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Microbial Aggregation

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PREFACE

Somewhere I have read that an author's prefatory notes are usually written last, if at all written, and that they are usually read last, if at all read. (It must have been in somebody's preface.) And yet they come, by definition, before the main text. First shall be last and last first. This one is no exception.

A preface has a number of uses. Here I will use the device for the most part as a means of acknowledging my debt to those who helped me assemble this book. For this is nothing more than an aggregation, conscious and unconscious, of the words and ideas of others and a bit of mine, chiefly the string to bind them together.

I originally attempted to cover all microbial aggregation systems known and studied to date, but halfway in the writing process, it appeared that to do just that, with justice, required at least two volumes. Thus, the present volume covers in detail bacteria and yeasts. A second volume will comprise the cellular slime molds, the filamentous fungi, algae, and protozoa. Nonetheless, an overall perspective of microbial aggregation as fundamental form and function is presented here to include systems still to be treated in detail. Such an overview, I feel, is demanded by the subject matter.

There is a long list of people I have to thank. First among them is Byron F. Johnson of the National Research Council of Canada in Ottawa. A good many of his suggestions, I have unabashedly appropriated. Without him around to read patiently the drafts as the typewriter spewed them, this little book would have been doubly difficult to write and shape. Among those I have worked with in the laboratory, Bong Yul Yoo of the University of New Brunswick in Fredericton has helped me much by way of many insightful discussions. Both Dr. Johnson and Professor Yoo have been most generous collaborators in other projects.

With deep appreciation, I like to bare my indebtedness to Teena Walker and Susan Levy-Rick. Without their help, intellectual and manual, I would have given up gathering the materials for this volume; instead, I would have reached for the nearest waste basket. Writing may be physically accomplished with one hand, left or right, but the making of a book of this kind takes more than a pair of hands. It requires friends, who do not just watch as the author bleeds. The more difficult references were collected with the kind assistance of Margaret Schade and Noreen Brady.

In addition, the book and I profited much from corrections, comments, and criticisms (some caustic, others kind, never with a hint of malice) from other colleagues, who took some of their precious time to read segments of the manuscript: Isabelle Boisclair, Jack Christ, J. R. Colvin, Patricia Douglas, Allen P. James, C. V. Lusena, Brian L. A. Miki, F. Moranelli, Alain Vaisius, as well as others who would prefer to remain anonymous for fear that their contribution might be only minor. I disagree that their contribution is small. Anne Daley, Chris Gobey, and Lynda Boucher took turns to type the final script for the printer. The original illustrations were drawn by Celia Clyde and Denise Ladoucer. The photographic plates were prepared by Harry Turner. I would like to thank them all.

As well, I would like to thank all the authors who have consented to the re-use of their original materials. They are individually acknowledged at the appropriate places in the text. Here I will just list their names in alphabetical order: M. Achtman, C. E. Ballou, J. T. Bonner, T. D. Brock, M. Crandall, J. P. Duguid, G. M. Dunny, G. Gerisch, R. J. Gibbons, R. B. Gilliland, R. N. Greenshields, B. D. Hartong, E. Helm, W. Heumann, J. Hodgkin, J. Kohli, R. P. Levine, V. L. MacKay, S. A. MacKay, D. Malchow, T. R. Manney, B. L. A. Miki, W. L. Orton, P. C. Newell, N. H. Poon, A. Pühler, H. Reichenbach, G. G. Stewart, N. W. Taylor, R. S. W. Thorne, A. Tomasz, Y. Tsubo, H. van den Ende, M. J. Vold, L. J. Wickerham, N. Yanagishima, B. Y. Yoo, and M. Yusa.

In spite of the many forms of assistance generously given by associates, the book is far from perfect. Of course, my friends are not responsible for the imperfections. That responsibility is greedily reserved by the author for himself. The fool and his book are never parted.

This book is not for my wife, Mia, and the children, Maria and Raoul — even as they suffered as I wrote. There is world enough, I hope, to write another book just for you. You are most welcome to read this one — or even to show it to friends, yours, mine, and ours — provided they are not minors or prudes.

Gode B. Calleja

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Chapter 1

GATHERING THINGS TOGETHER

I. INTRODUCTION

A first book on an ill-defined subject must begin with definitions. For, in spite of the literature it has generated, microbial aggregation remains an ill-defined subject. This book and a subsequent volume are an attempt to gather in two bundles the scattered literature on the subject.

II. AGGREGATION DEFINED

Aggregation is the gathering together of units to make a larger unit (Figure 1). The resultant larger unit is also called an aggregation. In addition, the state or condition of being gathered together is also known as aggregation. The clumping of cells in liquid culture is aggregation. The resultant clump is an aggregation, or an aggregate, of cells. In the first sense, aggregation is a process, a function, the gathering together. In the second, it is a product, a structure, the larger unit. Such a melding of function and structure, despite the mutual disrespect of the physiologist and the morphologist for each other, appears to be a common feature of many languages.

The gathering together and the units may be concrete or purely conceptual. A compilation such as this book is therefore an aggregation. A cell is an aggregation of organelles, an organelle of molecules, a molecule of atoms. Society is an aggregation of individuals. A forest is an aggregation of trees. A sentence is an aggregation of words, a word of letters. The mathematical operation of addition is aggregation. The resultant sum is an aggregation, too, and so is any collective noun or pronoun. However, we shall concern ourselves here not merely with either grammar or numerical manipulation, but primarily with the concrete and the palpable, in particular, the aggregation of microbial cells.

The Milky Way is an aggregation of stars, but only structurally and conceptually. Its origins are not likely due to an aggregation process. Concatenated paper dolls make an aggregation (Figure 2). They are the product of a process not of aggregation but of paper cutting. A chain of bacilli, another concrete example, may be thought of as an aggregation, conceptually, but even in the conceptual sense, the process of chain formation among bacilli is not aggregation. Instead, it is properly called cell division or even cell multiplication, which is outside our present concern. Aggregation is addition, mathematically and rhetorically.

III. CELL AGGREGATION DEFINED

With that in mind and with the unabashed assumption that aggregation is a fundamental condition, function, and structure in biology, we shall define "cell aggregation" as the gathering together of cells to form fairly stable, contiguous, multicellular associations under physiological conditions (Figure 3). To various investigators, it is also known as adhesion, adherence, agglomeration, agglutination, association, autoagglutination, clumping, coagulation, coherence, cohesion, flocculation, flocculence, flotation, isoagglutination, sedimentation, stickiness. The resultant structure is also called agglomerate, agglomeration, aggregate, aggregation, agglutinate, agglutination, clump, cluster, coagulum, coremium, film, floc, flock, grex, head, pellet, pellicle, plasmodium, rhizomorph, ring, sclerotium, slime, slug, strand, stroma, synnema.

