatively reversible process was examined in detail. Various methods were used to remove magnesium cation from magnesium activated cells and also from

agglutinated masses. The methods included washings with distilled water, dialysis against distilled water for 48 hours at 5° C and chelation with ethylenediaminetetraacetic acid (EDTA).

If magnesium activated cells were washed repeatedly in distilled water prior to mixing (and agglutinating), the magnesium cations in solution were removed and agglutinability (activation) was lost. As shown by Figure 3.1, the degree of agglutination of these inactivated cells reached a low plateau after the second or third washing. This ready loss of agglutinability was interpreted as the removal of magnesium ions weakly bound to the cell wall.

Attempts by Brock to use the chelating agent EDTA to remove magnesium cations (and thus agglutinability) from cation activated cells were unsuccessful (Brock, 1959). Tests with EDTA were abandoned in the present study when it was observed in a control that the EDTA solution alone activated the cells. The sodium salt of the EDTA probably caused the activation and thus masked any effect of the EDTA.

If the magnesium activated cells of both strains were first agglutinated and then the agglutinated mass was washed repeatedly with distilled water, the mass of cells remained agglutinated regardless of the number of washes (Figure 3.2). Thus, it appeared that repeated distilled water washes of

Figure 3.1 Effect on Agglutination of Repeated Distilled Water Washings of Magnesium Activated Cells

Activated cells washed prior to mixing (agglutinating). Low T_f (final turbidity) reading reflects high degree of agglutination and a high T_f value reflects low degree of agglutination.

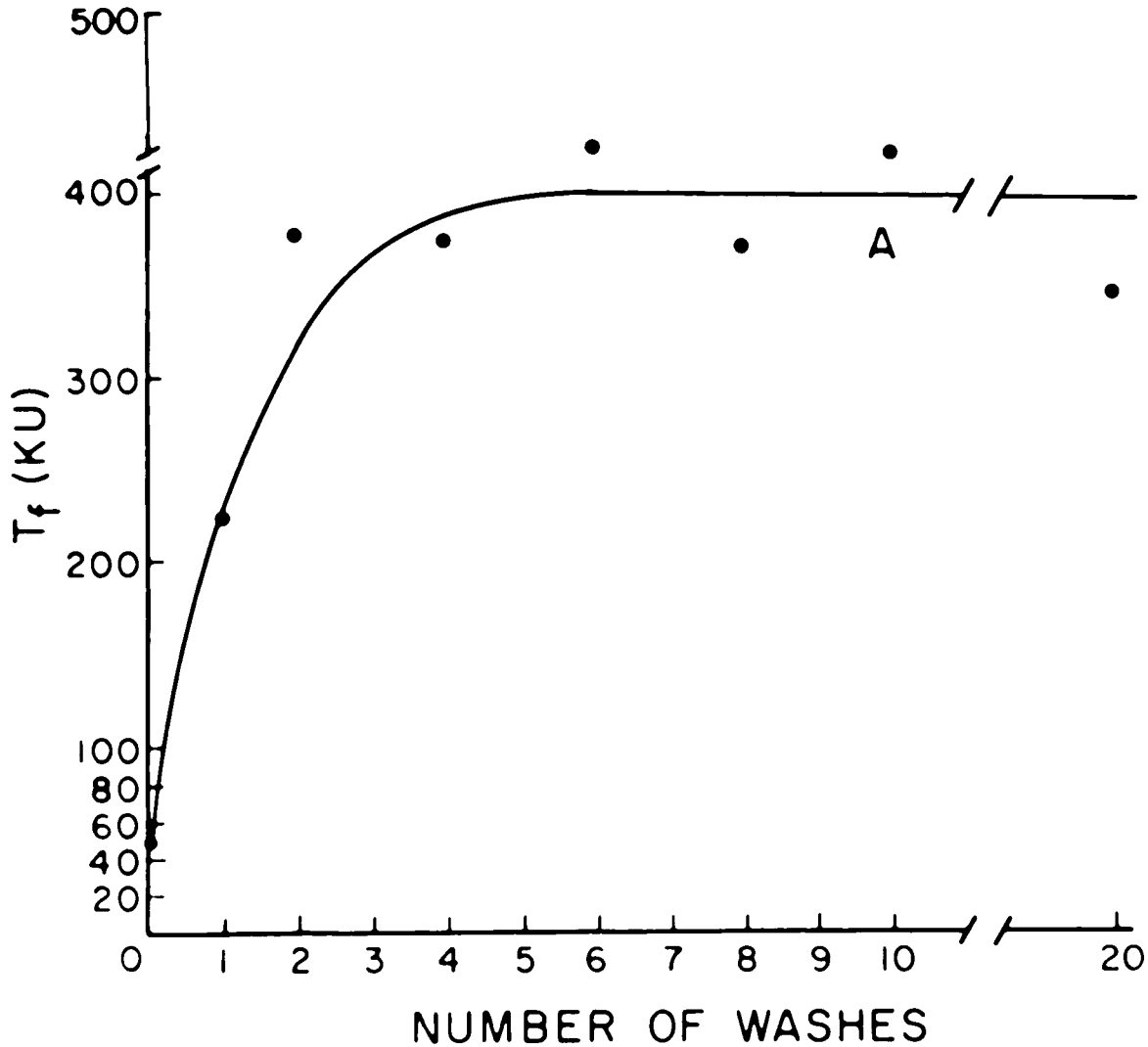
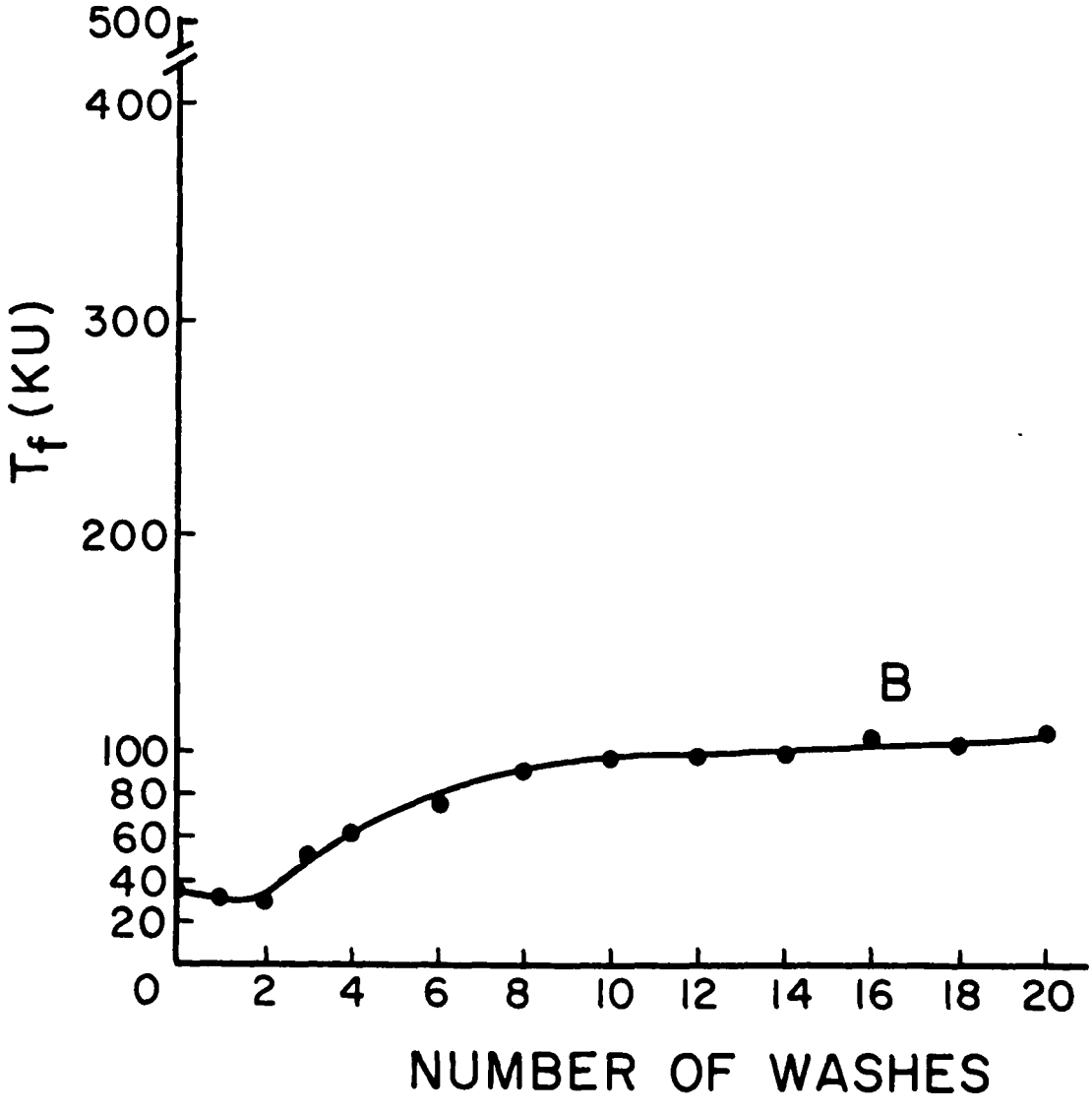

500
400
300
200
100
80
60
40
20
T_f (KU)
A
0
1
2
3
4
5
6
7
8
9
10
20
NUMBER OF WASHES

Figure 3.2 Effect of Repeated Distilled Water Washes of Magnesium Activated, Agglutinated Cells

Cells mixed (agglutinated) and then washed repeatedly.


500
400
300
200
100
80
60
40
20
0
T_f (KU)
B
2
4
6
8
10
12
14
16
18
20
NUMBER OF WASHES

magnesium activated cells removed about 80 percent of their agglutinability (Figure 3.1); whereas, distilled water washing of magnesium activated cells after mixing and agglutinating had no tendency to loosen the agglutinated mass. The magnesium ions appeared to be bound so tightly once opposite mating types were mixed and agglutinated that no degree of washing would remove them, but the magnesium ions apparently were removed rather easily from the cell surface prior to mixing and agglutinating.

When magnesium activated cells were rendered non-agglutinable by removing the soluble magnesium ions by either washing with distilled water or by exhaustive dialysis, resuspension of the cells in magnesium solution fully restored agglutinability. Thus, neither washing nor dialysis irreversibly inactivated the cells. Cationic activation was, therefore, mostly and easily reversible, but up to 20 percent agglutinability was not completely removable by washing or dialysis (Figure 3.1). This suggested initially that some of the magnesium may remain too tightly bound to be removed by gentle treatments. As will be brought out in the discussion, Chapter 6, this failure to lose completely agglutinating activity may be explained alternatively by changes in cell wall structure, involving partial loss of material.

Heat Activation

Brock (1958) indicated that cells from strain 5 and 21 would be activated to a greater extent with heat treatment than by cationic activation. In contrast to magnesium activation, heat activation was highly irreversible. Distilled water washings of heat activated cells, either prior to or after mixing and agglutinating opposite cell types, had no effect upon high agglutinability. Once heat activated, the cells remained fully agglutinable in the absence of externally supplied, solute cations. Heat activated, agglutinated cells could be made to deagglutinate in the presence of 8M urea, but, when resuspended in distilled water, would again show maximal agglutination.

Henderson (1966) demonstrated that strain-21, but not strain-5, cells were sensitive to prolonged heat treatment and would rapidly and irreversibly lose agglutinability when the cells were heated in the presence of magnesium ion. With a modification of the technique of Henderson, his observations on prolonged heating effects on strain-21 were extended. Washed, non-activated cells were heated to 100° C in the reaction apparatus shown by Figure 3.3. Samples were removed at intervals and agglutinated with heat activated cells of the opposite agglutinating type. As shown by Figure 3.4, prolonged heating of non-activated (distilled water washed) cells of strain-21 led to the complete and irreversible loss of agglutinability, whereas strain-5 cells showed no loss of agglutinability after

Figure 3.3 Reaction Apparatus for Heat Activation Studies

(a) Reaction mixture; (b) 1 liter, three port flask; (c) sampling port; (d) cold water reflux condenser; (e) heating mantle; (f) rheostat controlling voltage through heating mantle; (g) 110°C thermometer; (h) magnetic stirrer

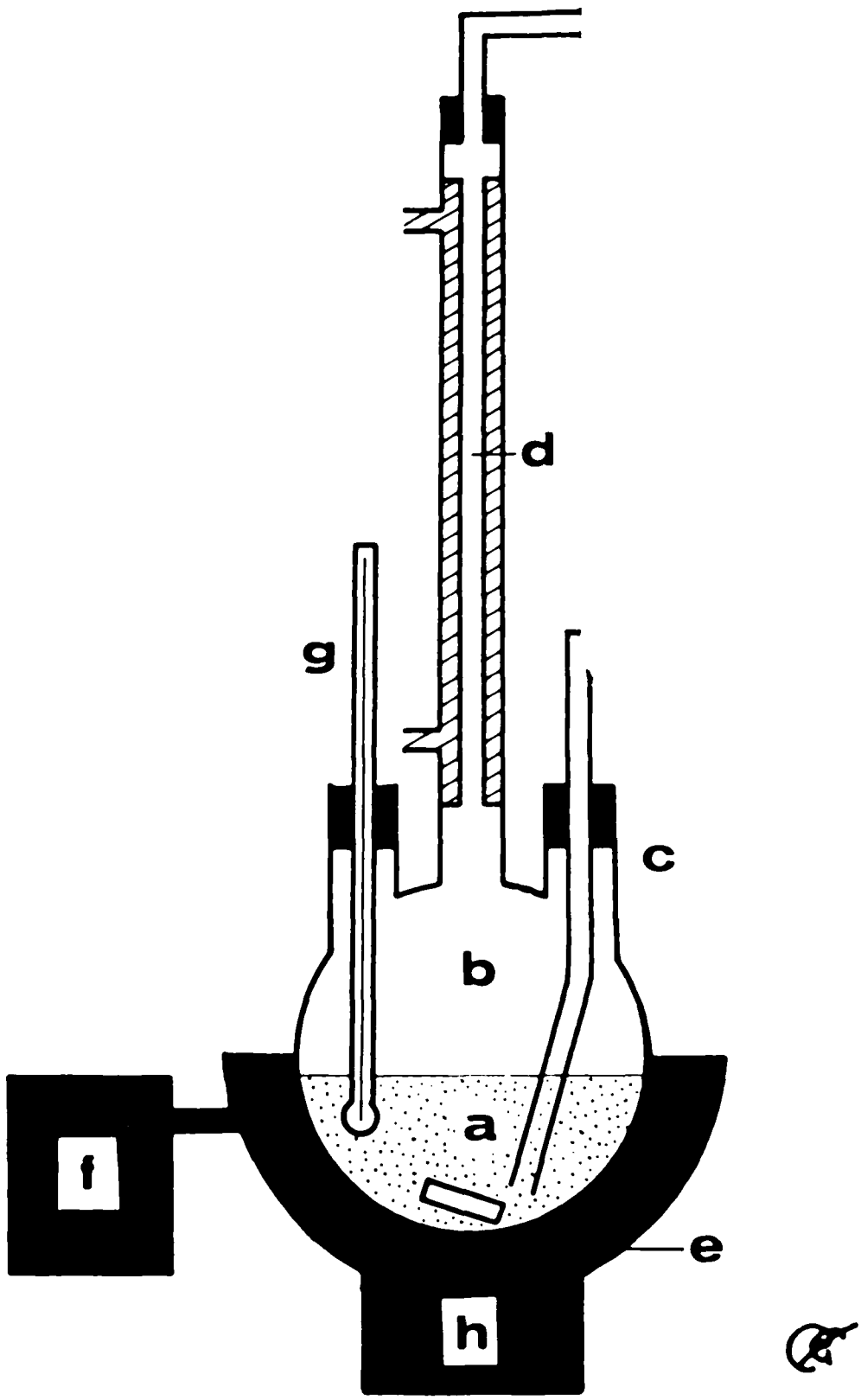
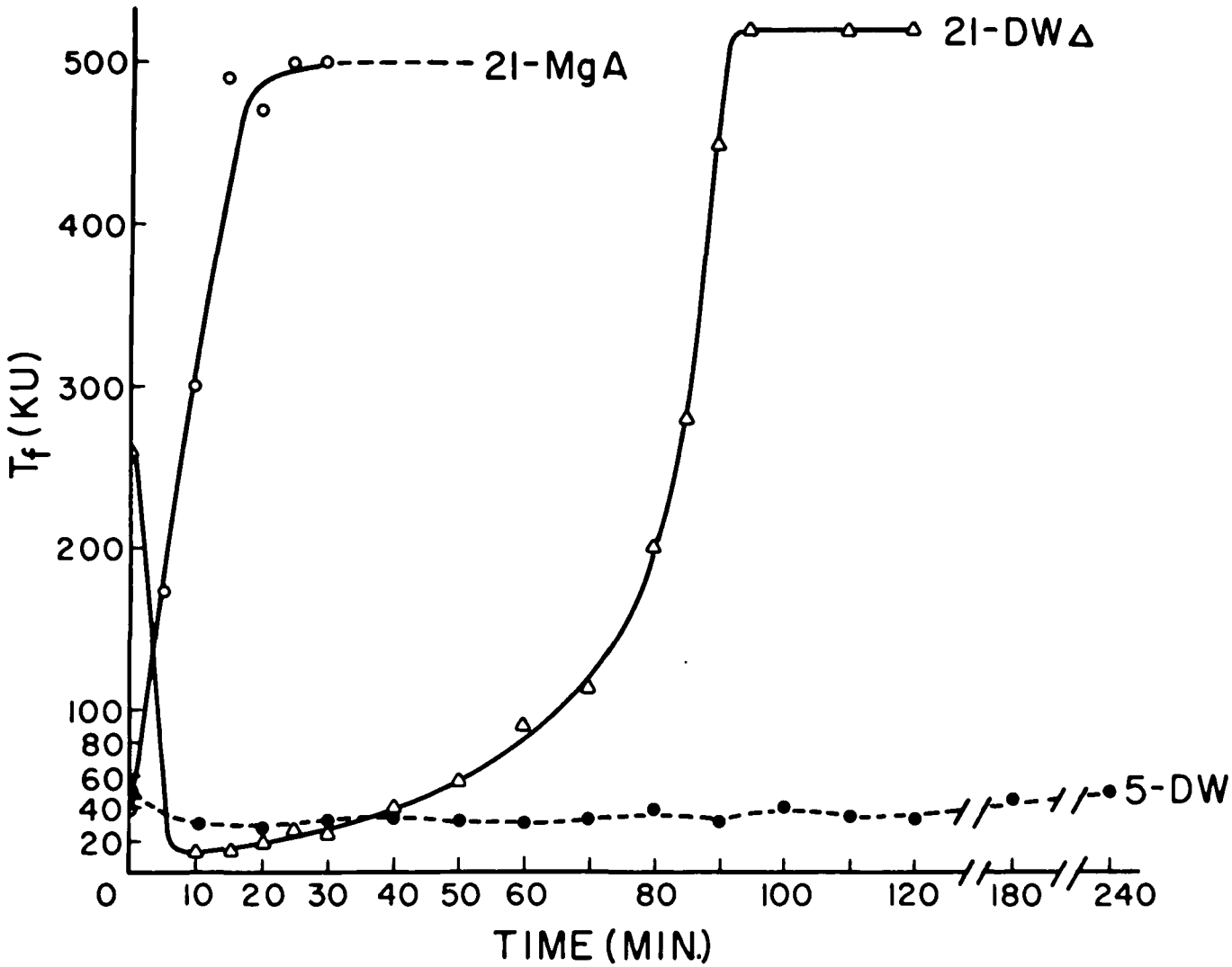

d
g
c
b
a
f
e
h

Figure 3.4 Effect of Prolonged Heating Upon Non-activated (distilled water washed) 2l and 5 Cells and Magnesium Activated 2l Cells


21-MgA
21-DWΔ
5-DW
T_f (KU)
500
400
300
200
100
80
60
40
20
0
10
20
30
40
50
60
80
100
120
180
240
TIME (MIN.)

four hours of heat treatment. Although non-activated 21 cells retained significant agglutinability for up to 90 minutes of heat treatment, magnesium activated 21 cells lost all agglutinability irreversibly after only 20 minutes of heat treatment in the presence of 1% solution of $MgSO_4$.