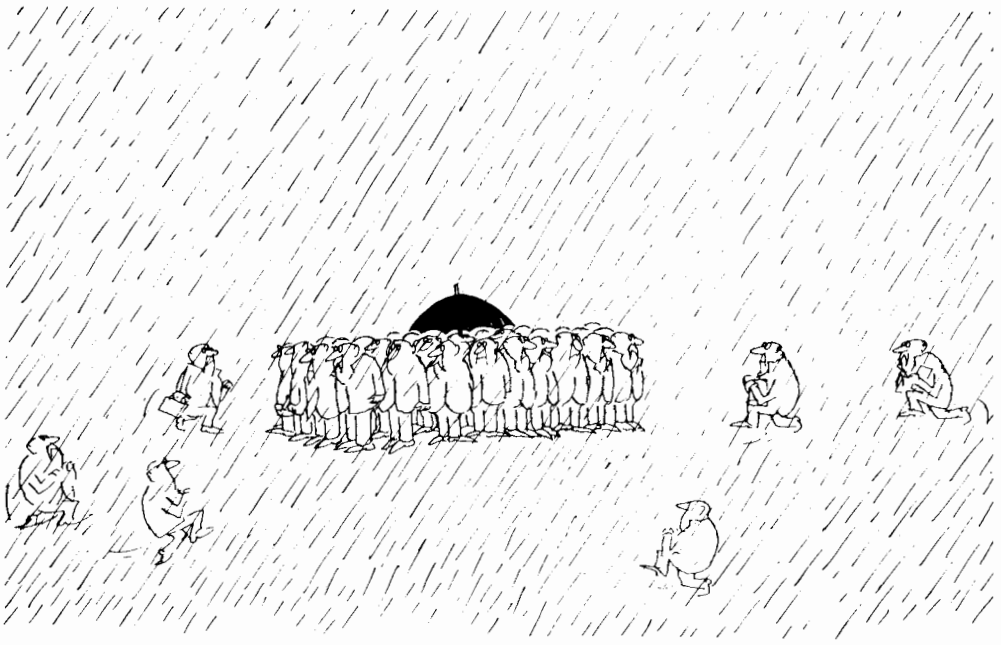

FIGURE 1. Aggregation, nonmicrobial. (Reprinted by permission of the *Bulletin of the Atomic Scientists*, a magazine of science and public affairs. Copyright (c) by the Educational Foundation for Nuclear Science, Chicago.)

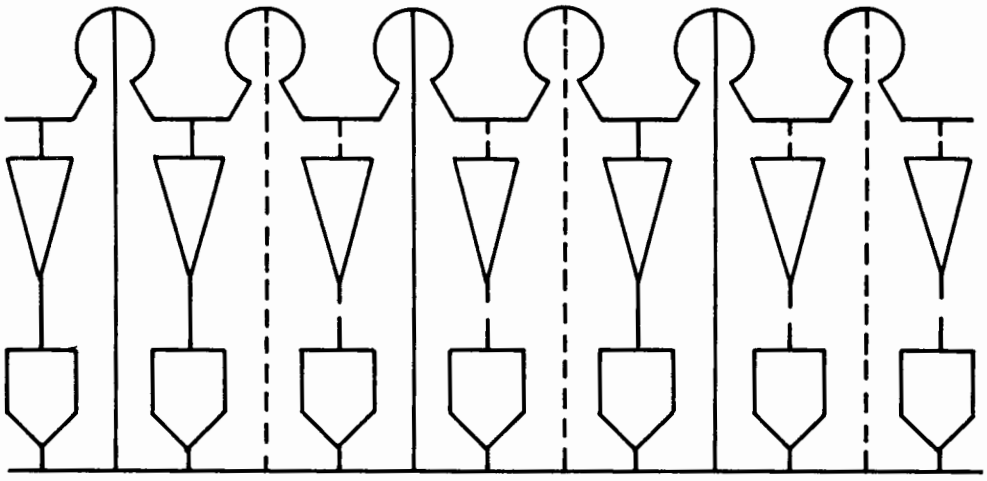

FIGURE 2. A catena of paper dolls. An aggregation structure that is not a product of an aggregation process.

The abundance of names, although a source of minor confusion, must not be viewed as a hindrance to the unification of the field of study. Rather, it is in keeping with the richness of the English language, especially with regard to words describing collectives or aggregations of animals. A few of these more colorful names are a bale of turtles, a charm of finches, a clutter of cats, a congregation of plovers, a cowardice of curs, a cry of hounds,

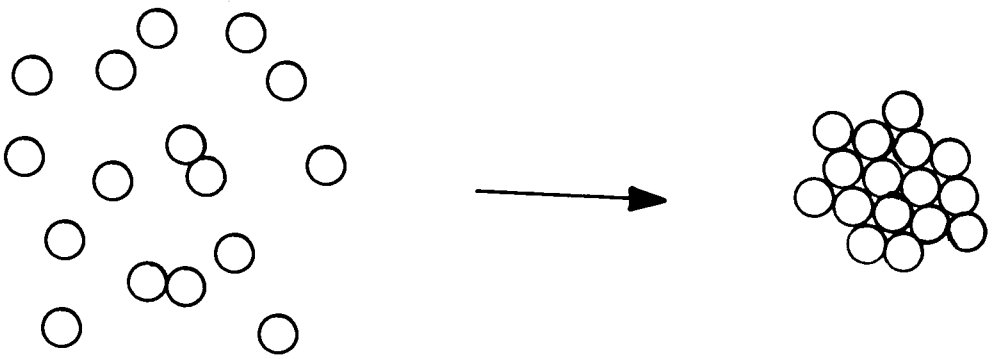

FIGURE 3. A generalized schema for cell aggregation. Cells are represented as spheres.

a drift of hogs, an exaltation of larks, a gaggle of geese, a leap of leopards, a murder of crows, an ostentation of peacocks, a parliament of owls, a pod of whales, a pride of lions, a shrewdness of apes, a skulk of foxes, a sleuth of bears, a spring of teal, an unkindness of ravens, a watch of nightingales.[1,2] In like manner, we may speak of an aggregation of myxobacteria, a clump of cells, a floc of yeast, a slug of amoebae, a strand of hyphae.

The definition contains two principal elements: physical movement and stable multicellular contacts. Both need to be present in any cell aggregation system.

Movement may be either directed (active), like chemotaxis in the cellular slime molds, or stochastic (passive), like random collision in a shaken liquid culture of bacteria (Figure 4). However it is achieved, there must be movement to allow cells to come together. This condition presupposes a state, prior to aggregation, in which the cells are disperse.

There must be actual physical contacts among the aggregated units, rather than merely conceptual grouping or lumping. Mere proximity of units, or even juxtaposition, is not good enough. Contacts must be more intimately close than close encounters of any kind. Moreover, they must be intercellular. Adsorption of cells onto inert surfaces fails to satisfy the definition. Furthermore, contacts must be multicellular. The minimally plural condition is not sufficient. Nor is the number of cells prior to aggregation strictly singular. The reason is more of convenience than of grammar: if we were to include pair formation (mechanistically, but not formally by our definition, the minimum aggregation), then all conjugation systems would have to be included, surely, not an easily managed lot. A lower limit of ten cells is a convenient, albeit largely arbitrary, size for a minimum group. It appears reasonable for certain systems, sex-directed flocculation in fission yeast, for example.

The multicellular condition connotes multivalency of the involved cells. It is clear that the final aggregated structure is determined by the combining power, or valence, of the individual components (Figure 5). Monovalent cells can only form pairs, divalent cells only chains (open or closed).

As in gametic agglutination of unicellular algae, contacts may be transient, but must last long enough and be strong enough to be experimentally describable. An aggregation that does not result in conjugation or cell fusion must be able to withstand Brownian buffeting under physiological conditions. A sediment of nonflocculent yeast cells, for instance, when gently agitated, disperses as a fairly homogeneous suspension in wort, but that of flocculent cells disperses as discrete flocs.

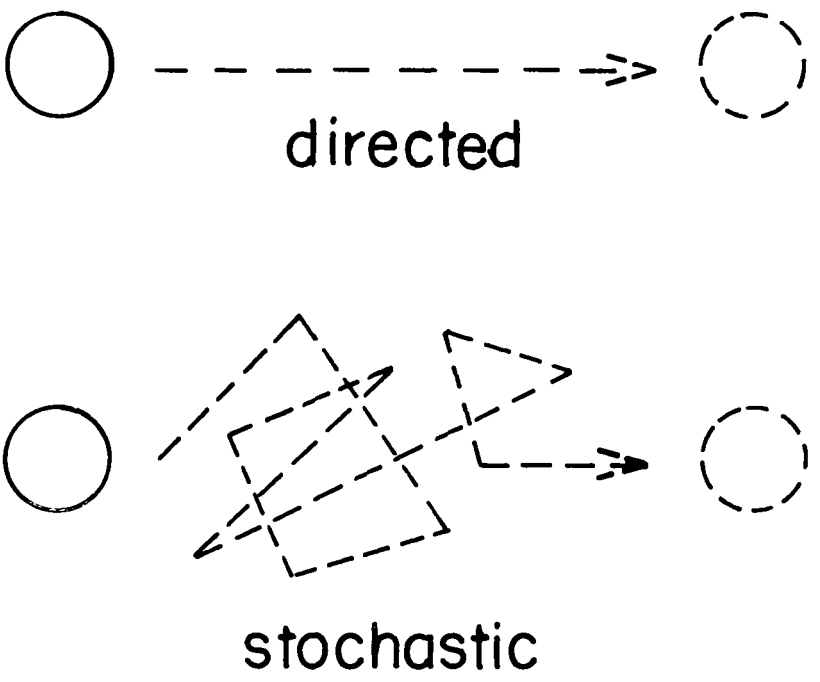

FIGURE 4. Movement of cells from one site to another.

IV. EXCLUDED PHENOMENA

Listed below are phenomena that may be, and have been in the literature, confused with cell aggregation. Some of them are graphically illustrated in Figure 6. They are things with which we shall not be concerned. For convenience, they are grouped as follows:

1. Noncellular aggregation: colloidal suspension, crystallization of viruses, gel formation, macromolecular self-assembly, polymerization, precipitation of molecules
2. Multicellular or multinuclear condition due to failure of progeny to effect complete separation after cell division or due to failure of cells to divide after nuclear division: chain, coenocyte, colonial form, filament, fruiting body, mycelium, packet, plasmodium, pseudomycelium, syncytium
3. Grouping (due to growth, buoyancy, or gravitation) under nondispersive conditions: bloom, colony on agar, focus of infection, pellet, pellicle, sediment
4. Association with noncellular objects: adherence, adhesion to glassware, adsorption onto solids, phage adsorption, stickiness
5. Interaction short of the multicellular condition: anastomosis, cell fusion, conjugation, copulation, heterokaryosis, mating, pair formation, palmella formation, zygote formation
6. Tropic response without subsequent stable contact: aerotaxis, chemotaxis, phototaxis, pellicle, swarm
7. Aggregation provoked by substances not usually found as components in the culture medium nor produced by the organism: agglutination, flocculation, and sedimentation provoked by antibodies, exotic lectins (e.g., concanavalin A, ricin, wheat germ agglutinin), native or denatured enzymes (e.g., lysozyme, ribonuclease), serum proteins (e.g., bovine serum albumin), artificial flocculants (e.g., bentonite, borate, undefined clays), and synthetic polymers (e.g., polyethyleneimine, polylysine)

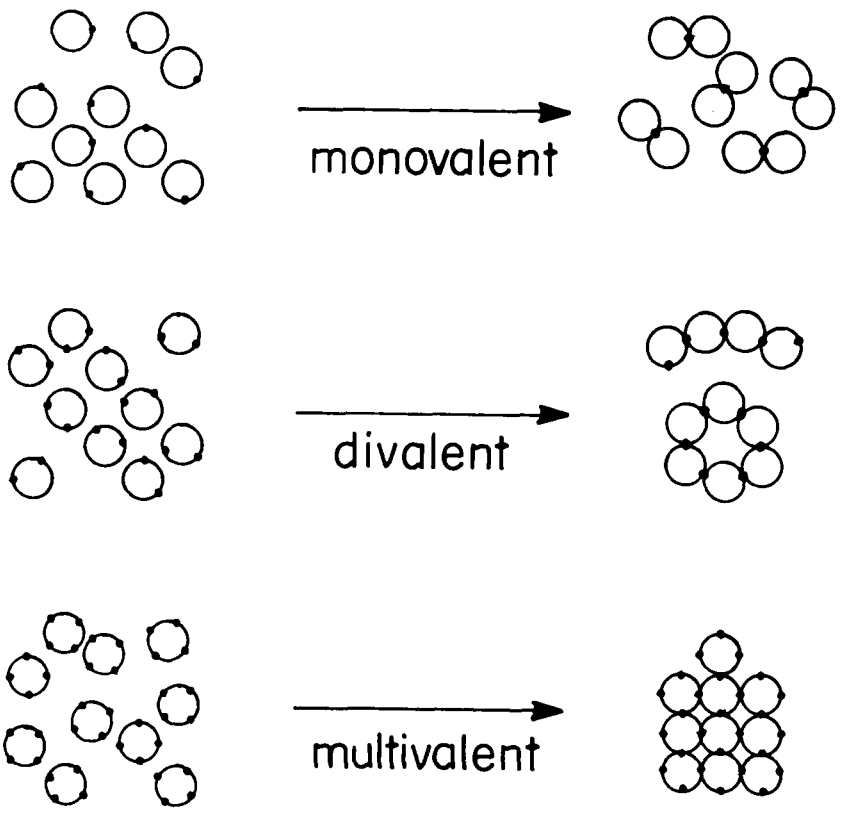

FIGURE 5. Valency of cells determines final aggregated structure. Dots represent potential and actual sites of union between cells.

8. Aggregation provoked under obviously nonphysiological conditions: agglutination, coagulation, denaturation, precipitation, and sedimentation due to strong acids and alkalis, organic solvents, centrifugation, dehydration, desiccation, heat, and lyophilization
9. Phenomena directly or indirectly associated with cell aggregation: anastomosis, cell fusion, competence, conjugation, copulation, fruiting-body formation, gametogenesis, growth, hyphal elongation, mating, meiosis, sexuality, sporulation, taxis, zygote formation

V. CRITERIA FOR INCLUSION

The following criteria are derived from the definition and the enumerated exclusions. To be included in a list of cell aggregation phenomena, a system must be (1) active (there is a change from a disperse condition to an aggregated condition), (2) inducible (there exists a physiological situation in which aggregation is absent), (3) stable (as an aqueous suspension), (4) intercellular (adhesion to noncellular surfaces is excluded), (5) multicomponent (a minimum group of ten cells), and (6) spontaneous (compatible with the life cycle of the organism and not due to obviously nonphysiological perturbations).