IV. CELL AGGLUTINATION

CHAPTER IV

CELL AGGLUTINATION

Assay Procedures

The standard agglutinating procedure used in this investigation was described in Chapter 2. Briefly, equal amounts of activated strain-5 and strain-21 cells were mixed, stirred vigorously, then pelleted by a short, low speed centrifugation. The pellet of cells was resuspended and allowed to settle for a fixed period before determining final turbidity.

An early concern over the possible effect of the centrifugation step on the final agglutination prompted a comparison with two other methods for facilitating agglutination, referred to as shake-agglutination and magnetic-stirred agglutination, in contrast to centrifuge-packed agglutination. These methods, two of them allowing the measurement of both the rate and extent of agglutination, also allowed comparison of the intensity, or strength, of the agglutinative binding with cells activated by different means, heat and cationic activation. In the shake-agglutination method the cells were mixed and then placed upon a rotary shaker and samples were withdrawn periodically, allowed to settle for 10 minutes and determined for final turbidity. In the magnetic stirred-agglutination method, suspensions of

activated 5 and 21 cells were agglutinated with the aid of magnetic stirrers (either in an Erlenmeyer flask or with microstirrers within the Klett tubes). Periodically, samples were removed and after the settling period, final turbidity was determined. A comparison of both heat-activated and magnesium-activated cells, agglutinated by the shaker method and the magnetic stirrer methods described above, is shown in Figure 4.1. In both methods of agglutination, heat activated cells showed a greater degree of agglutination as indicated by a lower final turbidity and also by a plateau of maximal agglutination reached faster, than did magnesium-activated cells. As seen in Figure 4.1, heat activated cells reached maximal agglutination by either of the non-centrifuge methods within two hours, whereas the magnesium activated cells required at least four hours for maximal agglutination to occur. A comparison of shake-agglutinated cells and centrifuge-packed cells is shown in Table 3. For heat activated cells there was only a slight difference in maximal agglutination (minimal T_f) between the shaker method and the single centrifugation method; the rate of agglutination could not be measured, of course, in the simple centrifuge technique employed. For magnesium activated cells there was a more pronounced difference in maximal agglutination between the two methods of agglutination than for heat activated cells.

Figure 4.1 Agglutination of Heat and Magnesium Activated Cells by Shaker versus Magnetic Stirrer Methods

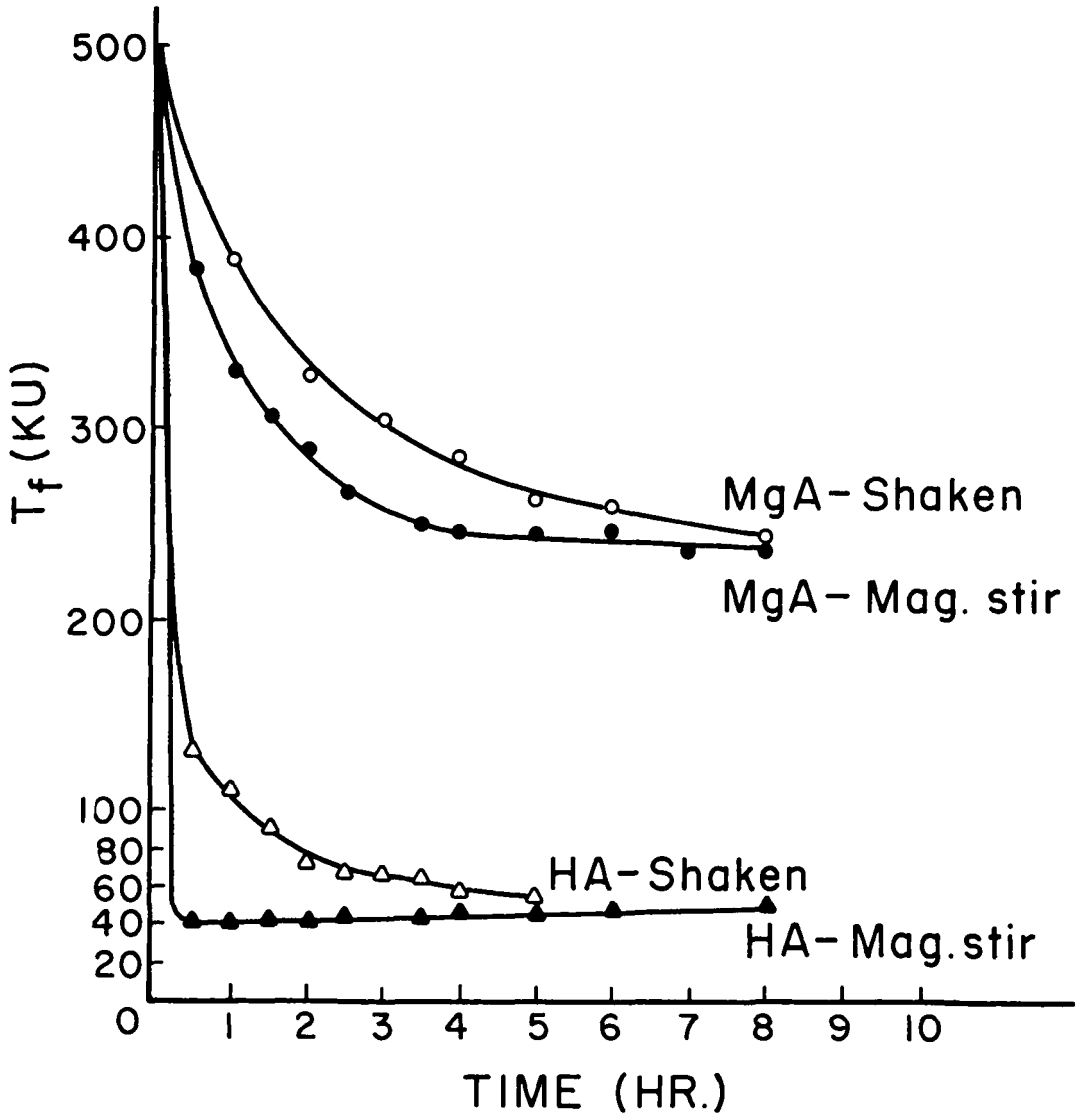

500
400
300
200
100
80
60
40
20
0
T_f (KU)
1
2
3
4
5
6
7
8
9
10
TIME (HR.)
MgA-Shaken
MgA-Mag. stir
HA-Shaken
HA-Mag.stir

TABLE 3

COMPARISON OF SHAKE-AGGLUTINATED CELLS AND CENTRIFUGE-PACKED AGGLUTINATED CELLS

Shake Agglutinated	Time on Shaker	T_f
Heat Activated	2 hrs	39
Mg Activated	4 hrs	284
	10 hrs	207
Centrifuge Packed Agglutinated*		
Heat Activated	----	16
Mg Activated	----	32

*Mean values for all controls

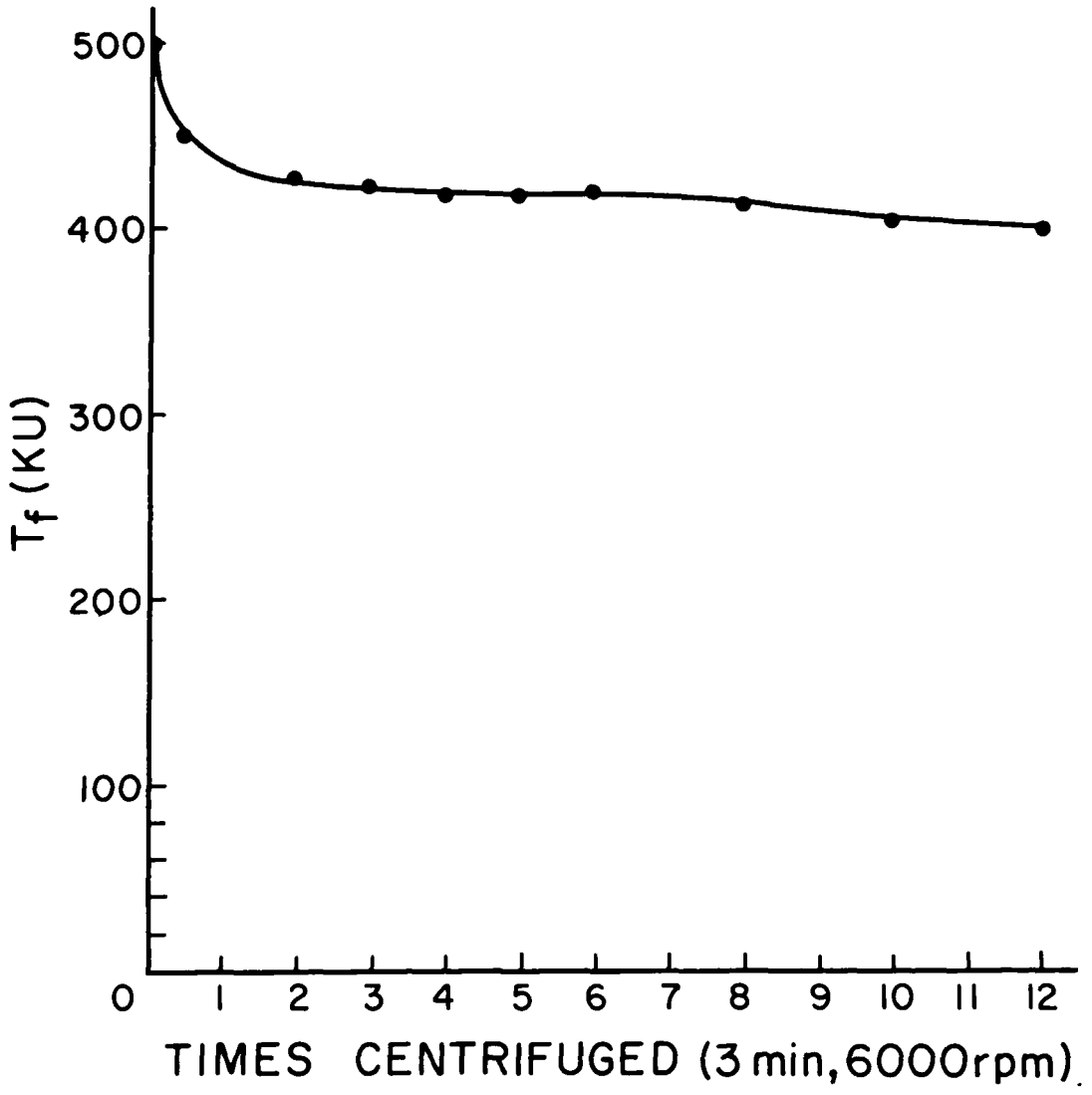
The possibility of the centrifugation step contributing by itself to the degree of final agglutination was investigated in a control experiment with non-activated cells. Washed, non-activated cells of both strains 5 and 21 in distilled water were mixed and then centrifuged repeatedly. After each 3 minute centrifuge period, the pellet of cells was resuspended and after a 10 minute period of settling, final turbidity was determined. The results of repeated short centrifugation periods upon non-activated 5 plus 21 cells are shown in Figure 4.2. Although repeated centrifugations may cause a slight increase in agglutination, due perhaps to forced cell packing, it was concluded that a single, low-speed, short period centrifugation would not contribute significantly to agglutination. With centrifugation for 3 minutes at 6000 RPM as the standard procedure, a final turbidity could be measured approximately 30 minutes after mixing the two strains together, a conveniently short time for a single set of measurements.

Most importantly in this section, evidence was presented showing that the binding force within an agglutinated mass of heat activated cells was evidently considerably stronger than in that of cation activated cells. An explanation of this difference in binding strength will be discussed in Chapter 6.

Cell Combining Number

Brock (1958) investigated the effect of varying the ratio of

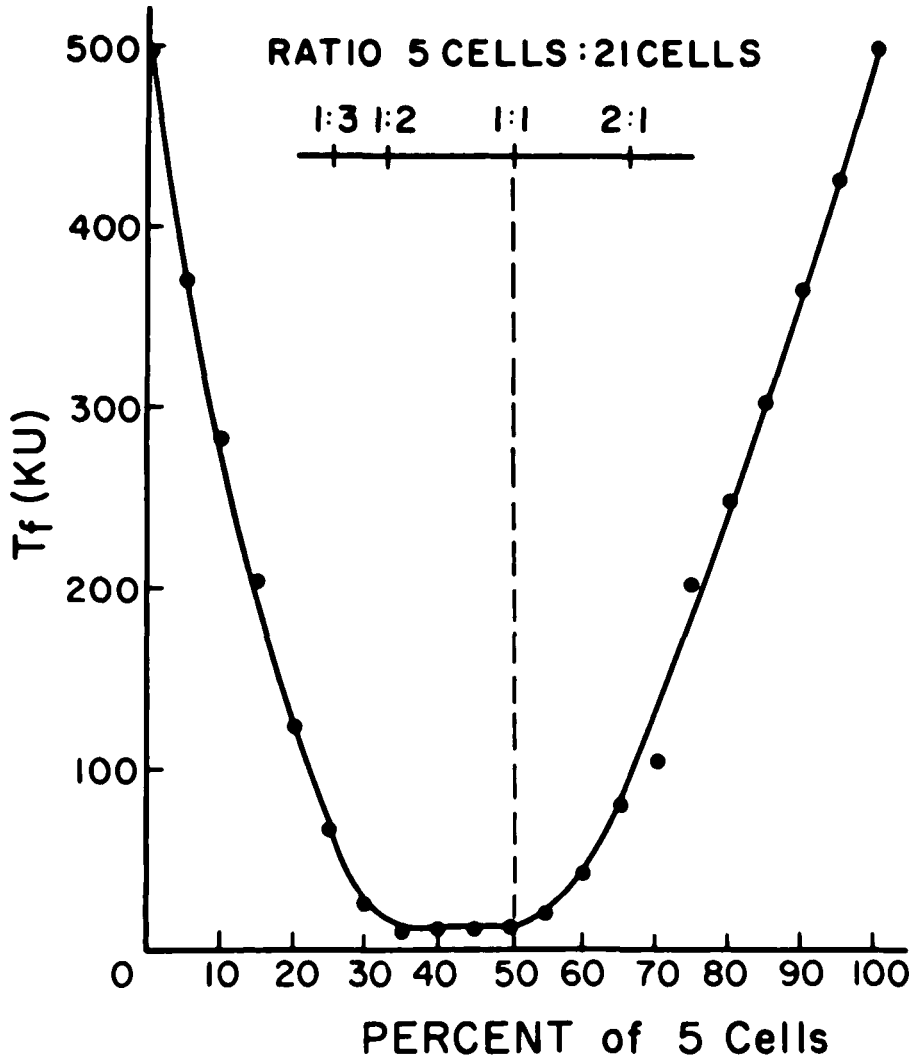
Figure 4.2 Effects of Repeated Short Centrifugations Upon Mixed Solution of Non-activated 5 and 21 Cells


500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
T_f (KU)
TIMES CENTRIFUGED (3 min, 6000 rpm)

strain-5 and strain-21 cells mixed for agglutination (the cell combining number) and concluded that when the cells of the two strains were present in equal proportions, an optimal degree of agglutination occurred. Held and Snider (unpublished results) thought it might be interesting to examine the combining number in greater detail and accuracy and found that optimal agglutination (96-100% of the cells) could be demonstrated for several ratios of 5 to 21 cells. Thus, the combining ratio was not an exact figure, such as 1:1, but more accurately a range of ratios. This range of optimal ratios was actually skewed, with optimal agglutination still obtained when the ratio of 5 cells to 21 cells was as low as 1 to 3 but not at a ratio of 3 to 1. A careful re-examination in another combining-number experiment here also showed definite asymmetry in the same direction and in approximately the same extent (Figure 4.3) as obtained by Held and Snider. The reproducible asymmetry of the curve was strongly suggestive that the strain-5 cell possessed more combining strength, or sites than the 21-strain cell. Even Brock's curve showed skewness although he made no comment as to whether or not the departure from symmetry might be significant.

Not only did the relative proportions of each cell type determine optimal agglutination, but also the manner in which the cells were mixed with each other. The experiments of Brock and those of Held and Snider consisted of pouring together and

Figure 4.3 Optimal Combining Ratios Observed Using Pour and Pack Method of Agglutination


RATIO 5 CELLS : 21 CELLS
1:3 1:2 1:1 2:1
500
400
300
200
100
0
Tf (KU)
10 20 30 40 50 60 70 80 90 100
PERCENT of 5 Cells

centrifuging varying proportions of each cell type to determine the degree of agglutination for that particular ratio. This method shall be referred to as the pour and pack method (Figure 4.3). A modification of this experiment, called the drip-stir method, consisted of a slow, dropwise addition of activated cells of one agglutinating type to a fixed volume of the opposite agglutinating type kept continuously and vigorously in suspension by magnetic stirring. The results of two variations of such experiments are shown in Figures 4.4 and 4.5. In Figure 4.4 pronounced agglutination occurred when the ratio of 5 cells to 21 cells was as high as 1:3. With this method for mixing of the two cell types, the 1:1 ratio was less than optimal. The increase in turbidity of the solution past the maximal agglutination point was due to excess of the cell type being added to the reaction vessel. This could be demonstrated by removing this cloudy supernate to a separate tube and showing clearing (complete precipitation) on adding fresh, activated cells of strain-21. Figure 4.5 shows the reciprocal pairing of strains in the drip-stir experiment: the slow addition of activated type 5 cells to a fixed volume of activated 21 cells. Again the standard 1:1 ratio proved not to be the optimal proportion of cells, as the maximal agglutination occurred when the ratio of 5 cells to 21 cells was 1:4. When adding 5 cells to strain-21 cells the range of maximal agglutination (i.e., the width of the

Figure 4.4 Optimal Combining Ratios obtained with Drip-stir Method

Dropwise addition of 5 cells to fixed volume of 21 cell suspension. ^ = shaken by hand, 30 sec., ° = centrifuge packed, 6,000 rpm x 3 min.