Notwithstanding the expedient limitations of the definition and the exclusion of many situations, cell aggregation remains a widespread condition in the microbial world. It is

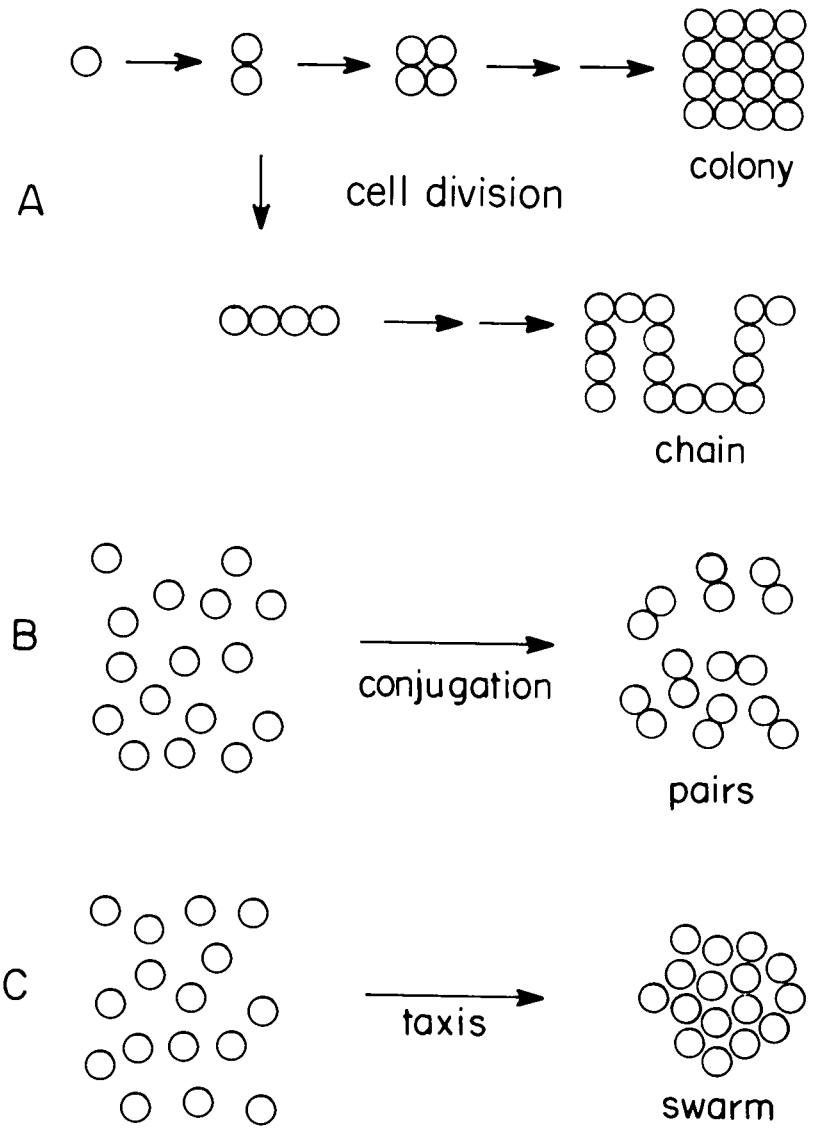

FIGURE 6. Phenomena that are related to, but excluded from, cell aggregation as defined in the text. (A) is excluded because the multicellular condition is achieved by a process that is more properly termed cell division; (B) because the final structures fall short of multicellularity; (C) because the final structures are units not in intimate material contact; (D) because the contacts are not intercellular; (E) because the process of achieving the multicellular condition is not physiological; (F) because the multicellular condition is elicited by exotic polymers; (G) because the final structure is achieved only under nondispersive conditions.

found among bacteria, yeasts, cellular slime molds, filamentous fungi, algae, and protozoa. In addition to well-known phenomena such as flocculation of brewers' yeasts, aggregation of cellular slime molds, and agglutination of gametes in chlamydomonads, the definition includes mating-aggregate formation during bacterial conjugation, bacterial star formation, agglutination associated with competence for bacterial transformation, dental plaque formation, aggregation in myxobacteria, sex-directed flocculation in fission yeast, and sexual agglutination in *Hansenula, Saccharomyces,* and other yeasts. It also includes mating re-

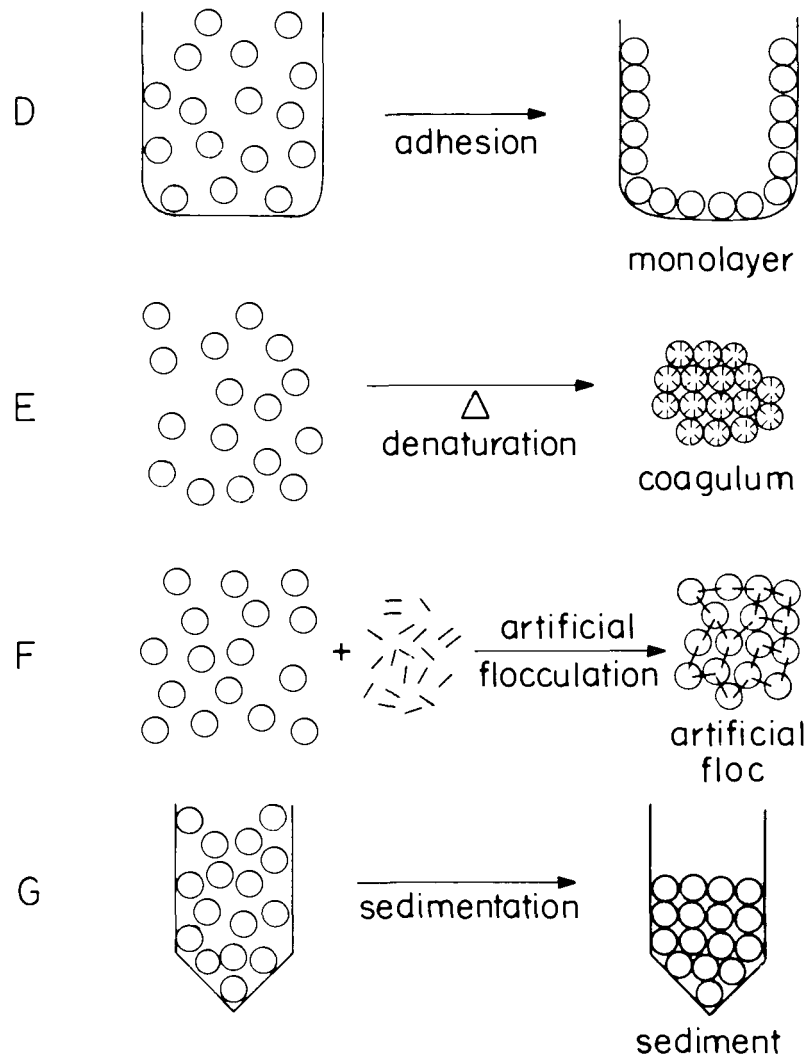

FIGURE 6.

actions in protozoa and the formation of strands, synnemata, rhizomorphs, sclerotia, and coremia in the filamentous fungi. As you well see, we have not run out of subject matter.

VI. REVIEWS REVIEWED

Of cell aggregation that is nonmicrobial, many reviews of general coverage have been written. A few of these must be mentioned, if for no other reason than to direct the reader to them.[3-7] Microbial systems, in contrast, have been less dealt with in broad terms, perhaps because of their apparent diversity.

Microbial aggregation has been reviewed from various vantage points. Hoffman[8] in 1964 treated bacterial aggregations as models of morphogenesis. His concept of aggregation, however, remains exactly that, that which we have dismissed as purely conceptual. The bacterial aggregations he reviewed are cell division, failure of cells to completely separate

after cell division, chain formation, and colonies on agar, items excluded from the definition. Indeed, the review virtually confines itself to these phenomena.

Morris's treatment,[9] in 1966, of aggregation in yeasts likewise includes colony formation in addition to flocculation and sexual aggregation. Strangely enough, the formation of pseudomycelia is also entertained as aggregation and labeled asexual conjugation.

The literature on the role of polymers in microbial aggregation was ably summarized in 1973 by Harris and Mitchell.[10] Systems discussed include bacterial aggregation by synthetic and natural polymers, formation of dental plaque, yeast aggregation during conjugation and fermentation, and aggregation in heterogeneous microbial communities, with emphasis on soil ecology and waste treatment. The ecological and practical import of microbial aggregation is underscored.

A different approach was made in 1974 by Reissig[11] in viewing the cell surface as a seat of cellular regulation and recognition. Touching on many different aspects of ektobiology,[12] his admirable synthesis covers the whole range of the microbial spectrum and, among other things, includes bacterial conjugation, sexual agglutination in yeasts, mating reactions in protozoa and algae, chemotaxis, and bacterial transformation. The emphasis is information content of the cell surface, but the significance of microbial aggregation comes out very clearly.

The survey by Atkinson and Daoud[13] in 1976 is from the standpoint of fermentation process engineering. It is quite useful because of its detailed handling of methodology, mechanisms, and process applications. However, a great majority of the flocculation systems described are outside the scope of our present concern.

Ottow's review[14] in 1975 on fimbrae and pili peripherally touches on bacterial aggregation, and so does Smith's review[15] in 1977 on microbial surfaces in relation to pathogenicity.

The subject of mating-type interactions in microorganisms was extensively reviewed in 1977 by Crandall[16] for the new series *Receptors and Recognition*. Most of the systems covered fall within bounds of our definition. The coverage, however, is limited to sexual systems.

In the same new series is an anthology, edited by Reissig[17] in 1977, of contributions from various laboratories on microbial cell-cell interactions. It includes aggregation in the cellular slime molds, bacterial chemotaxis, bacterial transformation, mating-aggregate formation in bacteria, and mating reactions in *Saccharomyces, Chlamydomonas,* and the ciliates. A summary by the editor gives the reader a broad overview of the subject.

Fungal aggregation in its various guises has been dealt with adequately by Burnett[18,19] in his book on mycology. Other reviews on fungal aggregation from various biased viewpoints are those by Hawker,[20] Garrett,[21] Butler,[22] Willetts,[23] and Chet and Innis.[24] A review by Carlile and Gooday[25] in 1978 on cell fusion in fungi and myxomycetes embraces a good number of fungal aggregation systems.

By far, the most adequately covered systems are those of brewers' yeast and the cellular slime molds. It is no accident that it is in these systems where there is most intensive research activity. Reviews exclusively concerning these systems will be dealt with in chapters devoted to them. For the moment, it is sufficient to note that, in Bonner's book on the cellular slime molds,[26] cell aggregation is treated as if it were a virtual monopoly of Acrasiales, among the eukaryotes, and of myxobacteria, among the prokaryotes.

VII. THE STRATEGY OF THE MONOGRAPH

The recent flurry of reviews, general or specialized, on microbial aggregation is a welcome sign of a growing interest in the subject among researchers of diverse prejudices. Still, there is the need to gather together the many scattered bits of information concerning the subject. The need to gather the bits into a monographic whole becomes more pressing as the clutter

and the confusion intensify. Because many associated and more complicated phenomena, including unidentified objects, have been mistaken for microbial aggregation, it has become imperative that a unifying treatment of the subject in rather broad and comprehensive terms be written, if only to put into proper perspective the extent and the meaning of a general biological phenomenon that has been too long neglected.

This monograph, an expansion of an earlier compilation on the nutritional aspects of microbial aggregation,[27] limits itself to microbes and to cell aggregation as defined above and as defined in the earlier review. The word "microbes" will be taken to mean organisms that are commonly accepted as cellular microorganisms by students of microbiology. Much space has already been spent on quibbling over definitions. There will be no further attempt to quibble in a taxonomic manner. Instead, the book will attempt to approach microbial aggregation as a unified subject and as a fundamental structure and a fundamental function in biology. As a way of emphasizing diversity, a number of chapters will be devoted in some detail to the better studied systems. Cellular slime molds, filamentous fungi, algae, and protozoa will be dealt with in a subsequent volume. In this volume, bacterial and yeast aggregation systems will be examined in detail. In a final chapter of this volume, these diverse systems will be gathered once again into an aggregated whole. The objective is to permit us, so to speak, to see the forest for the trees, the exaltation for the larks.

Chapter 2

CELL AGGREGATION IN THE MICROBIAL WORLD

I. CLASSIFICATION ATTEMPTED

The different forms of cell aggregation may be classified into "homotypic" and "heterotypic" systems (Figure 1). A homotypic system is made up of similar units, whereas a heterotypic system is made up of dissimilar units. Flocculation of brewers' yeast during fermentation is homotypic. Co-flocculation of yeast with bacteria is heterotypic. These examples seem to demonstrate the usefulness of the classification scheme. However, all is not that clear-cut.

If intergeneric interactions are obviously heterotypic, interspecific ones must no less be so. Homotypic systems are then limited to intraspecific interactions, but how does one classify interactions of mating types? Certainly, mating reactions involve cells of the same species, but cells that must differ in some way as to make them distinguishable from one another.

At the level of molecular structures, homotypic interactions may be achieved in two ways: (1) the interaction is mediated by a functionally symmetrical molecule and (2) the interaction is mediated in a lock-and-key fashion, both lock and key being found on the surface of the same cell (Figure 2). At this level, all systems involving mating types will have to be considered as heterotypic.

In the strict sense of the attempted classification, there is scarcely any cell aggregation system that has been shown to be truly homotypic. Perhaps, all interactions are among units that are dissimilar, in one way or the other, but more specifically at the level of the interlocking structures. To be truly homotypic, a system must be shown to be mediated by functionally symmetrical molecules or by interlocking structures that are found on the same cell.

The term "heterotypic" will be confined to systems that are obviously heterotypic and "homotypic" to those that do not formally require that the components be different. Many systems that will be described in detail are not obviously heterotypic and therefore, for the present purpose, classified as homotypic. Sex-directed flocculation or sexual agglutination, although among units within a species, is classified as heterotypic because it requires two types of complementary cells, however difficult it might be to distinguish them morphologically. Sexual agglutination aside, there are not many heterotypic systems that have been investigated carefully.

Many different ways of classification are conceivably useful. For instance, teleologic, according to the reason of a system for being, or mechanistic, according to how the interaction is mediated. Perhaps it is wiser to resist the temptation to classify, when the present state of the science does not warrant or even require classification, or failing that, to merely classify the systems taxonomically, according to the organisms involved.

Convenience, then, rather than logical insight, prompts us to group the various microbial aggregation systems according to taxa: bacteria, yeasts, cellular slime molds, filamentous fungi, algae, and protozoa. There will be a minimum reference to heterotypic systems, except to mean intergeneric and interspecific systems and interactions of mating types.

II. DISTRIBUTION AMONG MICROBIAL TAXA

Microbial aggregation systems are distributed among many microbial taxa. They are found in both prokaryotes and eukaryotes. A list of systems that will be discussed in detail is found in Table 1.

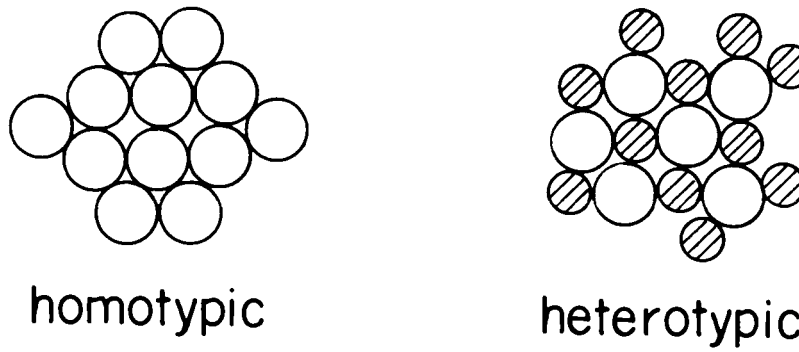

FIGURE 1. Homotypic and heterotypic aggregations. Cells are represented as spheres.