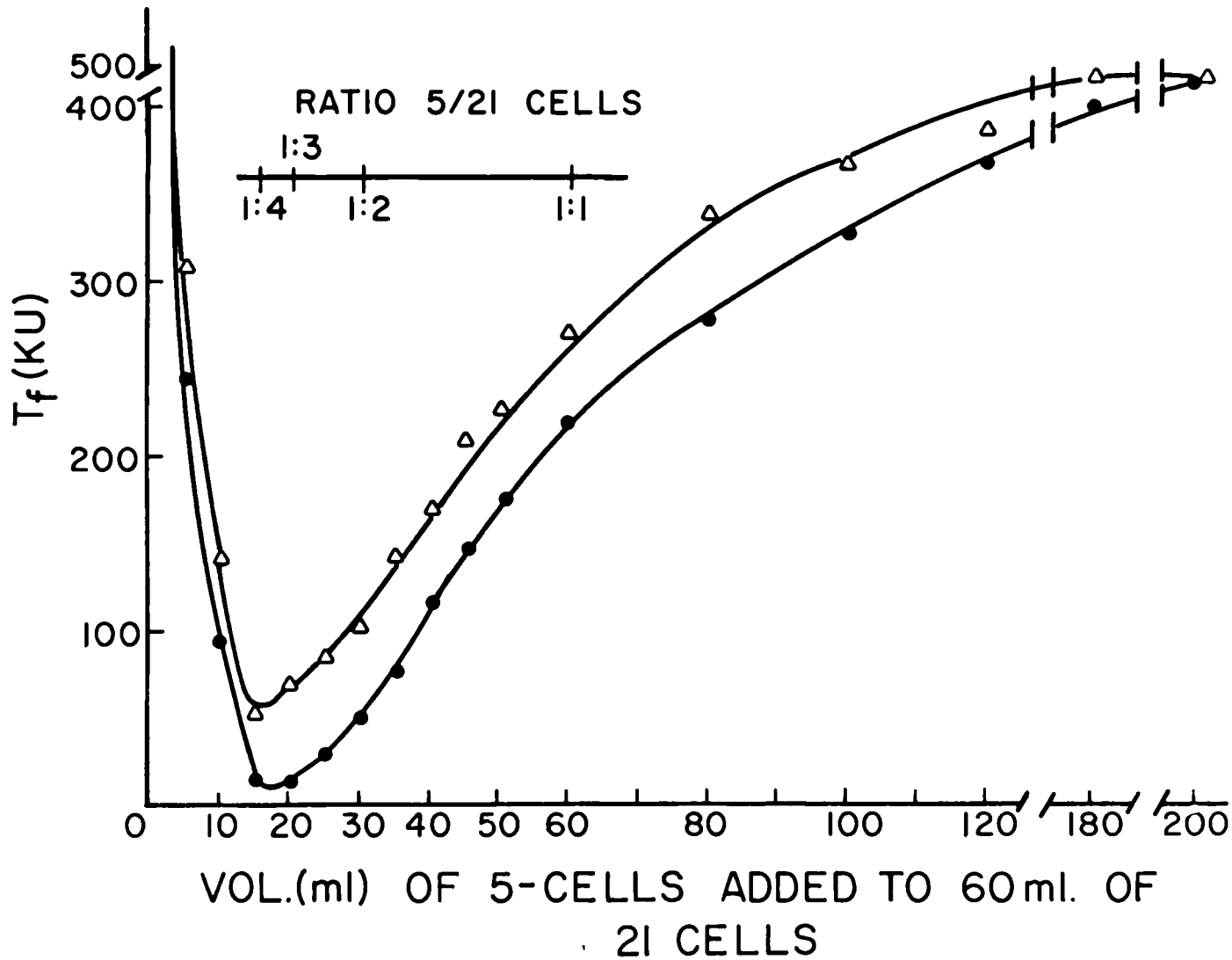
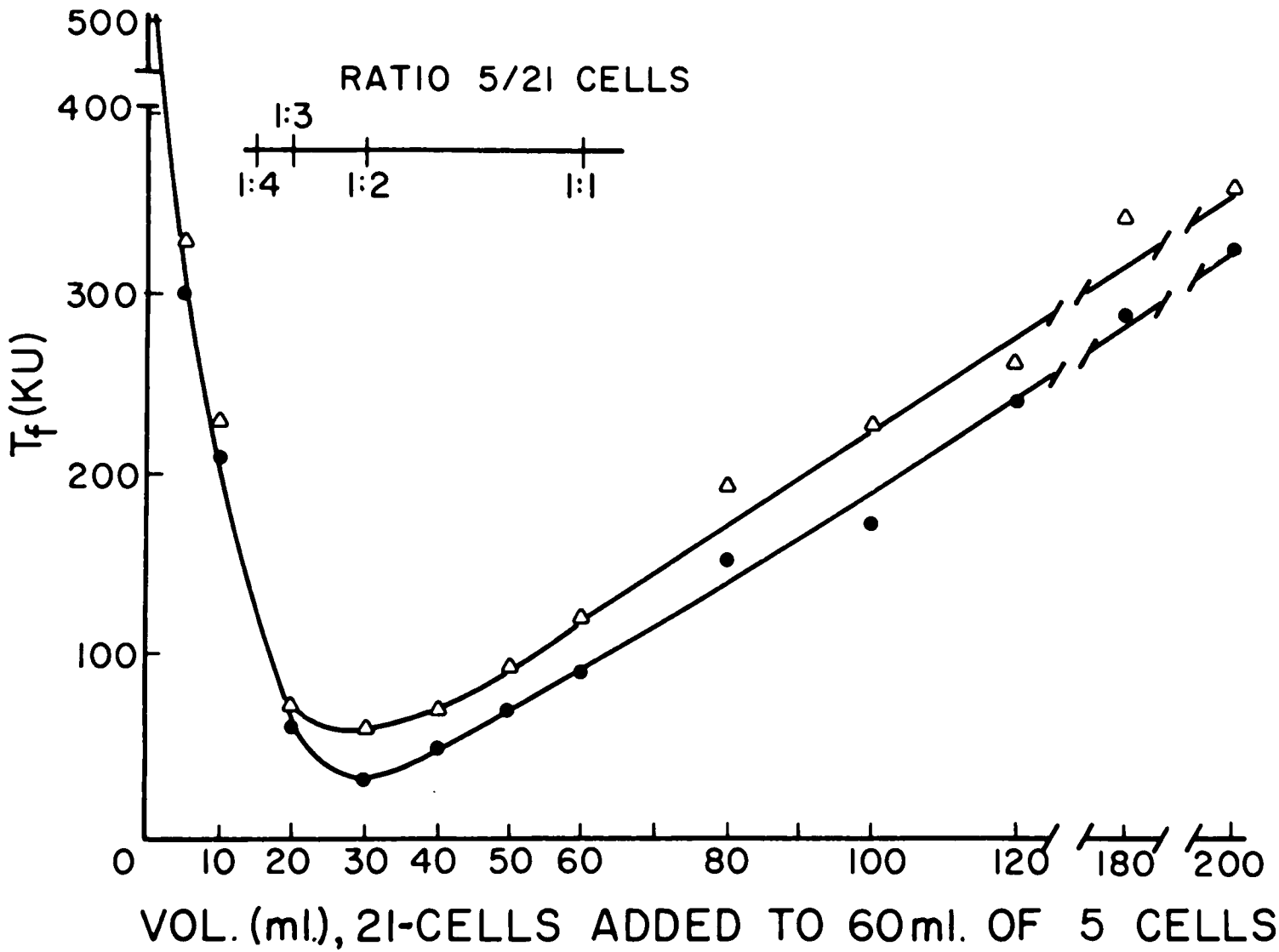

RATIO 5/21 CELLS
1:3
1:4
1:2
1:1
500
400
300
200
100
T_f (KU)
0
10
20
30
40
50
60
80
100
120
180
200
VOL.(ml) OF 5-CELLS ADDED TO 60 ml. OF 21 CELLS

Figure 4.5 Optimal Combining Ratios obtained with Drip-stir Method.

Dropwise addition of 21 cells to fixed volume of 5 cell suspension. Δ = shaken by hand, ● = centrifuge packed.


RATIO 5/21 CELLS
1:3
1:4
1:2
1:1
500
400
300
200
100
0
T_f(KU)
10 20 30 40 50 60 80 100 120 180 200
VOL. (ml.), 21-CELLS ADDED TO 60 ml. OF 5 CELLS

optimal plateau) was more narrow than when the reverse order of mixing was used. After mixing by the drip-stir method, there was no significant difference in the total amount of agglutination observed whether the cells were shake-agglutinated or centrifuge-packed agglutinated. The final amount of agglutination observed depended only upon the initial ratio of cell types at the moment they first interacted in the drip-stir portion of the procedure.

The results of these experiments were consistent with the idea that a strain-5 cell possessed more combining sites, or combining strength, than a strain-21 cell. One explanation for the differences observed between the pour-pack method and the drip-stir method was the possibility that in the pour-pack method all the combining sites on the 5 cells were not saturated by 21 cells. In addition, non-agglutinated cells could become randomly trapped in the matrix without agglutinating. One could easily obtain maximal agglutination from those sample tubes in the drip-stir procedures containing ratios of cells expected to demonstrate good agglutination, but not doing so probably because many combining sites were blocked prematurely in the process. This was demonstrated by the deagglutination of these samples in 8M urea and resuspension in distilled water. This latter step was equivalent to the pour-pack method and resulted in a very complete agglutination for this ratio of cells as would be

predicted by the pour-pack method. Results from one such experiment are shown by Table 4.

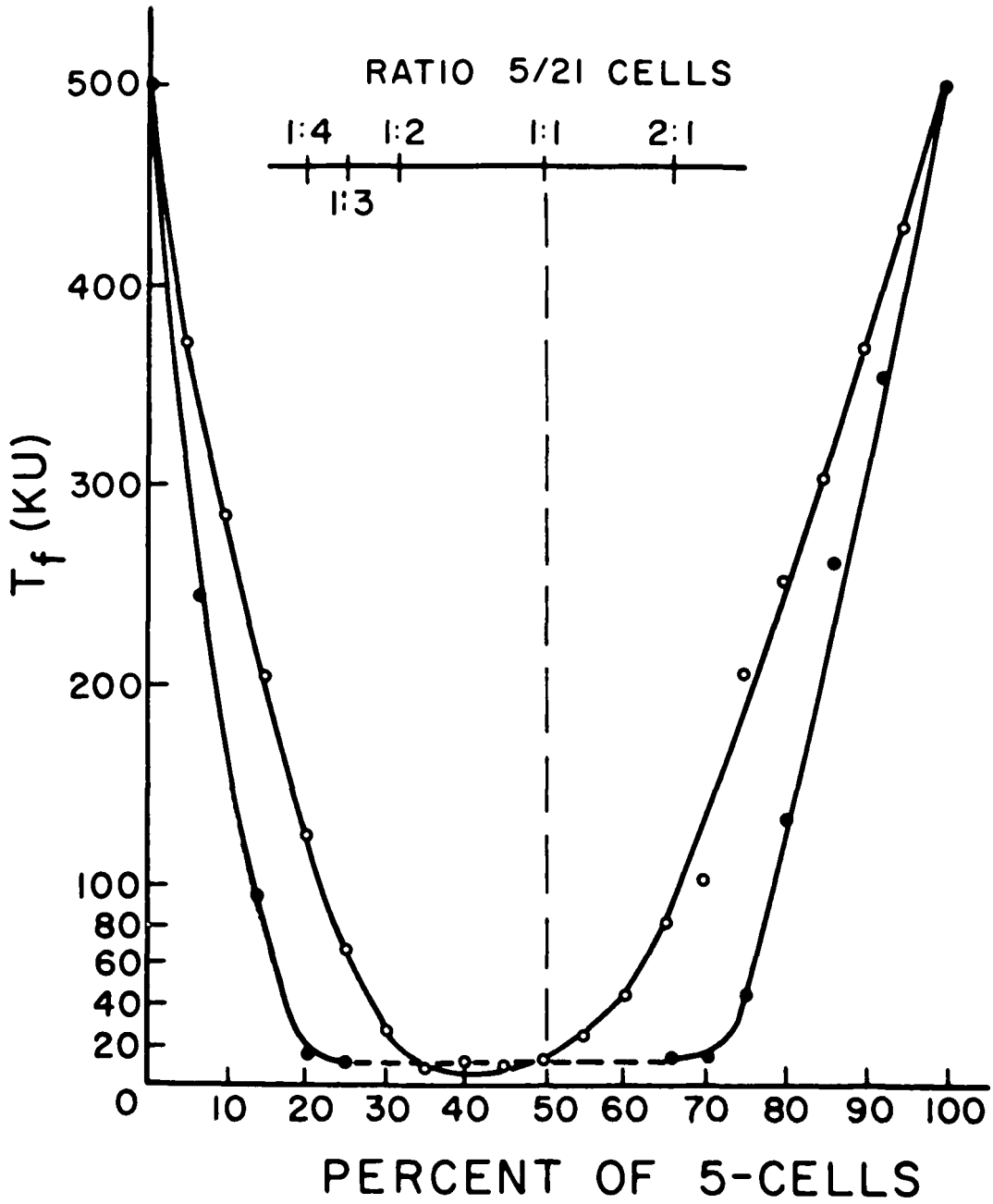
In the drip-stir method in Figure 4.4 and 4.5, the increase in turbidity after maximal agglutination has been obtained was actually an experimental artifact. As seen in Table 4, several ratios of cells not giving a low final turbidity by the drip-stir method, demonstrated good agglutination (low T_f) after deagglutination in 8M urea and resuspension into distilled water. When the data from the first portion of the curves of these two figures were converted into percent of 5 cells added to the mixture, a direct comparison of the pour-pack and the drip-stir method of agglutination could be made. This comparison (Figure 4.6) showed that maximal agglutination can be obtained with the drip-stir method over an even broader range of 5-cell:21 cell ratios (1:4 to 2:1) than by the pour-pack method in Figure 4.3. Thus the degree of agglutination obtained when mixing 5 cells with 21 cells was determined not only by a combining ratio of cells but also by the manner in which the cells initially made contact with each other. The full potential for agglutination and thus the full range of combining numbers for maximal agglutination was shown only with the proper procedure.

TABLE 4

COMPARISON OF DIFFERENT RATIOS OF 5:21 CELLS. AGGLUTINATION FIRST BY DRIP-STIR METHOD, DEAGGLUTINATION IN 8M UREA FOLLOWED BY AGGLUTINATION BY POUR-PACK METHOD.

Sample	Volume of 21 cells (ml)	Volume of 5 cells (ml)	Ratio 5/21	Final Turbidity		
				Drip-Stir in DW	Deagglutination in 8 M urea	Pour-Pack in DW
A	5	30	6:1	230		
B	10	30	3:1	34		
C	15	30	2:1	25		
D	20	30	3:2	16		
E	30	30	1:1	50	268	18
F	45	30	2:3	123		
G	50	30	3:5	180		
H	60	30	1:2	190	370	13
I	90	30	1:3	330	260	24
J	120	30	1:4	370	242	63
C_1	5	-	0:1	500	260	-
C_2	-	5	1:0	500	262	-
C_3	2.5	2.5	1:1	-	270	18

Figure 4.6 Direct Comparison of Optimal Combining Ratios from Pour-pack Method (Open Circles) and Drip-Stir Method (Closed Circles)


RATIO 5/21 CELLS
1:4
1:3
1:2
1:1
2:1
500
400
300
200
100
80
60
40
20
0
T_f (KU)
10
20
30
40
50
60
70
80
90
100
PERCENT OF 5-CELLS

Effect of Urea Upon Agglutination

Brock (1959a) first suggested that cell agglutination involved a weak chemical bonding, such as hydrogen bonding, on the basis of the effect of 8M urea on the agglutination process. Brock observed that agglutination of cells mixed in 8M urea was inhibited and that agglutinated masses could be deagglutinated with 8M urea. Treatment of cells with 8M urea did not irreversibly remove the agglutinability of these cells, as the inhibition was removed when the urea solution was removed from the suspension by washing and resuspending the cells in distilled water or magnesium solution. Brock's initial observations have been confirmed here and kinetic data has been obtained to show that the deagglutination of an agglutinated cell mass by 8M urea was truly a freely reversible process. The urea deagglutination procedure has then been used for another comparison of the strength of agglutination with different means of activation and agglutination.

As indicated by Brock, cell suspensions of either magnesium or heat activated cells suspended in 8M urea would not agglutinate. Repeated washings of the cells in 8M urea prior to mixing would not remove the agglutinability of either cell type; urea-washed cells always agglutinated fully if resuspended in distilled water (or magnesium solution, depending upon the means of activation) in the absence of 8M urea before mixing. The free reversibility of the deagglutination caused by 8M urea was shown

by sequential treatments in which an agglutinated cell mass was repeatedly first deagglutinated by suspending in 8M urea and then allowed to re-agglutinate upon removal of the 8M urea. These deagglutination-agglutination cycles may be repeated an arbitrarily large number of times, and typical results were shown for heat activated and magnesium activated cells (Figure 4.7 and Figure 4.8). When the second and subsequent urea dissociation (deagglutination) kinetics were compared to the initial dissociation kinetics, no significant difference was observed in the amplitude of the repeated responses for heat activated cells (Figure 4.9); whereas, the maximal, final amount of agglutination for magnesium activated cells appeared to drop somewhat with repeated cycling (Figure 4.10) and suggested once again that cation activated cells reacted with less intensity than heat activated cells. The initial deagglutination of magnesium activated cells and heat activated cells demonstrated essentially identical kinetics, both generally requiring approximately 60 minutes exposure to 8M urea to reach a plateau or occasionally, up to 140 minutes. (Note that a Klett reading of 260 for T_f in 8M urea was equivalent to a Klett reading of 500 in distilled water). Both of these curves for magnesium and heat activated cells in Figures 4.7 and 4.8 were data from the centrifuge-packing method of agglutination. Shaken agglutinated cells

Figure 4.7 Sequential Agglutination and Deagglutination Cycles Using Magnesium Activated Cells and 8M Urea

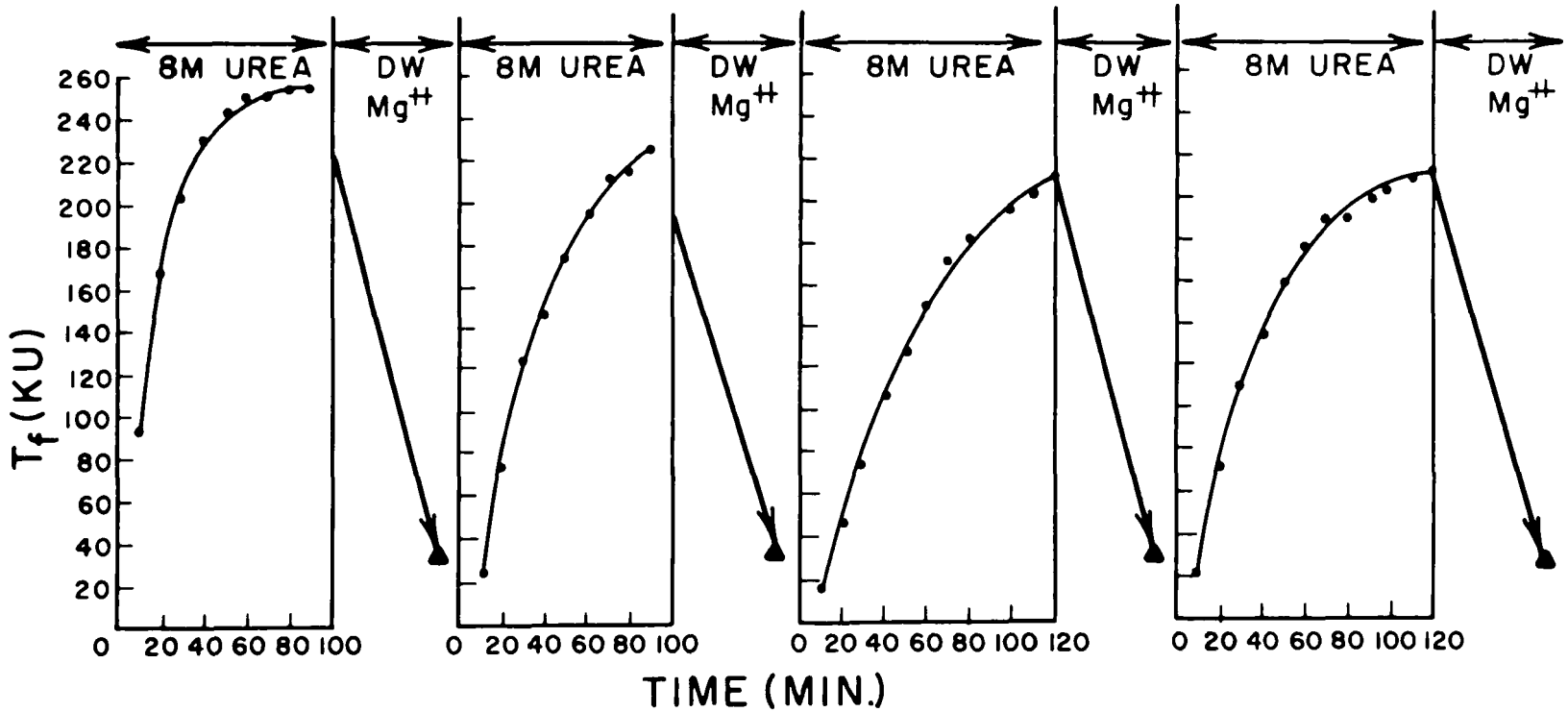

8M UREA
DW
Mg++
8M UREA
DW
Mg++
8M UREA
DW
Mg++
8M UREA
DW
Mg++
Tf (KU)
260
240
220
200
180
160
140
120
100
80
60
40
20
0 20 40 60 80 100
0 20 40 60 80 100
0 20 40 60 80 100 120
0 20 40 60 80 100 120
TIME (MIN.)