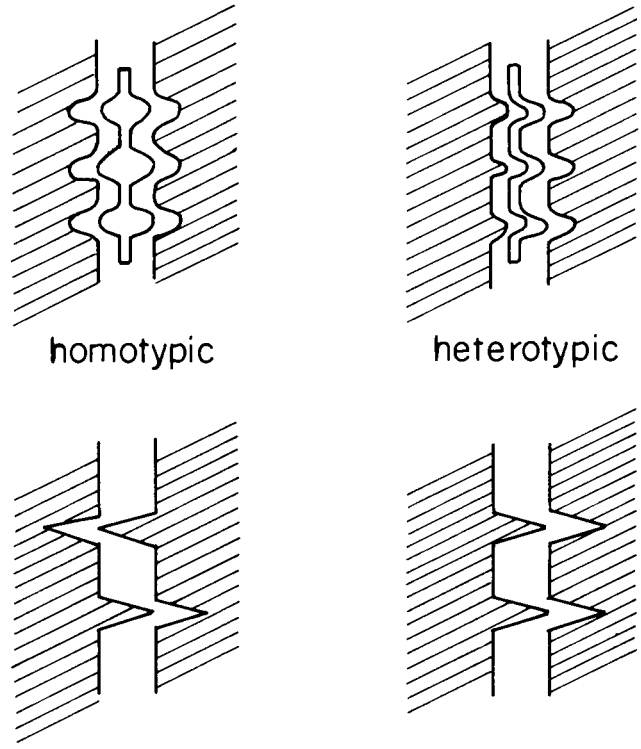

FIGURE 2. Cell-cell interactions. Hatched areas represent cell walls or cell membranes or wall appendages. Top: interactions mediated by a discrete molecule. Bottom: interactions mediated by lock-and-key structures which are integral parts of the cell wall or the cell membrane or the wall appendage.

The terminologies for aggregation as used by the authors of primary references are retained where possible. Such an arrangement, it is hoped, does not become a source of inconvenience to the reader. Rather, it lends some quaintness to the minor confusion, making the list look more like an oriental bazaar than a department store. Where there is lack of agreement as to the appropriate terminology among several laboratories working on the same system, the better known nomenclature, not necessarily the earliest, is chosen. Where there is no specific

Table 1
A LIST OF MICROBIAL AGGREGATION SYSTEMS

- Bacteria
  - Mating-aggregate formation during conjugation in *E. coli*
  - Star formation in *Pseudomonas, Rhizobium, Agrobacterium*
  - Pellicle formation in fimbriate bacteria
  - Agglutination associated with transformation competence in bacteria
    - *Streptococcus*
    - *Pneumococcus*
    - *Bacillus*
  - Plaque formation and agglutination in oral bacteria
  - Aggregation in *Streptococcus faecalis*
  - Aggregation in myxobacteria
  - Other bacterial aggregation systems
- Yeasts
  - Sexual agglutination in *Hansenula wingei*
  - Sex-directed flocculation in *Schizosaccharomyces pombe*
  - Sexual agglutination in *Saccharomyces cerevisiae*
  - Flocculation of brewers' yeast
  - Other yeast aggregation systems
- Mycetozoa
  - Aggregate growth in *Physarum*
  - Aggregation in cellular slime molds
    - *Dictyostelium*
    - *Polysphondylium*
    - *Acytostelium*
- Filamentous fungi
  - Strand formation: *Merulius, Helicobasidium, Agaricus*
  - Rhizomorph formation: *Armillaria, Marasmius*
  - Sclerotium formation
    - *Sclerotium*
    - *Sclerotinia*
- Microsclerotium formation in *Verticillium*
- Synnema formation: *Hirsutella, Isaria, Graphium*
- Coremium formation: *Penicillium, Sphaerocybe*
- Algae
  - Mating reaction or sexual agglutination in *Chlamydomonas*
  - Agglutination reaction in *Dunaliella salina*
  - Isoagglutination of gametes in *Pandorina*
  - Agglutination of gametes in *Ulva mutabilis*
  - Other algal aggregation systems
- Protozoa
  - Mating reaction in *Blepharisma*
  - Mating reaction in *Euplotes*
  - Mating reaction in *Paramecium*
  - Mating-aggregate formation during chemically induced conjugation in *Paramecium*
  - Clumping in *Mayorella palistinensis*
  - Other protozoan aggregation systems

terminology proposed, the author takes the liberty of inventing a descriptive title as a means of identifying a system and preventing its being mistaken for similar or related phenomena.

The making of this list is like making an all-star selection for a national hockey team. The choice and the order would be quite different if the list were made by somebody else. It would look different again, if it were to be submitted to a book of lists.

In spite of its innumerable contributions to general biology, the bacterium thus far has not found much favor among workers in the field of cell aggregation. Perhaps this is due to the fact that the field is barely past its phenomenological stage. Consequently, prokaryotes, being considerably smaller, carry with them a built-in disadvantage. Two bacterial systems,

nevertheless, have attracted a loyal following. These are dental plaque formation caused by streptococci and aggregation in myxobacteria. Studies on dental plaque formation show signs of being in the forefront even as the myxobacteria may prove to be as engaging as their eukaryotic analogs, the cellular slime molds.

The yeasts, especially brewers' yeast, have been the standard models for microbial aggregation. Ever since fermentation was proved in the last century to be due to cellular activity, yeast flocculation has earned the serious attention of the brewer. So far there is no evidence of it being sexual. Sexual agglutination in the same organism, however, has drawn much attention since the discovery of mating factors, and sex-directed flocculation in fission yeast has been advanced as a model for development. Among the yeasts, it is *Hansenula wingei* which has the best claim to being the organism of choice in the study of sexual agglutination as mediated by glycoproteins on the cell surface.

Very interesting findings have been made with the cellular slime molds, the best studied of the microbial aggregation systems, at least with regard to the chemotactic signals involved, and easily the most popular. Various approaches, theoretical and experimental, have been brought to bear on this intriguingly beautiful process. The morphology and the mechanics of the movement, the generation of cyclic-AMP pulses, and the reception of these signals have occupied the time of many laboratories. These, however, form a small part of the investigations that treat these organisms as models for morphogenesis and differentiation.

Despite their potential role in plant pathogenesis, the aggregation systems of filamentous fungi have not been exploited to the fullest. Except for studies on sclerotium development, there has been no sustained effort to unravel the intricacies of the aggregation process in filamentous fungi, a process known to the early mycologists. There are some inherent difficulties, not the least of which is the difficulty in separating the process of aggregation from that of growth. The coalescence of hyphae to form hyphal aggregates is normally achieved concomitant with hyphal elongation.

Research on algal aggregation has been centered on the mating reaction or sexual agglutination of species of *Chlamydomonas*. Gametogenesis precedes the mating reaction, mediated by gametangial flagella. The morphological description of this reaction has been pursued with intensity together with attempts to isolate the mating factors. There is hardly any other alga that has been carefully investigated with regard to aggregation. And, of course, the colonial forms are excluded from our definition.

The mating reactions of a number of ciliates have been investigated, but species of *Paramecium* remain the mainstay. Upon mixing, cells of complementary mating types agglutinate by their ciliary tips, a process analogous to flagellar agglutination in chlamydomonads. The ciliary reaction is not the only way agglutination may be elicited. Mating-aggregate formation is also observed when conjugation is induced chemically.

A few of the systems found in the list are somewhat unexpected and require justification. These are formation of mating aggregates during conjugation in *Escherichia coli*, agglutination associated with the development of transformation competence in bacteria, and the mating reactions in *Blepharisma* and *Euplotes*. Since its discovery, bacterial conjugation has been assumed to be uncomplicated pair formation. Recently, however, it has been convincingly shown that the mating structure is an aggregate of many cells.[28] Genetic transformation in bacteria eventually gave us molecular biology. It has been reported lately that associated with competence for transformation is an agglutination reaction.[29-31] The mating reactions in *Blepharisma* and *Euplotes* have been known for many years. Unlike those in *Paramecium* and in chlamydomonads, these reactions are so weak that they have been assumed to be mere pairing of gametes. Ways of bringing about the agglutination reactions have been found not too long ago.[32,33] However, there has not been any report as to the capability of *Tetrahymena* for multicellular aggregation, and hence its absence from the list.

The list, of course, is representative rather than comprehensive. It is safe to assume that a large majority of microbial taxa still await investigative attention with regard to their aggregative capabilities. When the preferred orientations of other secretively sensuous microbes are unmasked, the list will undoubtedly become much longer.

Too often, cell aggregation is not the most conspicuous (nor the most interesting) phenomenon in a complex series of events or structures. This has occasionally led to it being just about ignored. When it gets more than the perfunctory attention, what often is scored is the event that comes before (e.g., gametogenesis, chemotaxis) or the event that comes after (e.g., conjugation, fruiting body, sporulation) or the associated event (e.g., competence for transformation, competence for conjugation), rather than aggregation as such. These events and others will be discussed, but only insofar as they help clarify the principal subject matter.

III. THE IMPORTANCE OF BEING AGGREGATED

A. Practical Considerations

Many a culture in the laboratory gets thrown out because what has been expected to be a pure culture turns out to be seemingly mutated, or worse, contaminated: an erstwhile homogeneous suspension of cells has become a collection of clumps. Instead of being sidetracked into finding out the reason for clumping, the all-too-careful investigator decides to throw away the errant culture and start afresh, this time being more careful not to encounter again the clumping phenomenon. Of course, if it occurs again and again, the nuisance is then taken seriously. Then the investigator is hooked on studying microbial aggregation. If you can't lick it, you might as well get a research grant for it.

To the investigator, however, who has to grow a homogeneous suspension of cells, because he has to count the cells or to sample the culture for analysis, clumping is no more interesting than a nuisance that must be wished away.[34] If he loses sleep on it, it is merely to find ways to avoid it.

Microbial aggregation has more than just negative value. In the case of yeast, it is a case of beer. To the brewer, yeast flocculation makes or unmakes a beer. Flocculation influences the fermentation process to a very large extent. Its onset signifies the end of primary fermentation. When the cells fall in flocs to the bottom of the brewing vessel, their access to the nutrients in the wort becomes restricted. At racking, the beer from a flocculent strain is poorly attenuated, sweeter, and brighter. Furthermore, flocculation influences the maturing of beer and the processes after the primary fermentation. Because it effectively reduces the yeast population in contact with the wort, it leaves very few cells for secondary fermentation, and the yet unborn beer becomes prone to spoilage by unwanted contaminants which may outfeed the yeast on the rich medium. Thus, the choice of yeast strains is critical. The quality of the final product depends on the flocculation characteristics of the chosen strains.

The flocculation characteristics of a strain are exploited not only in the production of beer but in the production of metabolites and biomass as well. The recovery, by filtration or by centrifugation, of yeast cells after fermentation is better accomplished in flocculent strains because flocs are fluffier than a sediment of "powdery" cells. Flocculent strains are also required in tower-fermentation systems to maintain adequate cell concentration and prevent washout.

In general, flocculation and the flocculating capacity of an organism may affect the design, performance, and yield of a fermentation set-up by facilitating the recovery of biomass, improving the flow properties of the brew at high concentrations of biomass, increasing microbial hold-up, and thus, presenting opportunities for novel fermentation design.[11] On the other hand, flocs hinder substrate diffusion and thus reduce substrate uptake and retard the metabolic processes.

Much of the potential usefulness of flocculation to the fermentation industry, for instance, tower-fermentation design in the brewery, the production of single-cell protein, and the production of biochemicals, remains to be realized. One organism that has commercial possibilities is *Saccharomyces kluyveri*.[35] A fast and strong fermenter, it may be grown for food or feed in place of *Candida utilis*. When the two complementary mating types are combined in water, a dramatic sexual agglutination reaction occurs (Figure 3). The mating types may be grown in separate containers and, at the end of growth, allowed to mix in a collecting tank. The agglutinated mass may then be recovered at much reduced time and expense.

One may suspect that cell aggregation plays a decisive role in the pathogenicity or invasiveness of a pathogen, or at least in its ability to colonize tissues. The best studied model on which one may base this suspicion is dental plaque formation. The possible relation of the aggregative capacity of *Streptococcus mutans* to cariogenesis and periodontal diseases has been the principal incentive in the surge of research activity on this system. The evidence has been summarized by Gibbons and van Houte.[37]

The ability of aggregated hyphal structures to withstand adverse conditions aggravates the hardships in eradicating plant pathogens. The destruction of these resistant structures and the prevention of their development are targets of measures to control the outbreaks of plant diseases.

Flocculation by natural and synthetic polymers is part of waste treatment by the activated sludge process. It is of minor significance in the trickling filter process.

Heterogeneous microbial communities may include bacteria, fungi, and protozoa in an aggregated mass. It is known that soil aggregation is enhanced by its biotic component, but as to whether cell aggregation, as contrasted to mere microbial growth, is of significance is open to question. However, aggregative microorganisms, including the cellular slime molds, are a significant component of the flora and fauna of the soil.

The ecological importance of microbial aggregation appears to lie in its contribution to the microbiota of a habitat. The environment at large selects for organisms that can adequately respond to its whimsical demands and selects against those that cannot. As an alternative state of a microbial population, cell aggregation may add to the viability of a biotic community beleaguered by unpredictable stresses.

B. Fundamental Function and Structure in Biology

Regardless of whether microbial aggregation is of any concern to the experimenter, the brewer, the biochemical engineer, the dentist, the plant pathologist, the sanitary engineer, the agronomist, or the ecologist, the microbes have their own reasons for coming together. They do not socialize for our pleasure or our concern or our consternation. The biological meaning of microbial aggregation clearly goes beyond its contribution to man and his daily needs. Its widespread distribution and the variety of its forms emanate from an intrinsic realization of the irrepressible living plasm. The force that drives cells to come together springs from an eternal design no less profound than man's search for purpose and far more profound than man's search for profit.

Microbial aggregation systems may be grouped, according to their apparent *raison d'être*, into three teleological categories: (1) a prelude to morphogenesis and differentiation, (2) a prelude to sexuality and parasexuality, and (3) a mechanism for survival under trying circumstances. Most of the better studied microbial aggregation systems may be grouped into these three teleological categories. The cellular slime molds may be included in the first category, the many sexual systems in the second, and the filamentous fungi in the third. The categories are not mutually exclusive. Some systems, or possibly most, may embrace all three, for instance, the fission yeast or the myxobacteria. However classified, a microbial aggregation system is a developmental system, simply because it is an inducible component of the life cycle of an organism.