Figure 4.8 Sequential Agglutination and Deagglutination Cycles Using Heat Activated Cells and 8M Urea

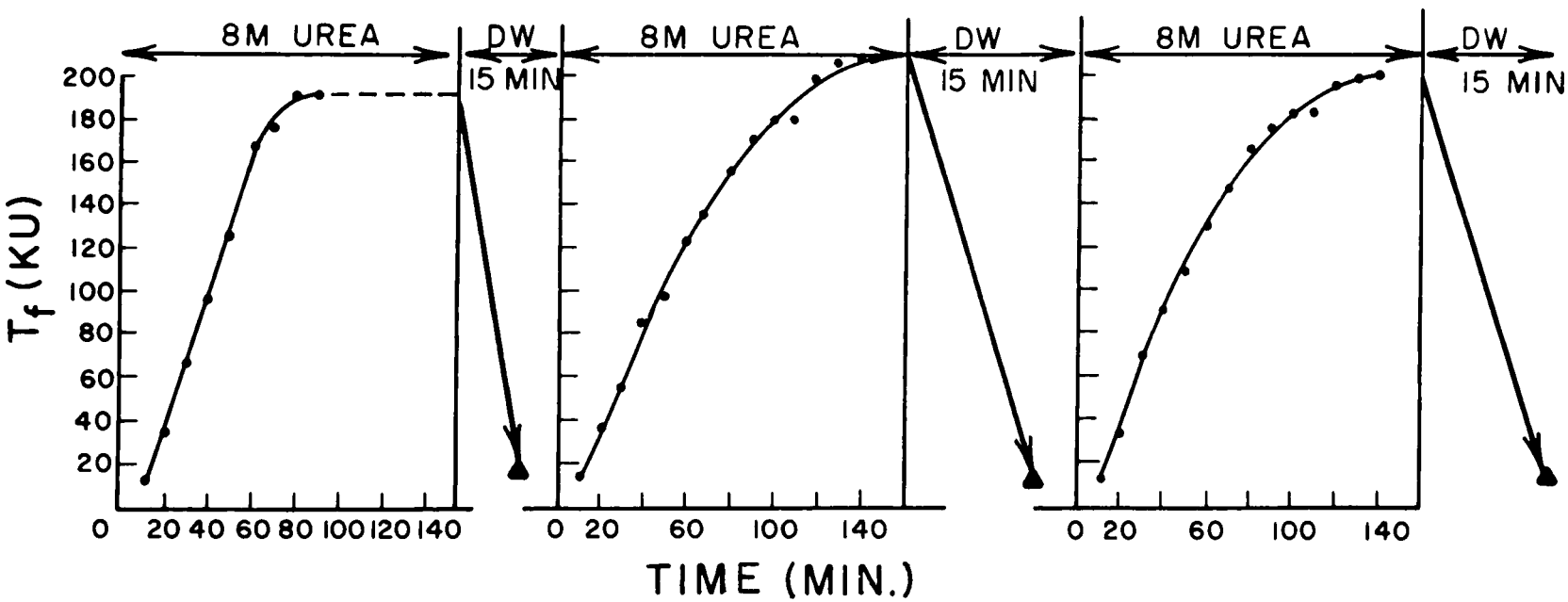

8M UREA
DW
15 MIN
8M UREA
DW
15 MIN
8M UREA
DW
15 MIN
T_f (KU)
200
180
160
140
120
100
80
60
40
20
0
0 20 40 60 80 100 120 140
0 20 60 100 140
0 20 60 100 140
TIME (MIN.)

Figure 4.9 Comparison of Deagglutination Kinetics of Heat Activated Cells in Sequential Agglutination-Deagglutination Cycles.

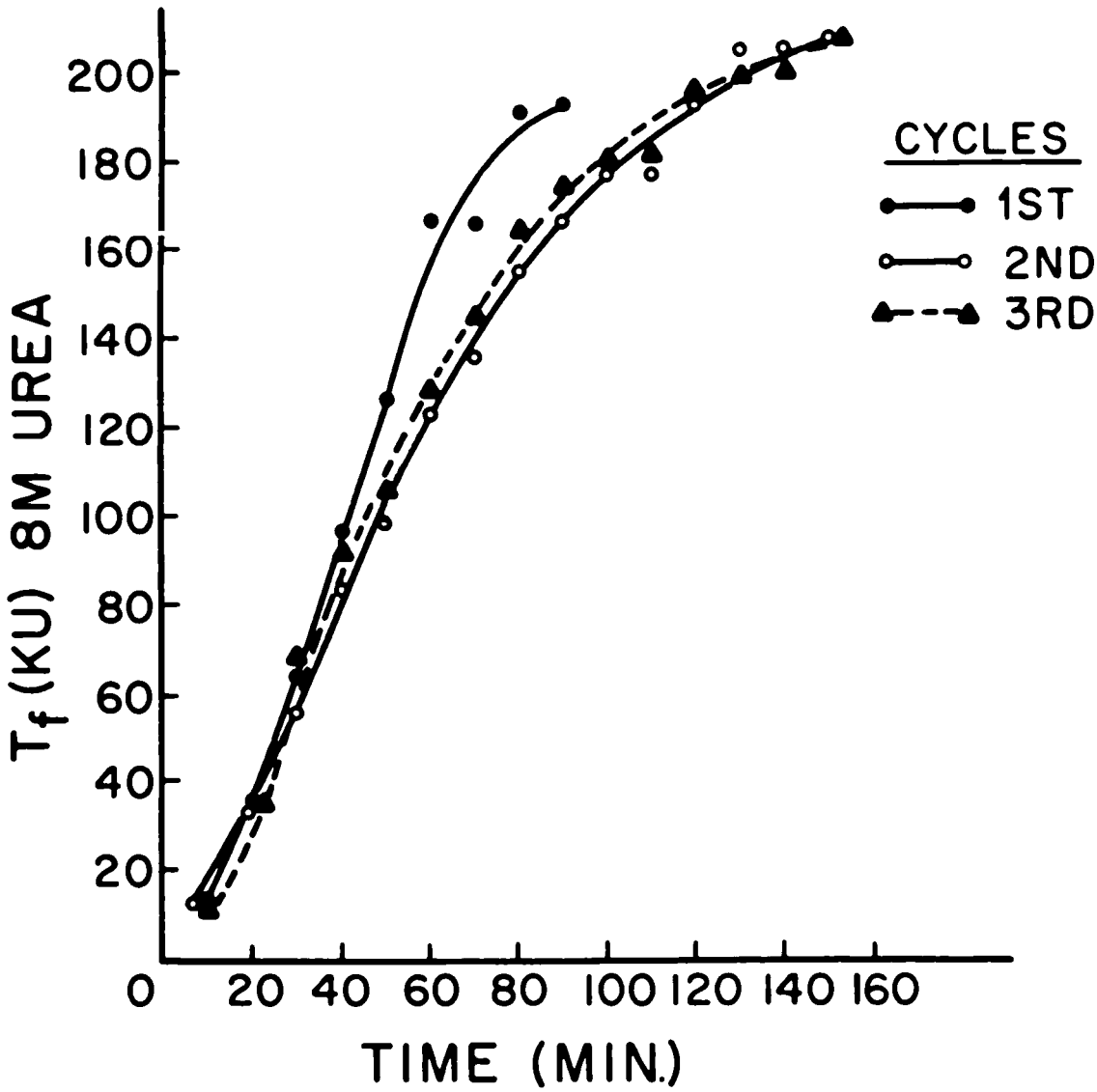
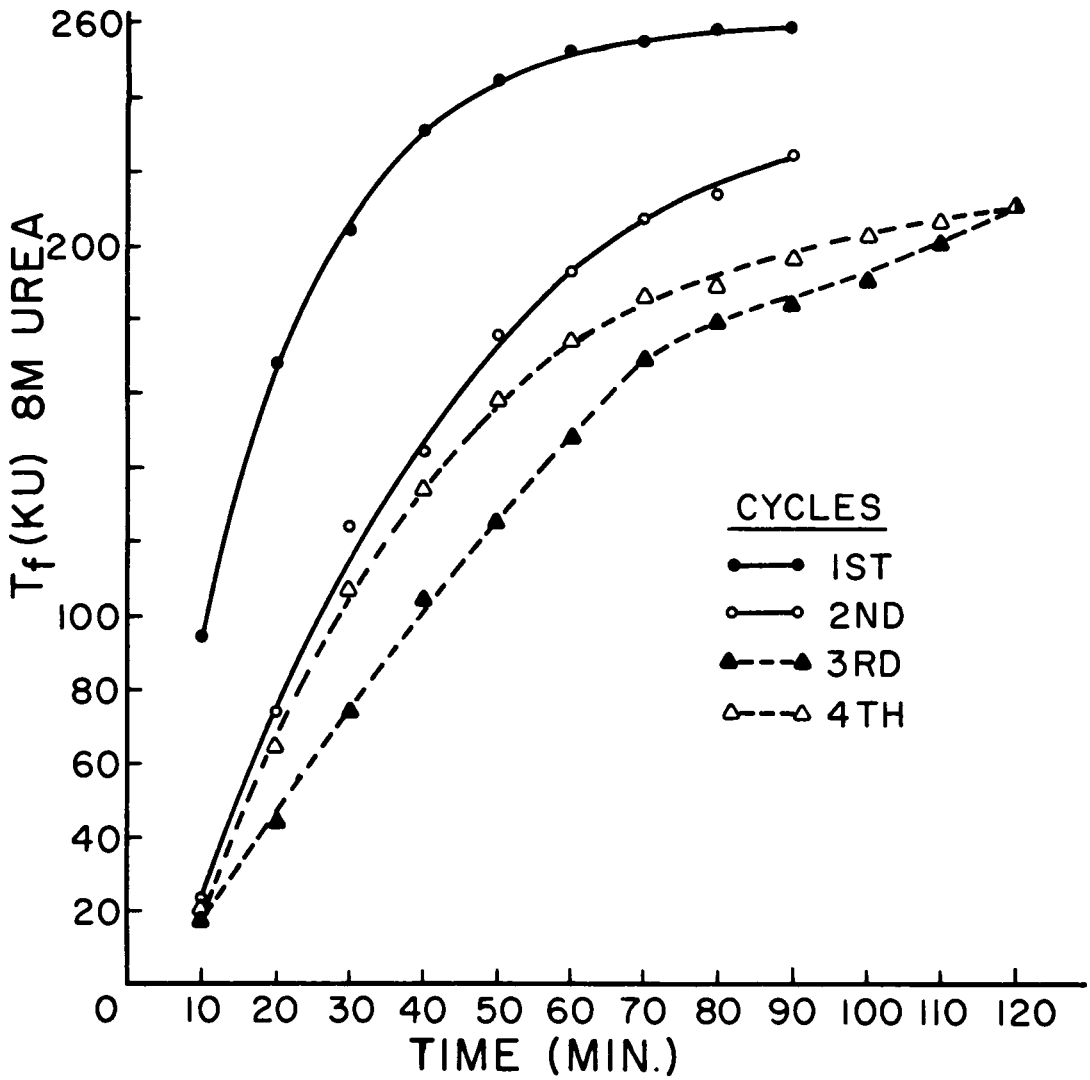

200
180
160
140
120
100
80
60
40
20
0
20 40 60 80 100 120 140 160
T_f (KU) 8M UREA
TIME (MIN.)
CYCLES
1ST
2ND
3RD

Figure 4.10 Comparison of Deagglutination Kinetics of Magnesium Activated Cells in Sequential Agglutination-Deagglutination Cycles

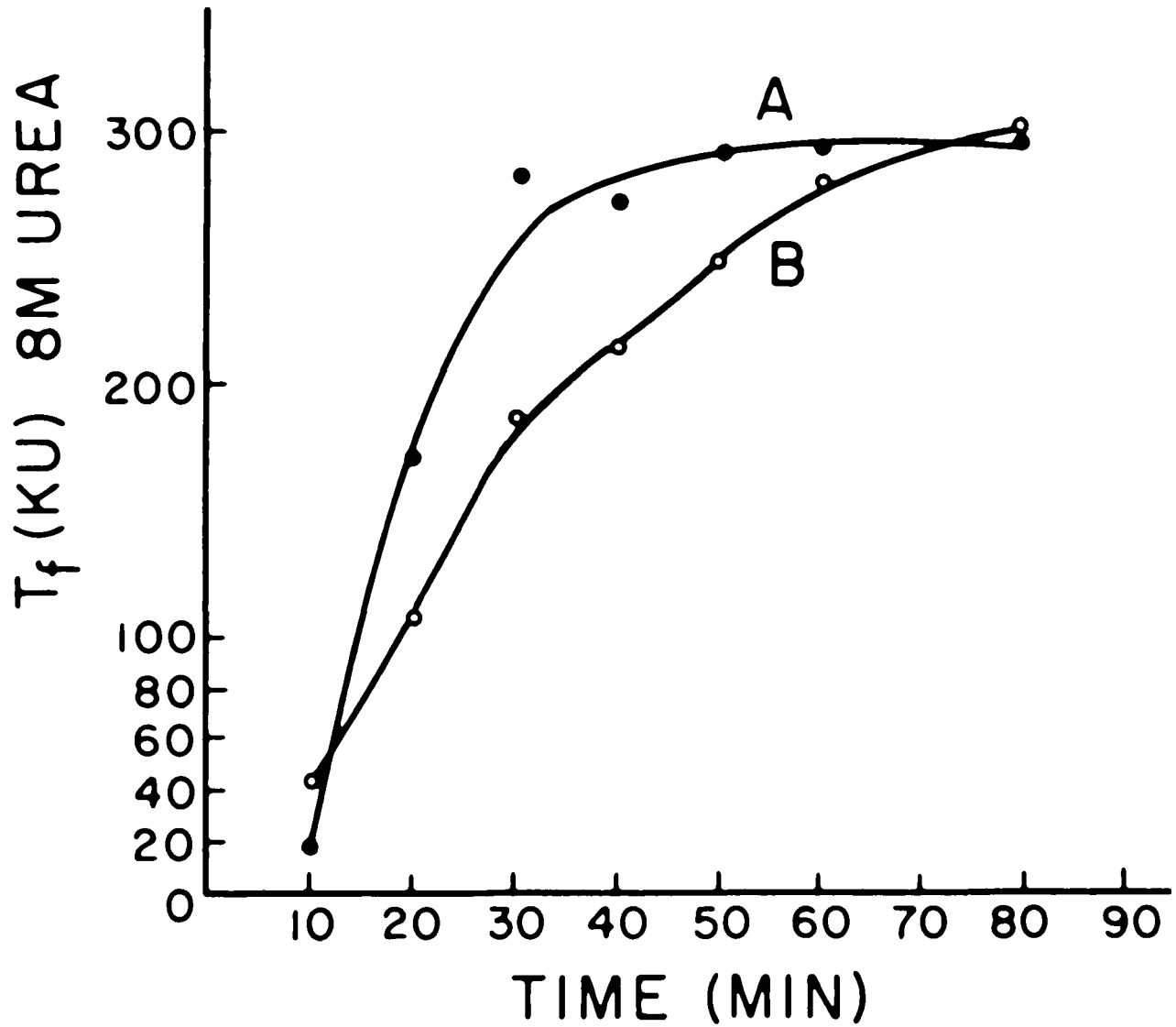

260
200
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
110
120
T_f (KU) 8M UREA
TIME (MIN.)
CYCLES
1ST
2ND
3RD
4TH

gave faster deagglutination kinetics than centrifuge-packed agglutinated cells (Figure 4.11).

Early in the electron microscope studies with ruthenium red staining, it was observed that this stain had an effect upon the agglutination process. Both magnesium activated and heat activated, ruthenium red-stained cells still gave strong macroscopically observable precipitations even though the results from the standard quantitative assay indicated that eight to twenty percent of these cells remained in suspension (T_f = 83KU for heat activated cells and T_f = 200KU for magnesium activated cells). A possible explanation for this partial inhibition will be developed in Chapter 6.

Figure 4.11 Effect of Agglutinating Method Upon Urea Deagglutination Kinetics

8M urea deagglutination kinetics of heat activated cells agglutinated by either centrifuge method (curve B) or shaken-agglutination method (curve A). Data represents means of three separate determinations.


T_f (KU) 8M UREA
300
200
100
80
60
40
20
0
A
B
10
20
30
40
50
60
70
80
90
TIME (MIN)

V. ELECTRON MICROSCOPY

CHAPTER V

ELECTRON MICROSCOPY

An electron microscopic study was undertaken in an attempt to correlate agglutinative activity with either gross or microstructural changes caused by treatments known to alter agglutinative activity. The electron microscope studies were limited here to heat activated and non-activated cells. Evidence has been obtained from which an explanation can be offered for the different intensities of agglutination observed between cation activated and heat activated cells. This principal result was made possible by a comparison of several different types of evidence, including electron microscope observations.

The fine structure of the yeast cell wall has been the subject of considerable electron microscopical research. Surface structure examined by shadow casting techniques were reported as early as 1952 by Northcote and Horne and bud scars on the cell surface were first reported by Barton in 1950. The newer technique of freeze etching has been used to study both the yeast cell wall topography and cytoplasmic ultrastructure (Moor and Mühlethaler, 1963; Moor, 1967; and Black, 1971). Investigations of sectioned cells have involved almost universally

the use of permanganate fixations (Havelková, 1969; Schmitter and Barker, 1966; McClary and Bowers, 1968, 1967; Havelková and Mensik, 1966; Monreal, Uruburu and Villanueva, 1967; Conti and Brock, 1965; Hawker, 1965; Marchant and Smith, 1968 a & b; Mundkur, 1960). Classically, yeast cells have been difficult materials for electron microscopical examination because the usual fixatives and stains penetrated the walls poorly. Species of yeast most commonly investigated with the electron microscope belonged to the general Saccharomyces, Schizosaccharomyces, Candida and Rhodotorula (see Ruinen for multiple references). Conti and Brock (1965), in their electron microscopical study of the conjugation process in Hansenula wingei, incorporated double fixation with potassium permanganate and osmic acid.

Thin sections of permanganate and osmium fixed yeast cells revealed generally three different layers or strata (Matile, 1969). Outermost was a dark staining (electron dense) region consisting of the outer layer of the cell wall proper plus a dense, hair-like fringe in which the filamentous material was oriented vertically to the cell wall surface. The innermost, plasmalemma-apposed layer of the cell wall was also relatively electron dense, with a wider, more electron transparent middle layer. The outermost region (outer cell wall layer and fringe component) was probably composed of mannan-protein (Northcote

and Horne, 1952), whereas the middle, electron transparent region was mainly glucan (Forman, 1967). Not all investigators have been able to confirm the presence of distinct layers in the cell wall of yeast (Rogers and Perkins, 1968). In the present studies the innermost layer was apparent only under certain conditions (see prolonged heating of 5-cells). The presence of well developed fringe appeared to be dependent upon cultural conditions and the preservation and visualization of the fringe depended upon the fixation and staining procedures utilized.

Ruthenium red stain, used classically by botanists to stain pectin (Jensen, 1962), was shown by Luft to react specifically with acidic mucopolysaccharides, and, when used in conjunction with osmium tetraoxide fixation, resulted in a specific increase in the electron density of structures containing acidic polysaccharides (Luft, 1964; Gustafson and Phil, 1967). The red stain contained a complex oxide of the heavy metal ruthenium (atomic weight 101), which accounted for the electron density of the stain.

Black, 1969, using double fixation with permanganate and osmic acid in the presence of the stain ruthenium red, obtained a highly intense staining of the fringe on Hansenula wingei vegetative cells. Black also observed less fixation damage when sodium salt was used rather than the usual potassium salt of permanganate introduced by Luft (Luft, 1956).

Results

Preliminary studies to determine the optimal fixation and staining conditions with H. wingei indicated that the double fixation with sodium permanganate and osmium tetroxide in the presence of ruthenium red (Black's modification of the method of Ordal and Pate) was superior to either the double fixation with potassium permanganate and osmic acid (method of Conti and Brock) or to the double fixation with gluteraldehyde and osmic acid (Sabatini, 1963). Sodium permanganate fixation alone gave good internal membrane preservation but the presence of both ruthenium red and osmium tetroxide was required for optimal staining of the fringe material on the outermost region of the cell wall (see below). For maximal fringe preservation and staining, cells were fixed and stained while still in the culture medium, with washings kept to a minimum.

Standard preparative and fixation methods revealed the typical cell organelles found in other yeasts, such as mitochrondria, the nucleus, a large vacuole and occasionally a storage granule (Plates 1 and 2). The plasmalemma was highly convoluted. The cell wall showed only two major layers; the innermost, dark staining, third layer reported in the literature was not discernable under these conditions. The outermost layer of the cell wall was darkly stained(electron dense) and had a definite hair-like fringe, with hairs of variable length oriented at

right angles to the layers of the cell wall proper. Optimal fringe development occurred when the cells were grown on YM medium and when the cells were grown either on agar plates or allowed to remain unshaken for six hours after growth in shake cultures. Crandall and Brock (1968) reported that cells of both mating types agglutinated poorly or not at all when grown in media containing 1% malt extract instead of yeast extract. This was not observed in this laboratory with YM media which contained both malt extract and yeast extract.

A typical shaker-grown cell with single permanganate fixation is shown in Plate 5. If the yeast were grown on agar plates (Plates 1, 2 and 6) or allowed to remain stationary for at least six hours after being grown under shake conditions (Plate 3 and 4), the fringe material on the outer surface of the cell wall became much thicker and more uniform than in continuously shaken cells. Plate 4 demonstrates the presence of a thick and uniform fringe but this fringe is relatively unstained in the absence of osmic acid fixative. Both Plates 4 and 5 represent permanganate fixed and ruthenium red stained cells but the outermost cell wall material does not stain as well as in Plates 1, 2, and 3, in which osmic acid fixative was included with the ruthenium red stain. There was no difference observed when the cells were stained before fixation with ruthenium red for twenty minutes or twenty hours. (Compare cell wall and fringe of Plate 3 with Plates 1 and 2). As reported by others

(Luft, 1966; Gustafson and Phil, 1967), staining with ruthenium red of fixed sections had no effect in increasing the electron density of the cell wall material. The fringe material also appeared best developed in the more actively growing regions of the cell, such as buds (Plate 6 & 7). Plate 7 is an enlargement of the cell in Plate 6 and shows a section of the cell wall near the bud. Magnesium activated cells appeared morphologically indistinguishable from washed, non-activated cells.

Fine Structure of Heat Activated Cells

Typical heat activated cells of strain-21 and strain-5 are shown in Plates 8, 9, 10, and 11. Both strains showed a loss of the fringe but both showed a retention of the deeply staining outermost layer of the cell wall. The standard ten minute heat treatment was sufficient to kill the cells (Henderson, 1966; and Chapter 3), and there was complete loss of internal organization. In many places the cell contents were separated from the innermost portion of the cell wall. Commonly seen in heat treated cells were concentric rings which probably were membrane artifacts. Although these membranous structures were occasionally seen in the central portion of the cell, most often they were observed near the cell wall and associated with the remains of the plasmalemma, which was usually fragmented. There was no observable difference between strain-21 cells and strain-5 cells

under either non-activated (i.e. typical conditions) or normal heat activated conditions.

When cells of strain 21 and strain 5, exposed to prolonged heat treatment (180 minutes), were compared, there was a distinct difference in cell wall structure. Walls of strain-5 cells still retained an outermost dark staining layer as seen in Plates 12, 13, and 14. Plate 15 is an enlargement of the 180 minute heat treated strain-5 cell wall and both a darkly staining inner layer and outer layer are evident. There was some indication for dark staining layers or strata within the middle, normally homogenous, electron transparent region. In contrast, strain-21 cells have lost all darkly staining layers, and the remains of the cell wall stains uniformly as is seen in Plates 16 and 17. As was brought out in Chapter 3, prolonged heating affected strains 5 and 21 differently. Strain-21 cells irreversibly lost the ability to agglutinate after prolonged heating and also lost any indication of an outer layer of the cell wall. Strain-5 cells still agglutinated quantitatively after 180 minutes of heat treatment and still retained a darkly stained outer cell wall layer. Both strains, of course, lost the fringe early in the heat treatments.

EXPLANATION OF PLATES

Standard preparative methods (refer to Chapter 2 for details), involving pre-fixation staining with ruthenium red followed by double fixation with permanganate and osmic acid in the presence of ruthenium red and post-section staining with uranyl acetate and lead citrate, were used in all plates unless indicated otherwise. All plates were photographed at either 20,000 x or 30,000 x magnification.

Abbreviations: Nu, nucleus; Nm, nuclear membrane; P, plasmalemma; F, fringe region; G, storage granule; OCW, outermost layer of cell wall; MCW, middle layer of cell wall; ICW, innermost layer of cell wall.

Plate 1 Section illustrating typical *Hansenula wingei* strain-5 cells grown on an agar plate.

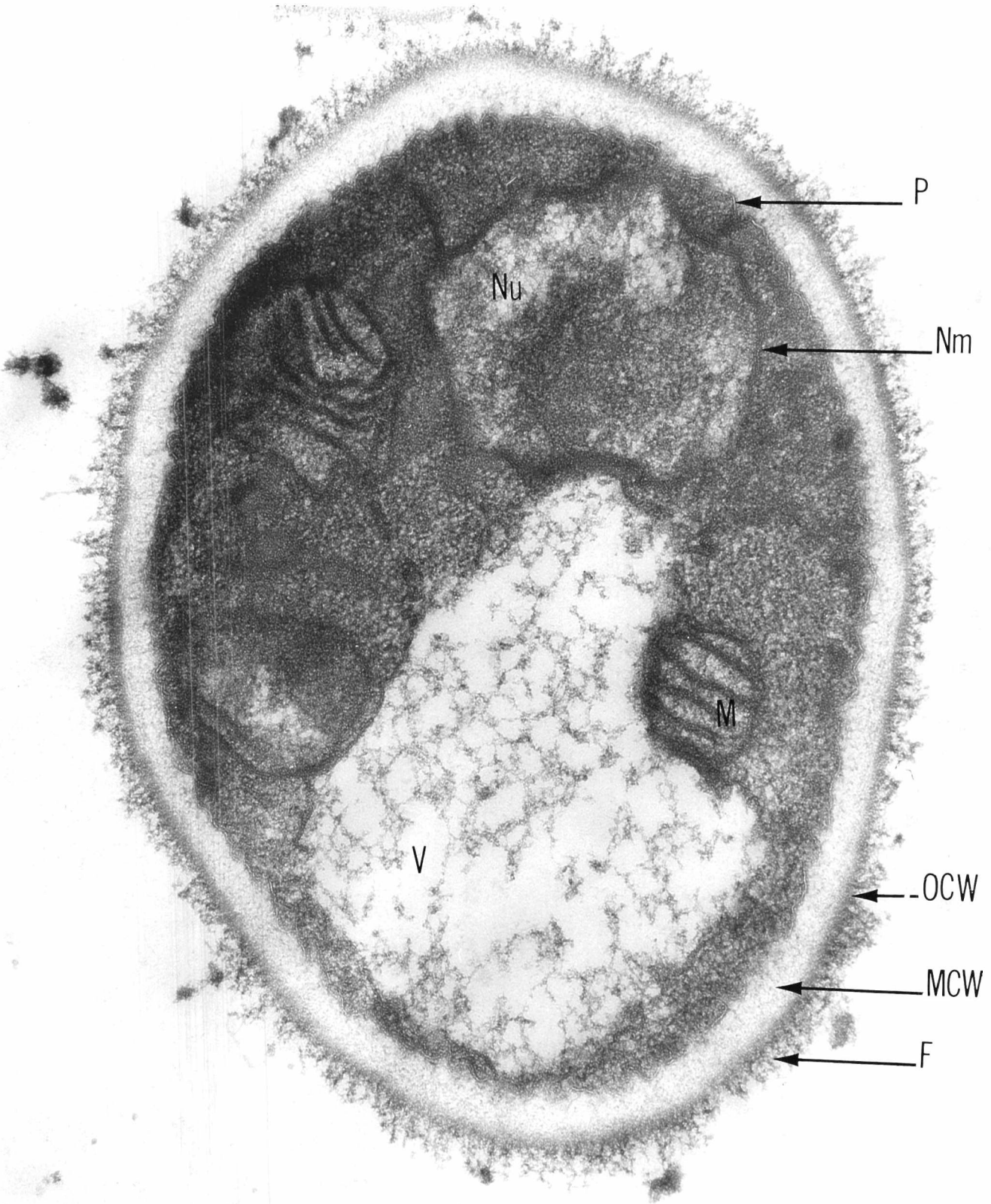

P
Nu
Nm
M
V
OCW
MCW
F

Plate 2 Section illustrating typical strain-21 cells grown on agar plate.

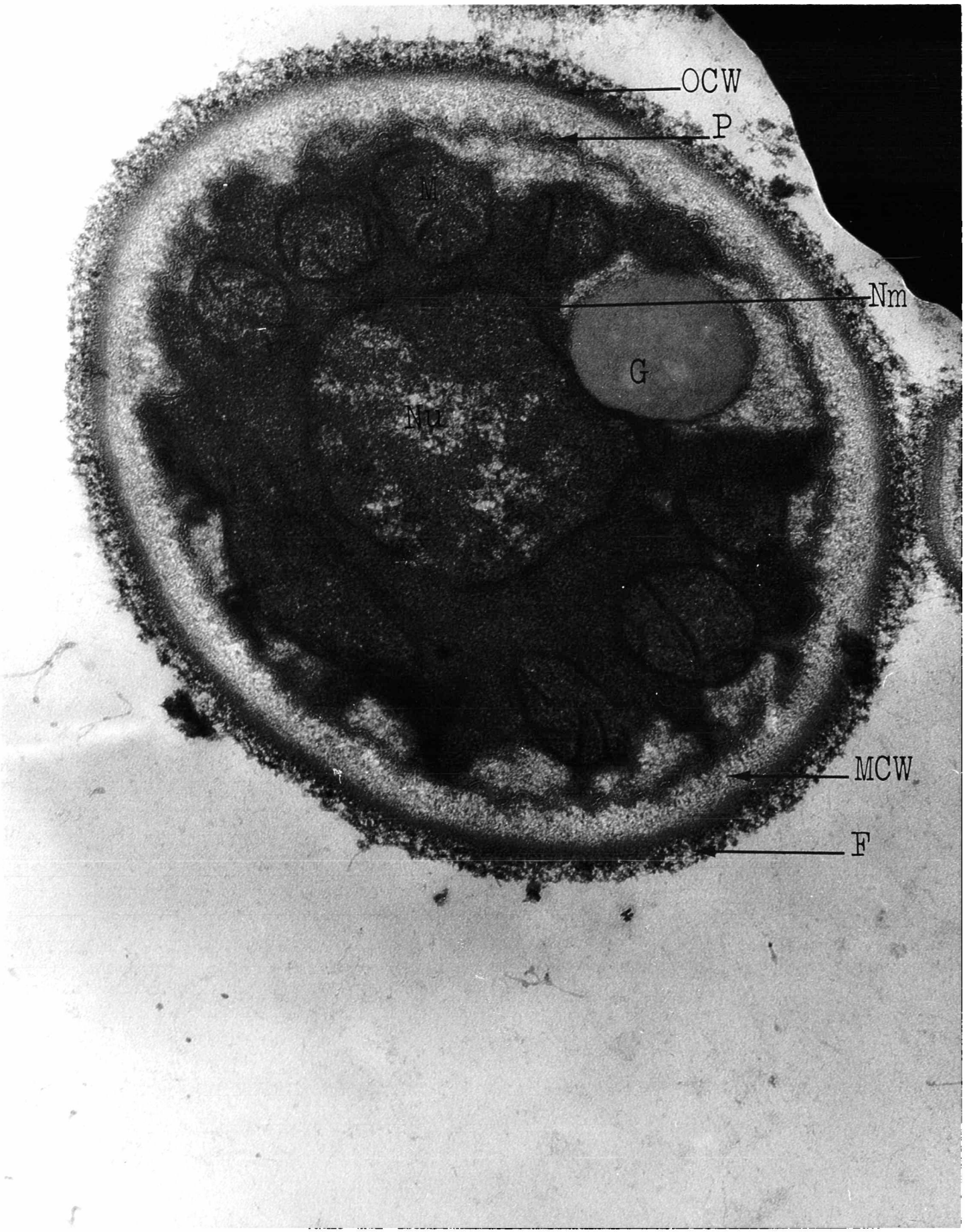

OCW
P
M
Nm
G
Nu
MCW
F

Plate 3 Section illustrating optimal fringe preservation and staining. Strain 21 cells stained in ruthenium red for prolonged period prior to standard fixation treatment.

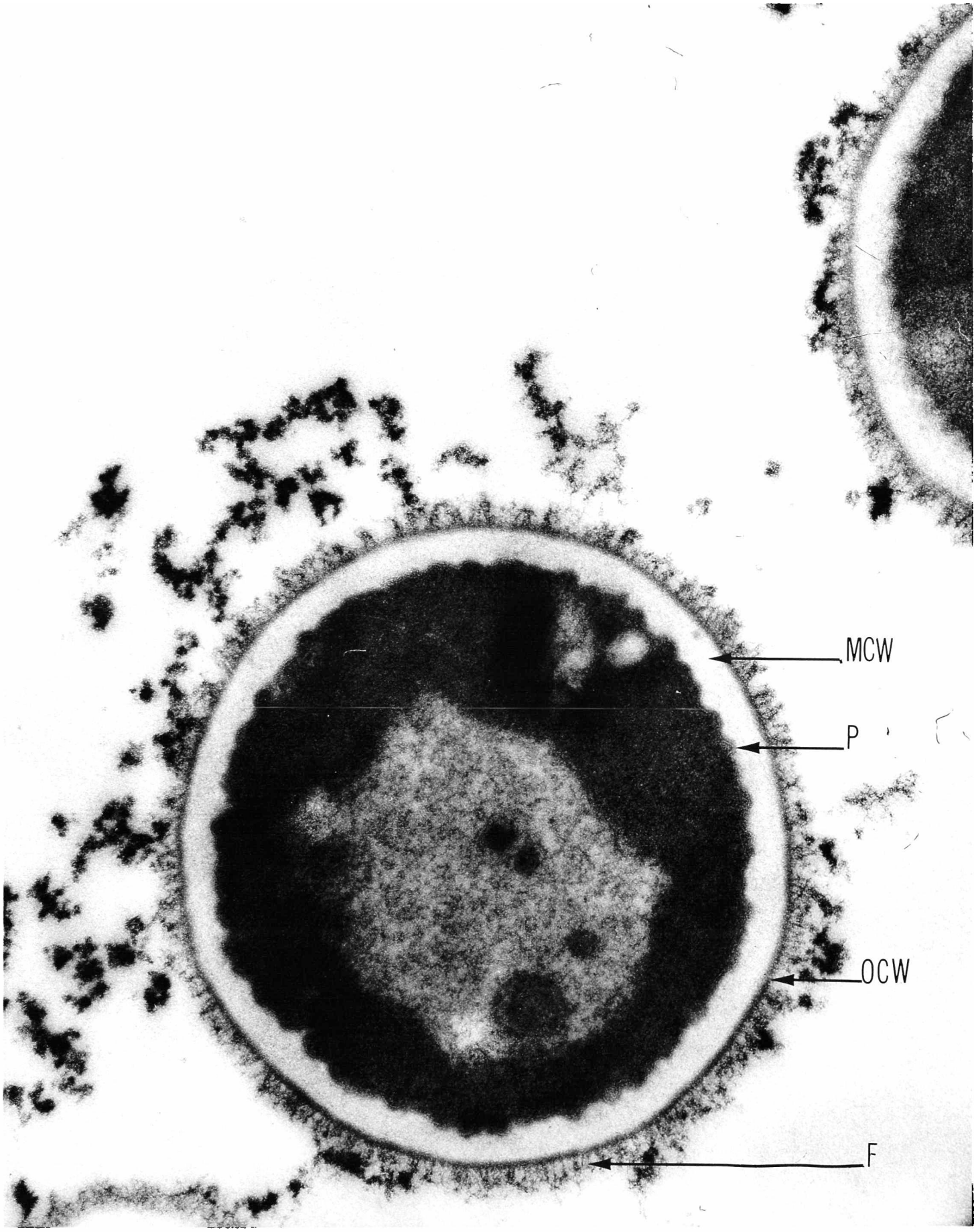

MCW
P
OCW
F

Plate 4 Section illustrating optimal fringe preservation but not optimal fringe staining. Cells fixed with only permanganate in presence of ruthenium red.

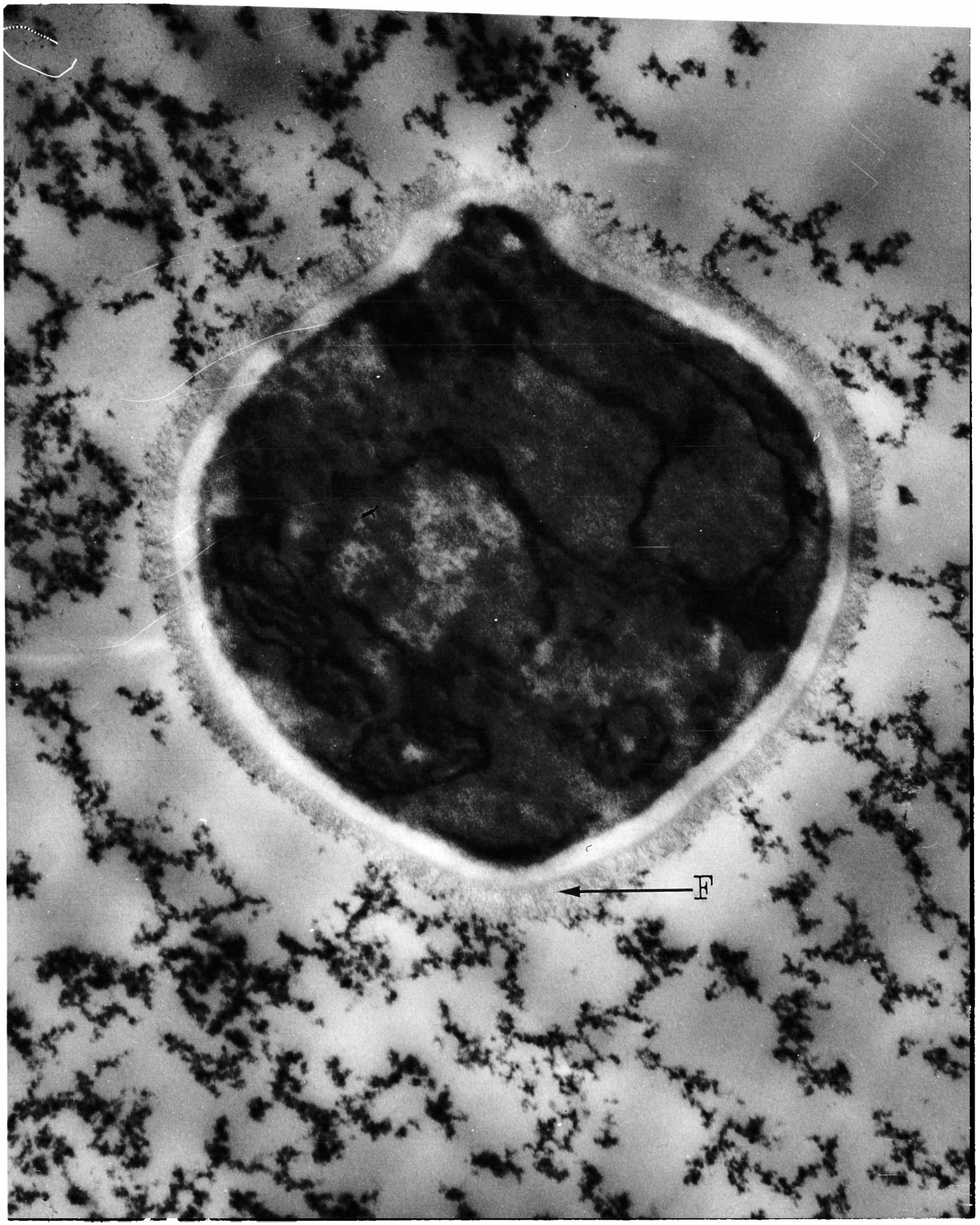

F

Plate 5 Typical fringe preservation and staining of permanganate fixed shaken cultured cells.

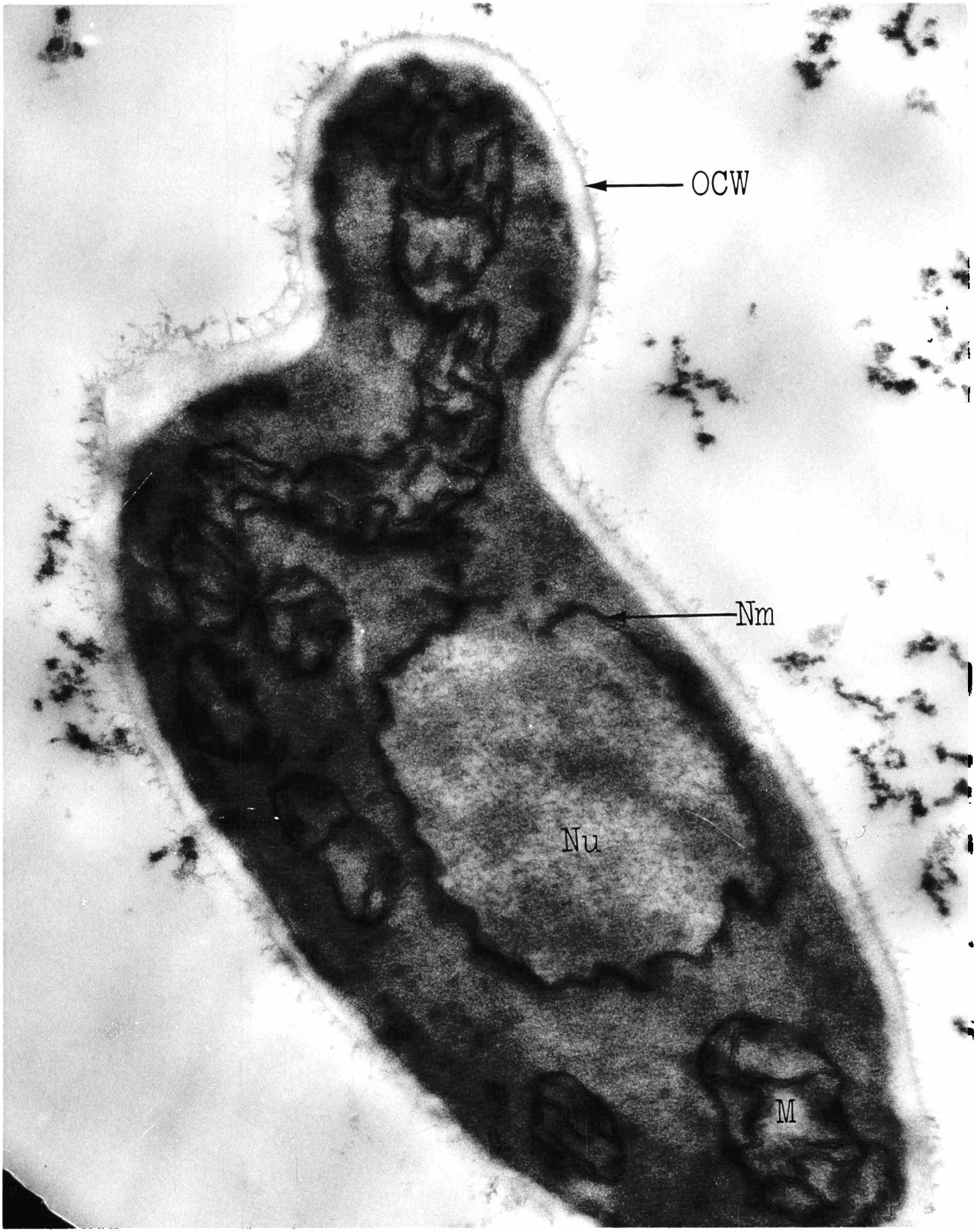

OCW
Nm
Nu
M

Plate 6 Typical agar plate cultured strain-5 cells showing better development of fringe region in actively growing regions of the cell.

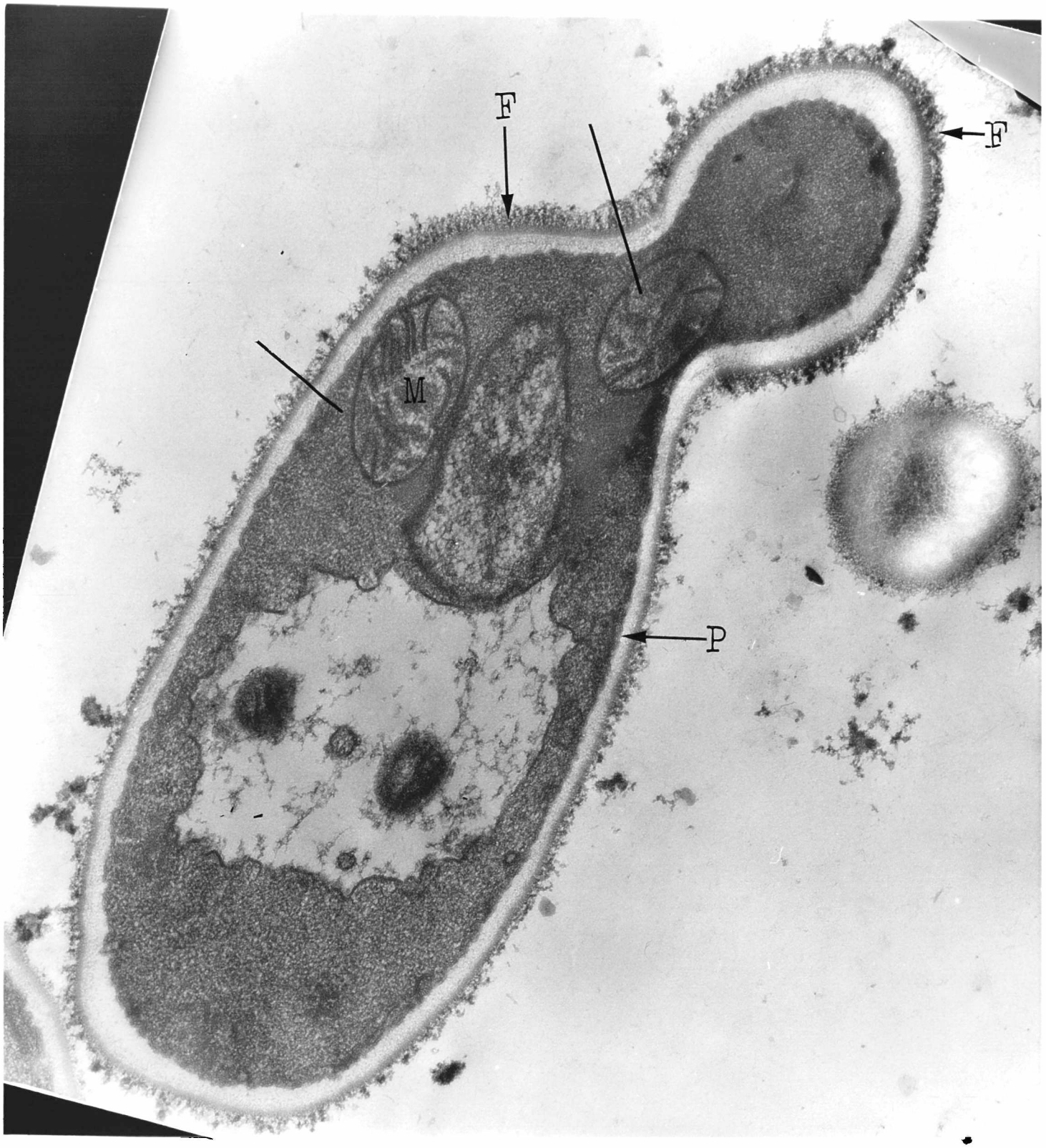

F
F
M
P

Plate 7 Enlargement of region of cell wall near a bud showing fringe and the two layers of cell wall demonstrable using standard fixation and staining methods.

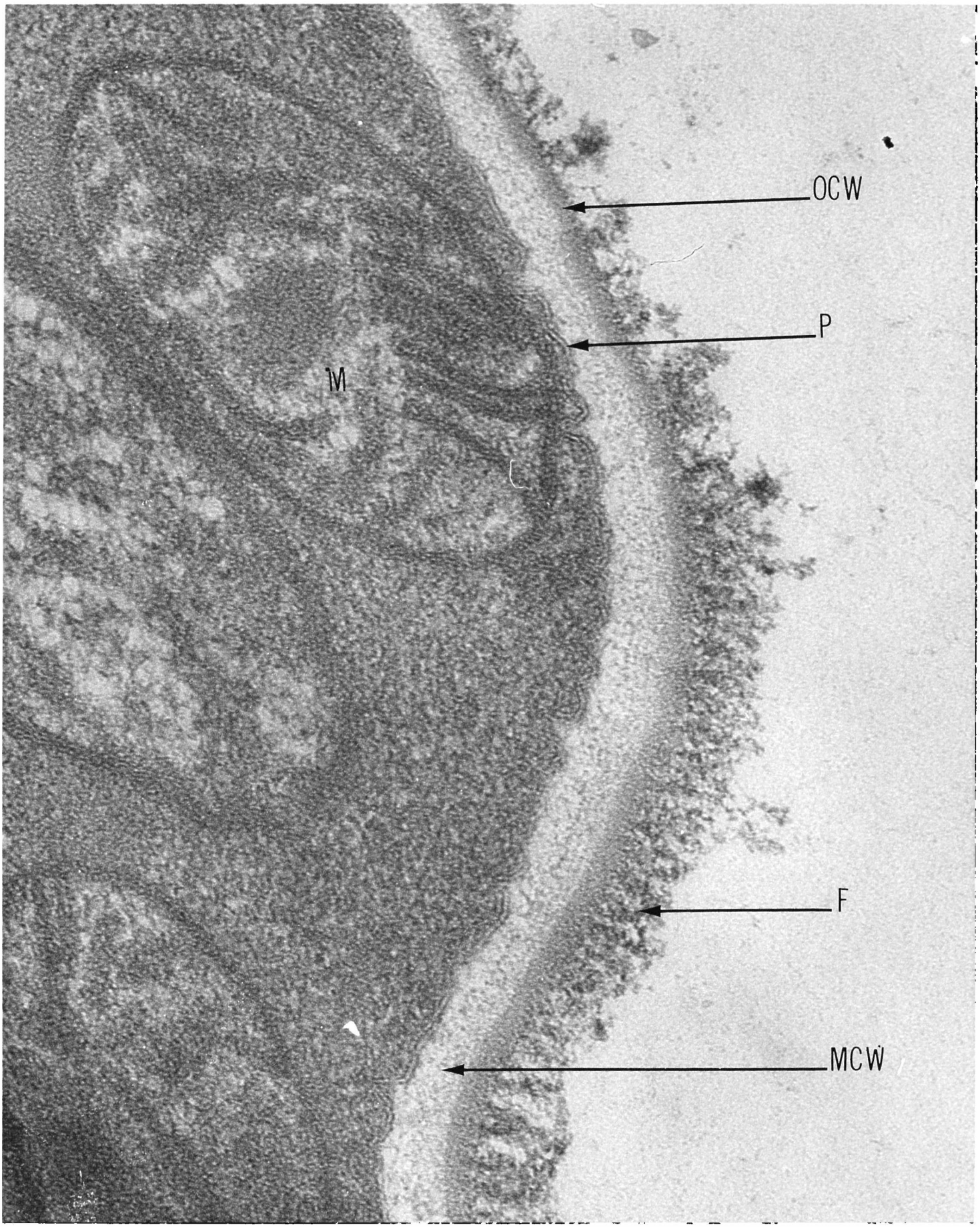

OCW
P
M
F
MCW

Plate 8 Section illustrating typical heat activated (10 min heat treatment) strain-21 cells.

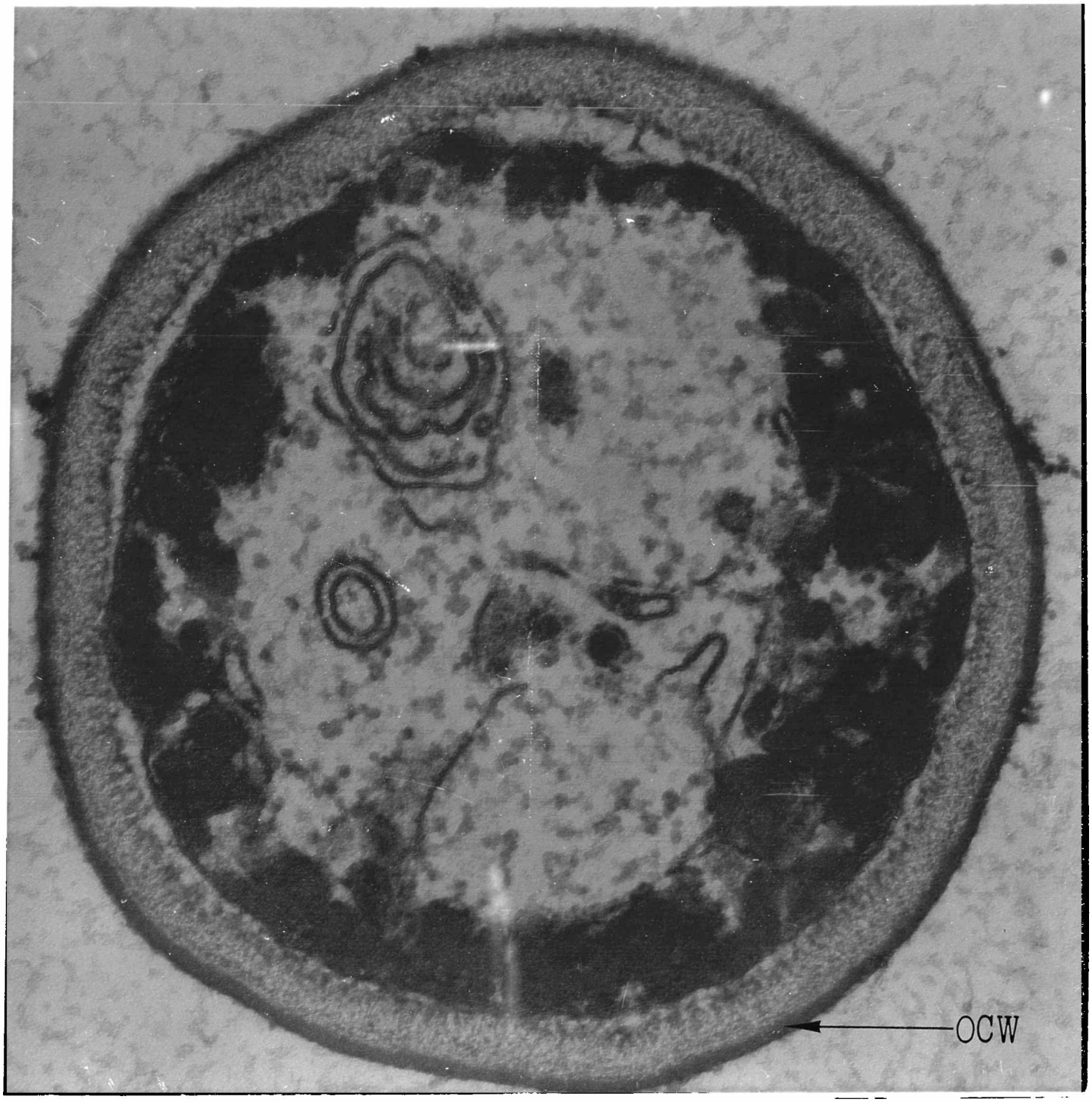

OCW

Plate 9 Section illustrating typical heat activated (10 min heat treatment) strain-21 cells.

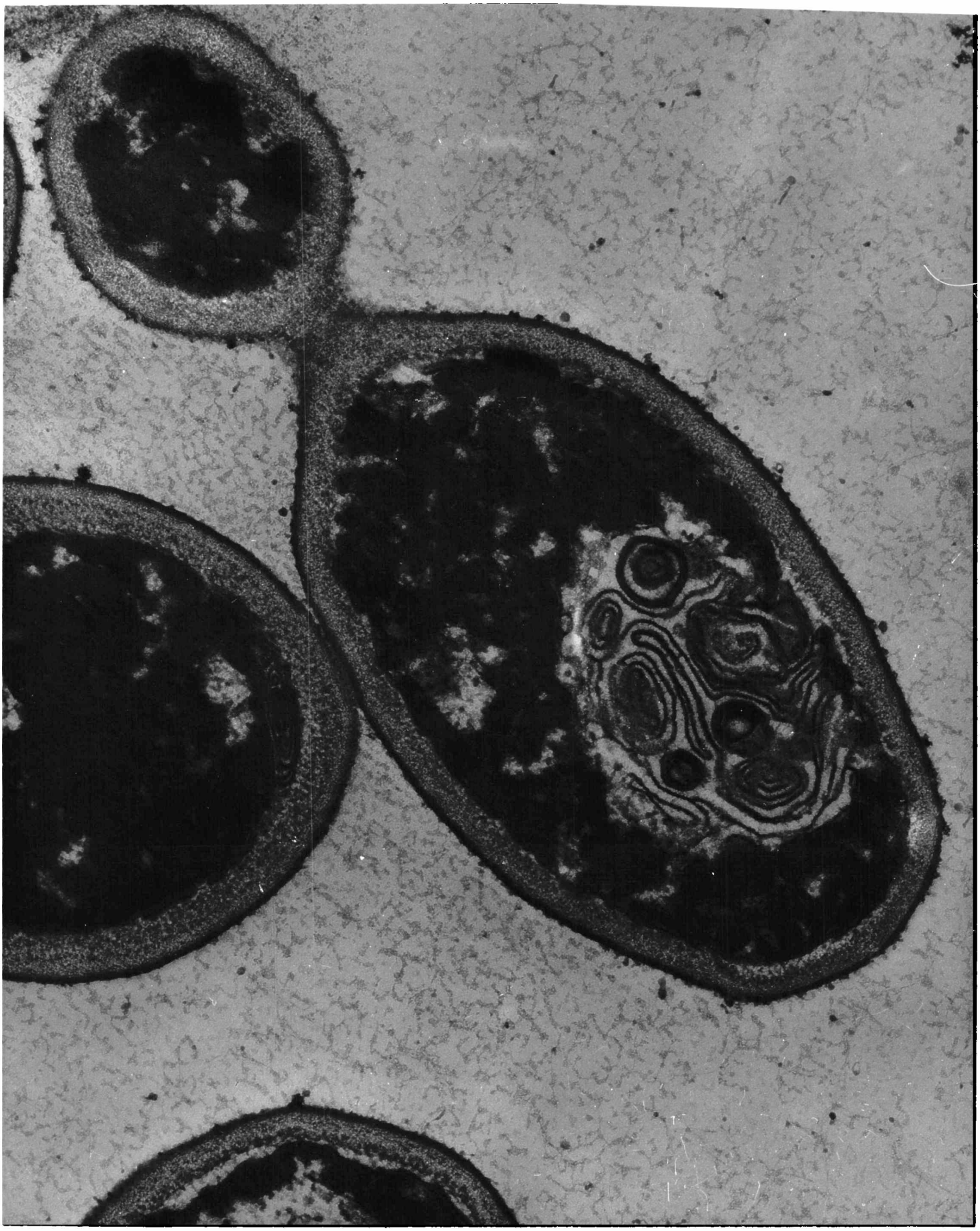


Plate 10 Section illustrating typical heat activated (10 min heat treatment) strain-5 cells.

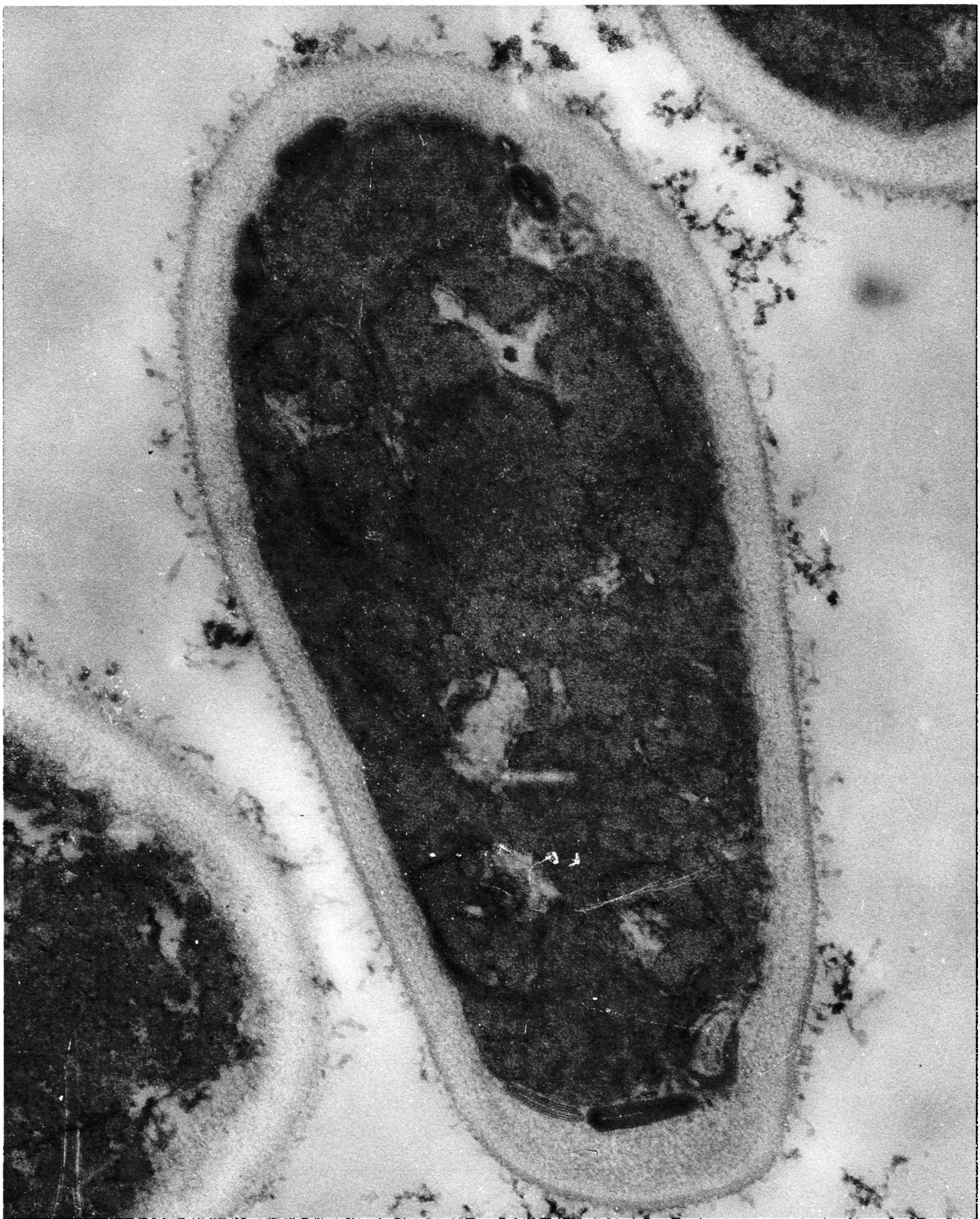


Plate 11 Section illustrating typical heat activated strain-5 cells with a single post-sectioned stain of lead citrate.

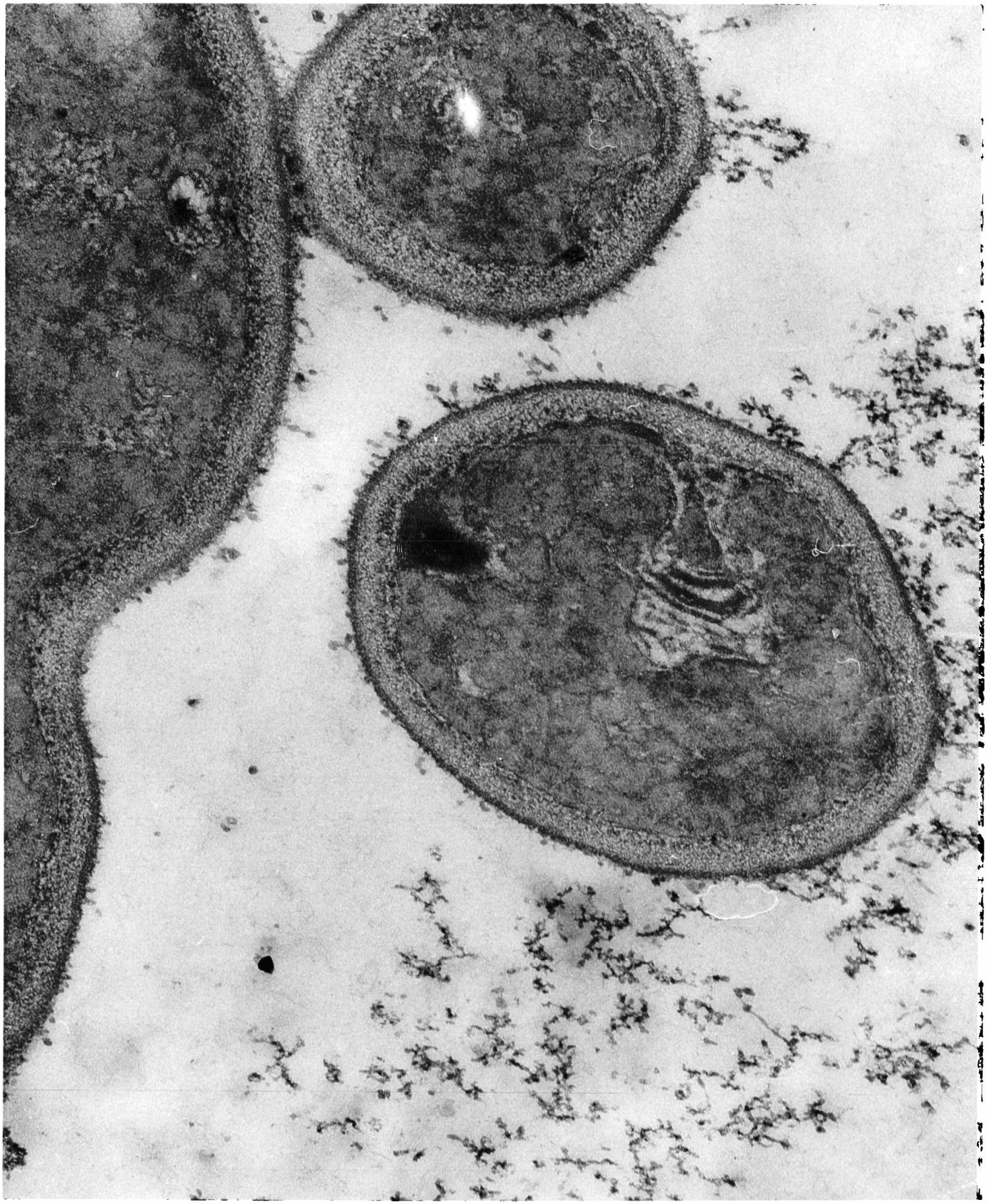


Plate 12 Section illustrating typical strain-5 cells after prolonged heat treatment (180 min at 100° C).

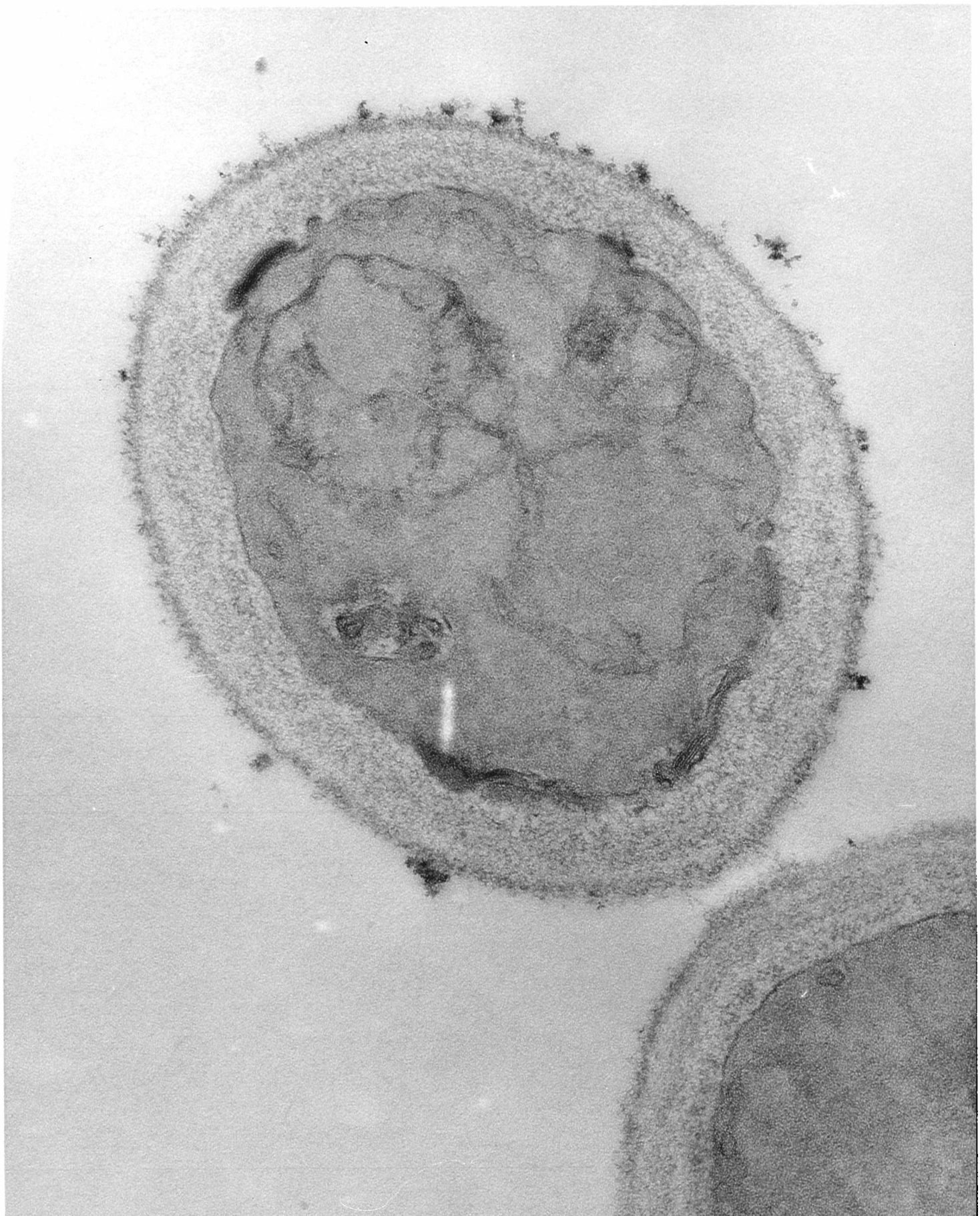


Plate 13 Section illustrating typical strain-5 cells after prolonged heat treatment (180 min at 100° C).

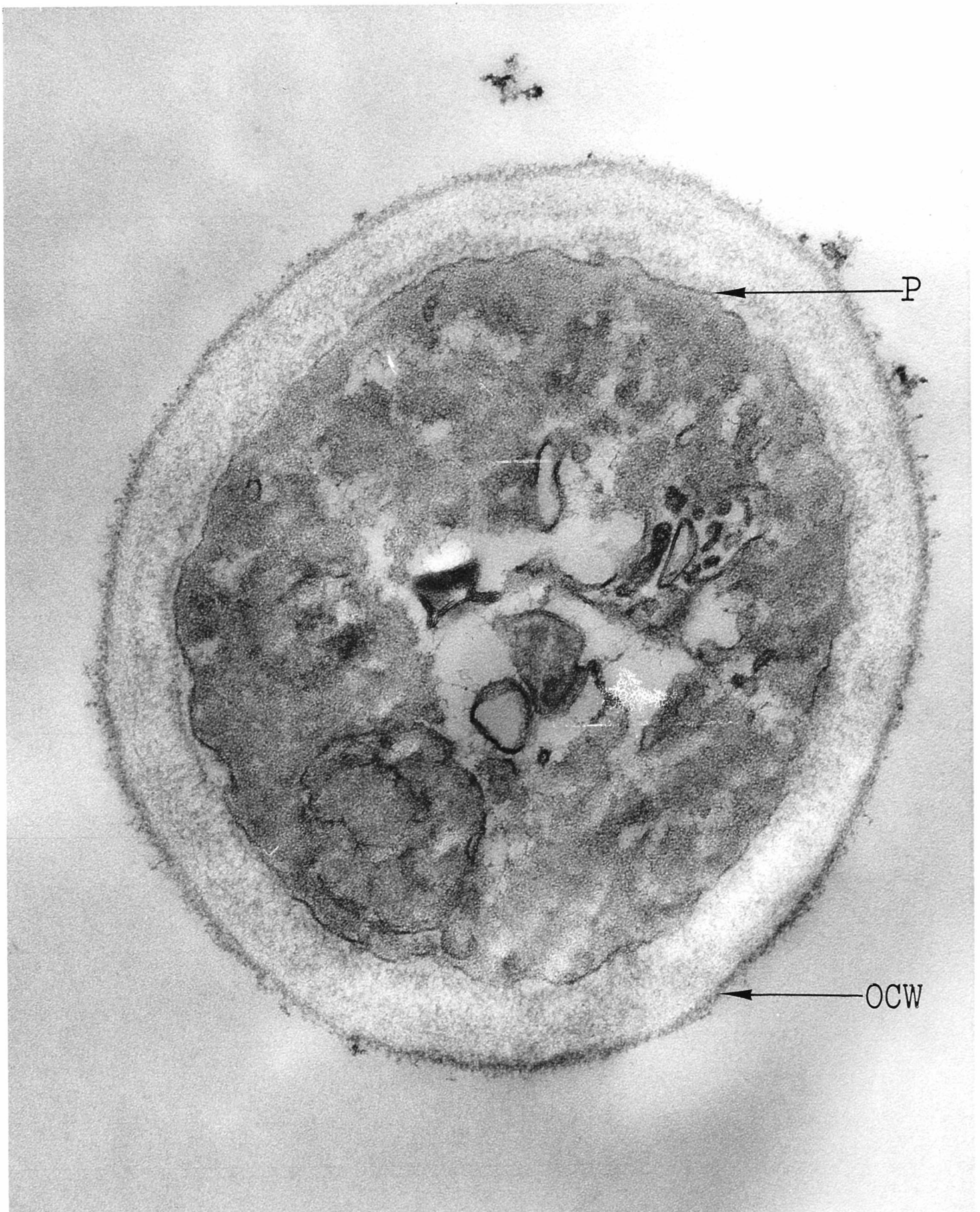

P
OCW

Plate 14 Section illustrating typical strain-5 cells after prolonged heat treatment (180 min at 100° C).

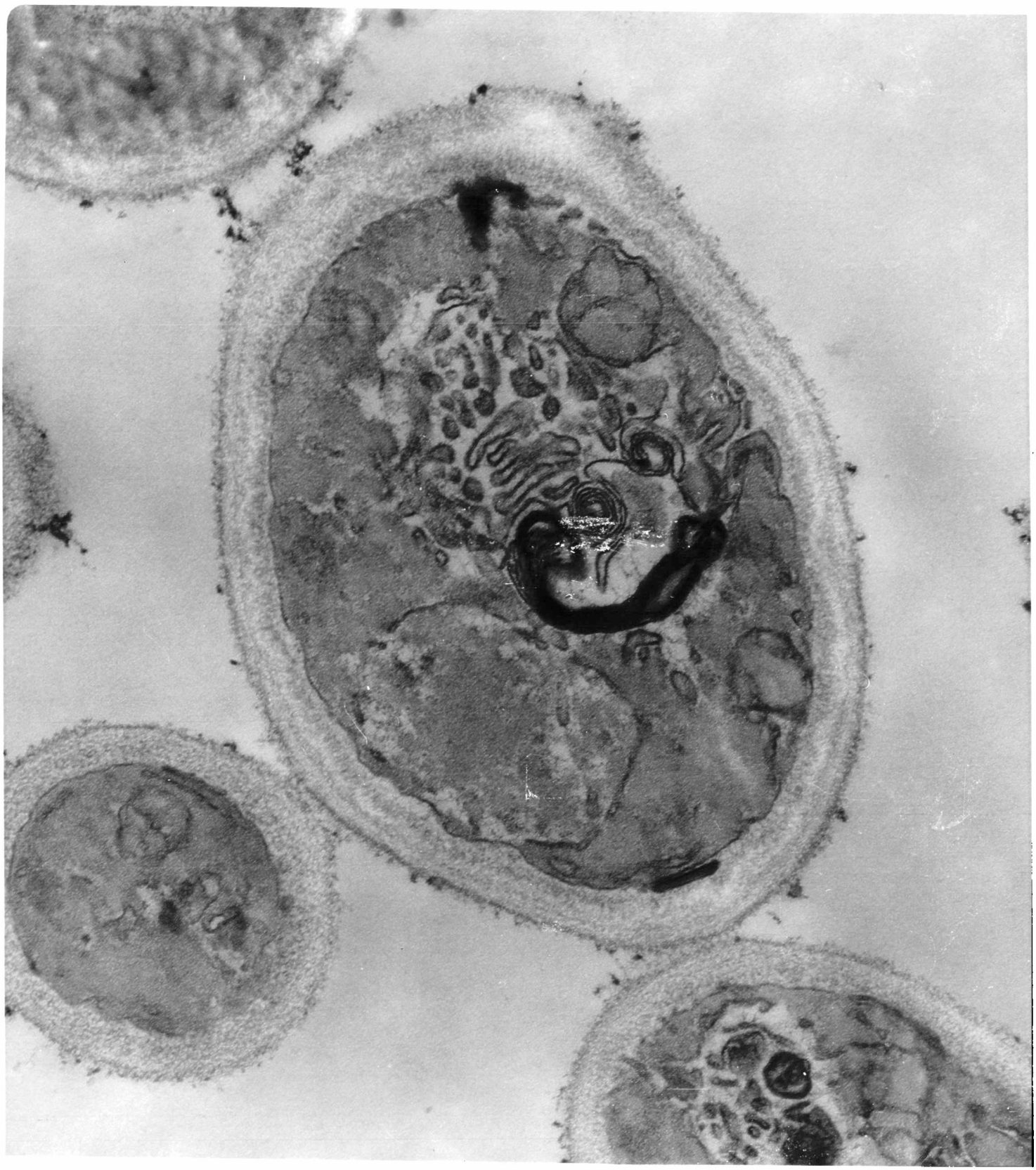


Plate 15 Enlargement of 180 min heat treated strain-5 cell showing innermost cell wall layer.

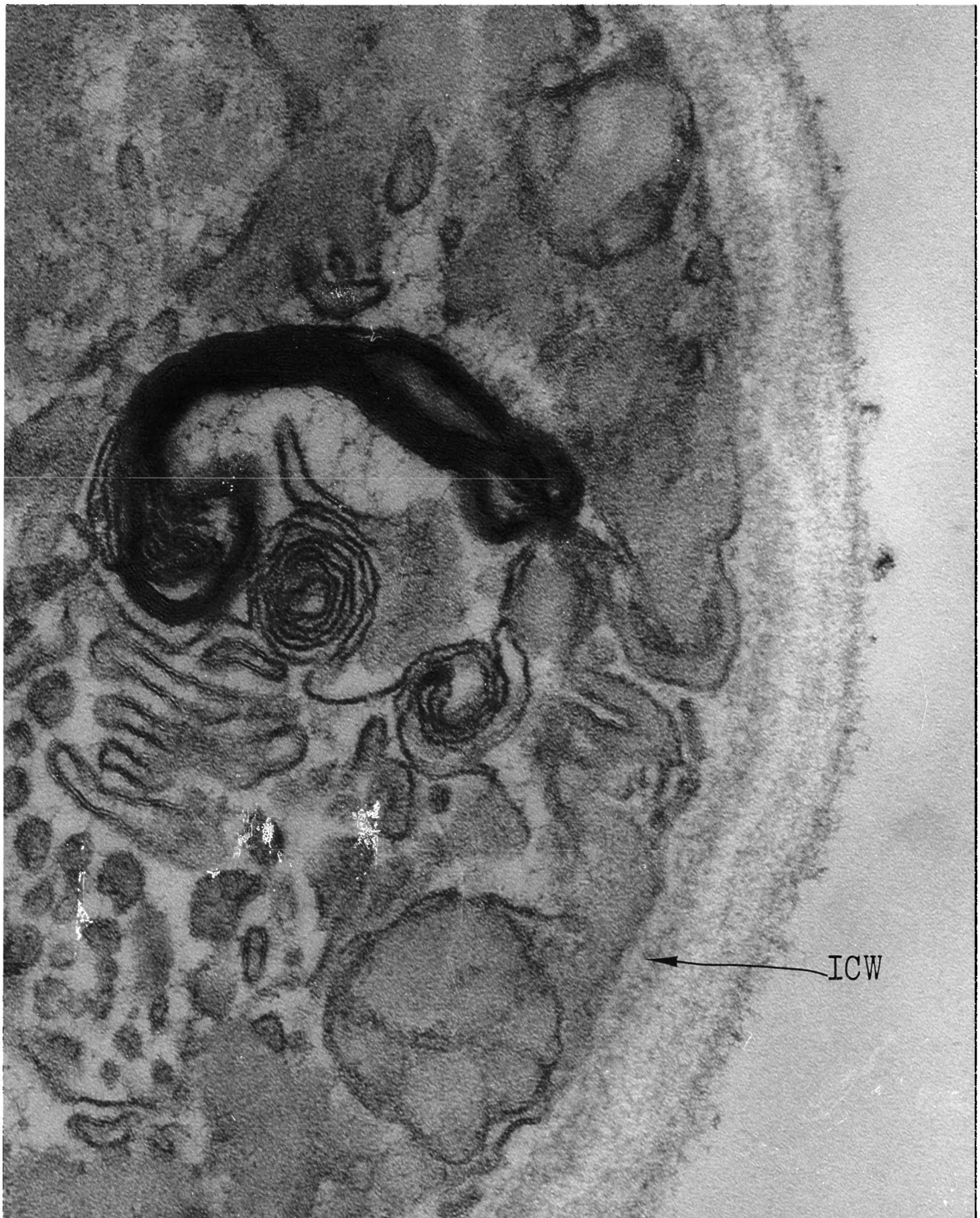

ICW

Plate 16 Section illustrating typical strain-21 cells after prolonged heat treatment. Note absence of distinct cell wall layers and electrondense staining material along outermost border of the cell wall.

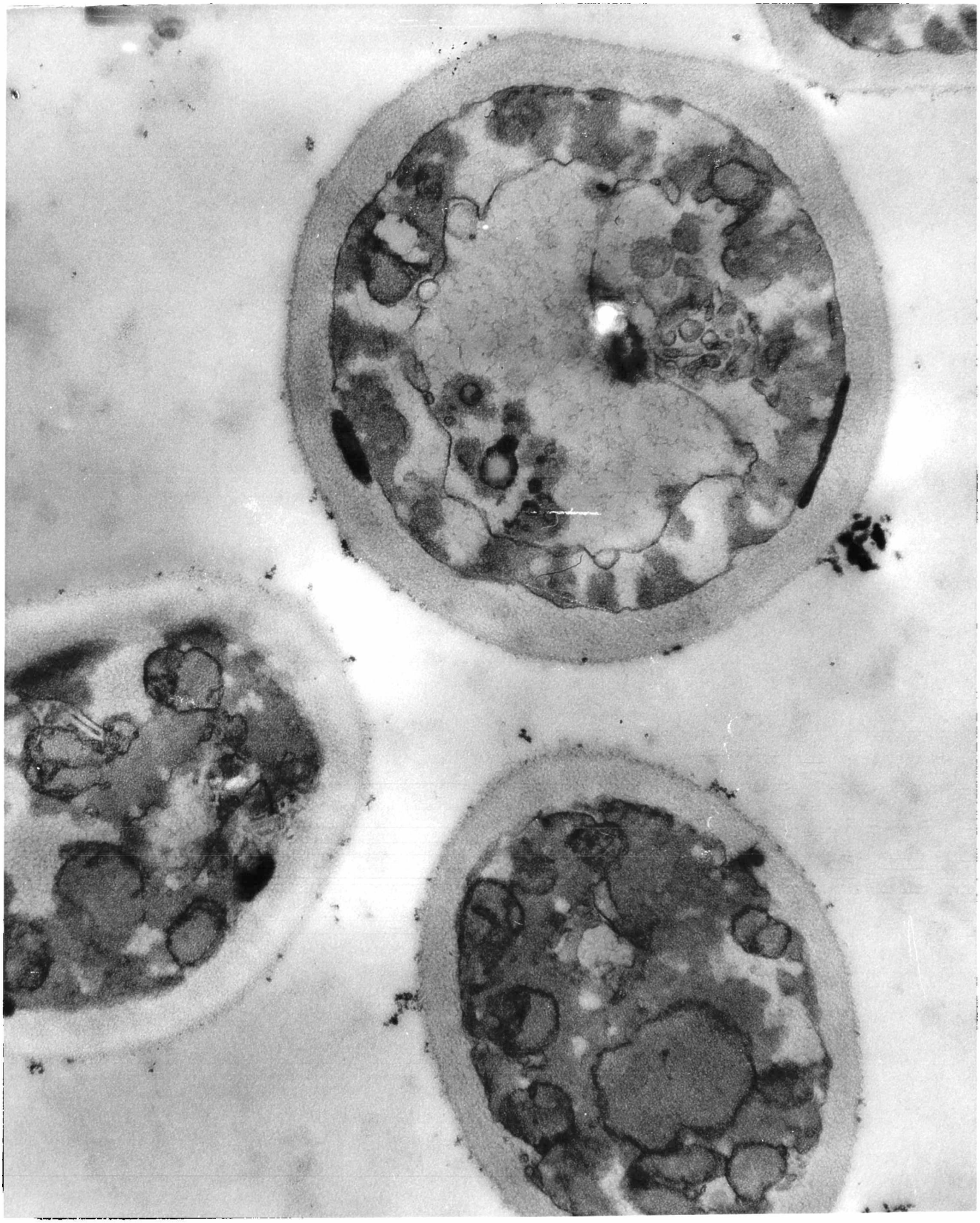
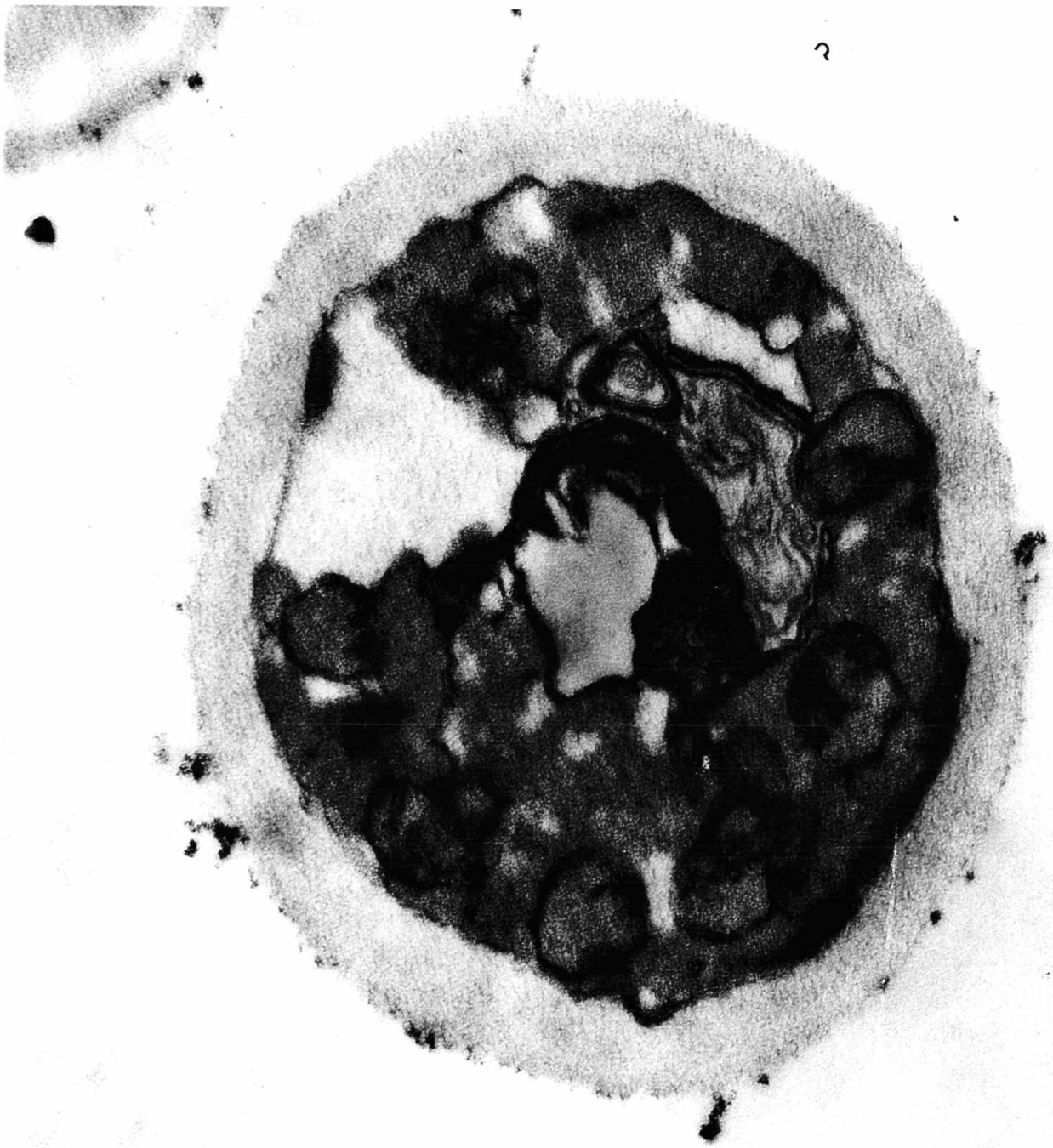


Plate 17 Section illustrating typical strain-21 cells after prolonged heat treatment. Note absence of distinct cell wall layers and electron dense staining material along outermost border of the cell wall.



VI. DISCUSSION

CHAPTER VI

DISCUSSION

The observation in Chapter 3 (Figure 3.4) that prolonged heating of washed cells in distilled water resulted in different responses for strain-5 and strain-21 cells led to the examination of strain-5 cells and strain-21 cells for gross or microstructural changes. The irreversible loss of agglutinability of strain-21 cells caused by prolonged heating could be explained by any of the following hypotheses. First, prolonged heating could cause the destructive fragmentation of the cell with a concomitant loss of agglutinability. Second, prolonged heating could result in a microstructural change in the agglutinative layer. Third, heating could cause a molecular change not observable at the electron microscopic level. These three possibilities were not necessarily mutually exclusive. The first hypothesis was eliminated by light microscope observations. Although cells heated exhaustively (180 min) were nonviable, they nevertheless remained intact in the sense that an overall form persisted still recognizable as a yeast cell. The yeast cell wall was seemingly an extremely rigid and resistant structure, although the cell contents were deteriorated almost beyond recognition. A comparison of prolonged heat-treated cells of

strain-5 and strain-21 indicated a microstructural change in the outer layer of the cell wall and it was possible to correlate this change with the loss of agglutinability of strain-21 cells. The third alternative was not by any means eliminated but was not testable with the electron microscope. Undoubtedly, many molecular changes resulted in the microstructural change observed with the electron microscope.

Several lines of evidence helped to localize the agglutinative material. Since agglutination was a cell surface phenomenon, one would expect that the agglutinative material was probably located either in the fringe, in the outer layer of the cell wall or in both. Brock (1958b) demonstrated that exhaustively washed preparations of isolated cell-wall materials retained their agglutinability. The observation in these studies that prolonged heat-treated 21-cells lost the outermost staining layer, along with a loss of agglutinability, was consistent with the involvement of the outermost layer in the agglutination process. From the present electron microscope studies, no structural difference was noted between non-activated and magnesium activated cells. It was not thought necessary to include photographs here of this negative result. Conti and Brock (1965) presented electron micrographs of sections through agglutinated masses of cells activated with magnesium. Their photographs, while probably not sufficiently extensive to support

a firm conclusion on this point, did indicate that the fringe zone, not the cell wall outer layers, were directly in contact between agglutinated cells. The tentative impression gained was that the agglutinating components are probably associated with the fringe material. Heat activated cells, however, lacked the fringe, which appeared to be quantitatively removed by heat activation (Plates 8, 9, 10, & 11); but since heat activated cells also agglutinated quite strongly, most probably the agglutinative molecules are also located in the outer layer of the cell wall.

Crandall and Brock (1968a, 1968b) have suggested that the process of cell agglutination in H. wingei was analogous to immunological reactions. The complementary glycoprotein macromolecules present on cell surfaces associated in the manner of antibodies and antigens. The precipitate of agglutinated cells in the agglutination reaction was a two phase process: the initial step, occurring immediately upon cell contact involved the clumping of small clusters of cells; and the second phase, being relatively slower, caused the macroscopically observable precipitation of massive clumps of cells. The assay technique used in these studies measured only the second reaction. The final turbidity (T_f) can be used to compare the degree of agglutination under described conditions but was not always the best parameter for comparison of intensity of agglutination.

It was noted earlier in Chapter 4 that heat activated cells demonstrated a more intense and complete agglutination than did magnesium activated cells (Figure 4.1 & Table 3). A comparison of T_f values indicated a slightly lower mean value for heat activated cells than for magnesium activated cells. The heat activated cells seemed to have a more intense and/or complete degree of binding, but, of course, there was little difference in the percentage of cells agglutinated, which was essentially 100 percent in both methods of activation. The physical appearance of the agglutinated masses also suggested rather strongly that the heat-activated cells were bound more tightly than the magnesium-activated cells. Another parameter to measure and compare differences in intensity of binding would be to compare the rates of agglutination. As was seen in Figure 4.1, the heat activated cells reached maximal agglutination (T_f) more rapidly than the magnesium-activated cells. In addition to the rates of agglutination, the kinetics of sequential urea deagglutination (Figures 4.9 - 4.10) suggested that the cation activated cells were bound less strongly. In these studies several independent observations suggested that heat activated cells demonstrated stronger binding intensities than magnesium activated cells.

Another major difference between cation activated and heat activated cells was the degree of reversibility. In Chapter 3 it was noted that the activation of magnesium-

activated cells was freely reversible, whereas heat activation was not freely reversible, since prolonged heating of 21 cells resulted in the irreversible inactivation of agglutinability.

Another major difference between heat-activated and cation-activated cells was determined in electron microscope studies. Morphologically, as mentioned (p. 53), the fringe layer in magnesium activated cells appeared no different from washed, non-activated cells. In heat activated cells the fringe was either removed completely or modified beyond recognition. In these cells there was no longer a fibrous layer with filaments perpendicular to the layers of the cell wall proper. The fringe had probably been quantitatively removed by the heat treatment but this could not be confirmed completely to one's satisfaction, since both the fringe and the outer cell wall layer stained readily with ruthenium red. Ruthenium red uptake was observed both with nonactivated cells as well as heat or magnesium activated cells. As mentioned before, ruthenium red demonstrated a staining specificity for acidic glycoproteins, and Crandall and Brock (1968a, 1968b) demonstrated the isolated agglutinating factors to be glycoprotein in nature. The conclusion suggested by the evidence was that the agglutinating material was associated with the ruthenium red staining sites. The observation that ruthenium red inhibited agglutination to some degree, suggested that the stain may have been combining with at least part of the agglutinating material. This observation also strengthened

the idea that the combining component, or agglutinating material, was located in both fringe layer and the outer cell wall, as these were the layers that stained specifically with ruthenium red.

The difference in degree of agglutination of magnesium activated and heat activated cells could then be explained in view of the effect of heat treatment on the cell wall. In both cell strains, heat treatment altered the fringe layer which could be acting simply as a physical barrier to the agglutinating sites in the outer cell wall. The fringe material could possibly still be attached to the cell surface as a highly modified, much flattened residue, but probably not. Whether or not a residue of fringe remained, it probably did not present the same physical barrier to the agglutinating sites as in non-activated or cation-activated cells. The increased intensity of binding of heat-activated cells was thus understandable if the heat treatment, in fact, exposed more reacting components or active sites. This interpretation was favored.

Crandall and Brock (1968a, 1968b) have presented evidence for the presence of a non-specific inhibitor on the surface of both strain-21 and strain-5 cells. It was non-specific because it was released from both haploid strains 5 and 21, their diploid non-agglutinative hybrid, as well as from other non-agglutinative strains of H. wingei. This inhibitor was removed from the cell surface by heating and had been purified

and characterized as a heterogeneous glycoprotein. These authors felt that the removal of this non-specific inhibitor allowed the heat-activated cells to agglutinate more strongly. This non-specific inhibitor material of Crandall and Brock could easily be parts of the fringe that had been removed and/or greatly altered.

The role of this non-specific inhibitor in cation activated cells was not clear at all. The reversibility of activation and agglutination with cationic solutions as described in Chapter 3 was inconsistent with the removal of a non-specific-inhibitor explanation of activation. In the present electron microscope studies it was observed that shaken-grown cells as well as cells washed repeatedly had a very sparse fringe layer. The observation that repeatedly centrifuge, non-activated cells did exhibit a measurable degree of agglutination (see Figure 4.2) was consistent either with the removal of an inhibitor from the cell surface or with the removal of the fringe itself, which exposed the more intensely binding material in the outer layer of the wall. Attempts to remove magnesium ions in magnesium activated cells by repeated washes of distilled water could have been complicated by the concomitant removal of fringe material, which could then expose additional agglutinative sites on the cell surface. Thus in the interpretation of Figure 3.1, the failure to obtain complete loss of agglutination after repeated

washes may not reflect only tightly bound or inaccessable magnesium ions but also may reflect increased availability of agglutination sites as the fringe material was removed by repeated washings.

VII. SUMMARY

CHAPTER VII

SUMMARY

The heterothallic haploid yeast, Hansenula wingei, exhibited a pronounced agglutination of opposite mating types prior to forming zygotes in sexual reproduction. This agglutination of cells in mass had been studied extensively by several investigators. Inhibition of the agglutination by 8M urea suggested that this process involved weak chemical bonding, such as hydrogen bonding or Van der Waals interactions. Genetic studies have indicated that cell agglutination and mating types were determined by two closely linked genetic loci. The two haploid, agglutinative isolates of opposite mating type, strains 5 and 21 of Wickerham, possessed complementary agglutinating factors. Several chemical and physical treatments affected the agglutinability of these two strains differentially. The agglutinating factors from both strains have been isolated and partially purified. Both factors appeared to be low molecular weight mucopolysaccharide-protein complexes.

The studies reported here have shown the following:

1. Activation of agglutinability with magnesium cation was freely reversible. Up to 20 percent of the magnesium activated cells, however, remained activated, and thus agglutinated even

after exhaustive washings. There was no observed change in microstructure of the cell wall material with magnesium activation.

2. Activation by heat treatment was not freely reversible. Brief heating fully activated the cells, but no amount of washing removed the heat-activated state. Prolonged heating of one strain, strain-21, resulted in the irreversible loss of agglutinability. Brief heating altered the ultrastructure of the fringe and appeared to remove it quantitatively. Prolonged heating also caused demonstrable change in the fine structure of the outer layer of the cell wall proper.

3. Cell agglutination of magnesium activated cells appeared to involve only fringe to fringe contact, whereas agglutination of heat activated cells involved contact of adjacent cell-walls proper.

4. Cell agglutination of both heat and magnesium activated cells was freely reversible in 8M urea, as shown kinetically in repeated agglutination--deagglutination cycles.

5. The optimal combining number was not a single ratio of 5:21 cells but, more accurately, a range of cell ratios depending upon the manner of initial cell contact.

6. The agglutinating factors appeared to be located both in the fringe region and in the outer cell-wall layer. The fringe region and the outer layer both stained uniformly with ruthenium red, an electron dense stain.

7. The staining sites of ruthenium red in the fringe and outer cell wall may be closely associated with the agglutinating sites in these regions, as suggested by the partially inhibitory action of this stain on agglutinability.

8. Heat activated cells demonstrated a greater binding strength than magnesium activated cells. This unequal binding was apparent in several independent lines of study, viz. the percentage of cells agglutinated, rates of agglutination, the rates of deagglutination, and also qualitative visual observations.

9. The difference in degree of agglutination of magnesium activated cells and heat activated cells was correlated with microstructural changes in the fringe of the cell wall. Brief heat treatment removed (or greatly modified) the fringe material, to an extent that more agglutinating sites were exposed in the denser, outer layer of the cell wall.

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