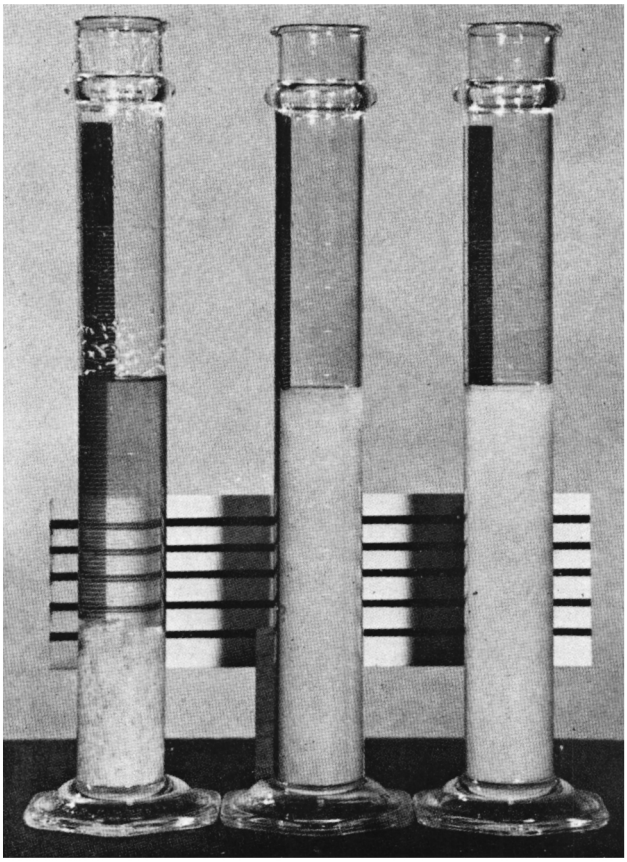

FIGURE 3. Sexual agglutination in *Saccharomyces kluyveri*. The leftmost graduate contains a 3-min-old mixture of complementary mating types. Separate cultures of mating types are shown in the other two graduates. (From Wickerham, L. J., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 35(Suppl. Yeast Symp.), 31, 1969. With permission.)

That aggregate formation is a prerequisite to sex has been shown in many sexual systems. In instances where sex does not obviously follow, one might still argue that some parasexual function is accomplished during the process of aggregation, or that the competence for aggregation remained untouched even as mutation followed by selection had dropped the sexual function.

Sexual agglutination reactions in yeasts tend to become more intense as species evolve from haploid to a higher ploidy.[35,36,38,39] Wickerham surmises that haploid species developing strong agglutination reactions could hardly remain haploid for long. He believes that the evolutionary import of sexual agglutination is its contribution to the increase of ploidy in yeasts.

Microbial aggregation may be viewed as a reaching out by lower forms for the multicellular condition. In some systems, a truly multicellular condition is achieved, in that the aggregate, until now a collection of individuals, now behaves as an integrated organism made up of functionally differentiated cells, a myxobacterial fruiting structure, for instance. Like the proverbial girl who goes to Hollywood or the proverbial boy who goes to Broadway, a population of cells after aggregation is never the same again. The population is embarked on a new program. The events after aggregation normally follow a sequence inimical to

individual cell proliferation. In this sense, the multicellular condition exists, however loose the integration and the coordination of functions might be.

Precisely because it is a structure derived from an erstwhile homogeneous collection of units, an aggregation seemingly defies the universal tendency toward an increase in entropy. It confers upon itself a higher degree of order, at the expense of its immediate environment, of course. Although it might not be bound by a new set of rules, it certainly has more information at its disposal than its components had.

In the filamentous fungi, the aggregation of hyphae leads to the production of a network which serves as nutritional pipelines, as alternate routes for communication and repair, and as an organized system for special functions.[40,41] In times of stress, a bundle of hyphae is better able to cope than a community of nonaggregated hyphae. The aggregate is a microenvironment less hostile and less variable than the larger environment in which it finds itself.

Alternatively, one may look at microbial aggregation as the mere consequence of some other events, instead of attempting to identify its survival value. Microbial aggregation is a surface phenomenon. The cell surface, be it membrane or wall or mural appendage, must undergo some change to allow and promote cell-cell interaction. Whether or not the change is in fact for the purpose of bringing the cells together is another matter. It is sufficient for the sake of argument that the change leads to a fairly stable aggregation structure. It may be solely for the purpose of facilitating genetic transformation, but the change in the structure of the cell surface confers on the cells a greater capacity to stick together.

How come sexual agglutination, not mere pair formation, not even just a *ménage à trois,* but a *mênage formidable*? The teleological doctrinaire will see in the agglutination structure the enhancement of mating efficiency. The enhancement is due to mass action, the result of the increase in collision frequency and in cell population density within the microcosm of the agglutination structure. Or it may be due to the increase in contact time, the result of mass immobilization of cells with respect to one another in a Brownian boiler. Or perhaps, the aggregate creates an ambiance, a microenvironment conducive to the mating game. The hedonist has a different explanation. He sees in the orgy a Maxwellian demon of sorts in the role of Eros, or better still, as group therapist whipping up the cell population into a frenzy. Passion uncontrolled precipitates into an exaggerated reaction: an agglutination. In yeast, the reaction is a veritable bacchanalia in ethanol. The mechano-materialist views sexual agglutination in a puritanic light. It is no more than a superficial change (in the literal sense) with more than superficial consequence (now both literally and figuratively): sexual agglutination is the consequence of a structural and functional change on the cell surface.

Whatever its reason for being, a motive force or a mere consequence, a microbial aggregate is a society of cells. The advantages that accrue to social organization may be thought of as applicable even to a society of cells. What cannot be attained by many individuals severally may be attained by the synergistic effort of a union of many individuals. It is the Marxist paradox: in uniting, individuals have nothing to lose but those that bind them, their common weaknesses as individuals. The union may be a means to a particular function, but it may be for no other reason than pure and naked survival.

C. Microbial Aggregation Systems as Models

Microbial aggregation systems make good models for cell aggregation of higher forms, for just the reasons that make microbes the organisms of choice for working out more complicated biological problems. Microbes are simpler, easier to grow, easier to handle. They require very little bench space. They have very short generation times. As a consequence, it is possible to work with a very large sample of cells. Mutations are easier to come by, genetic manipulations convenient, but more important, what is true for *E. coli*, according to Monod's act of faith, is even truer for the elephants.

Because of the fundamental nature of microbial aggregation and because of the diversity of its many guises, there is a microbe for everybody's needs. There will always be a microbe

from which to learn the rules of sexuality, differentiation, morphogenesis, and development of higher organisms, including man. Fertilization in higher gametes, for example, has its counterparts in the conjugation systems of the algae, the protozoa, the yeasts, the filamentous fungi, and the bacteria. The problems of embryogenesis may be simulated in the cellular slime molds. One sees in the sexual agglutination of *Hansenula* the designs of an antigen-antibody reaction, an enzyme-substrate complex. In many microbial aggregation systems may be seen the patterns for cellular adhesion, sorting-out of cells, intercellular communication and recognition, the generation and reception of molecular signals, cell fusion, the emergence of cells into tissues. As a spin-off, several aggregation systems may serve as back-up systems for the study of genetic expression, induction of protein synthesis, catabolite repression, metabolic regulation, infection, mitochondrial development, allosteric interaction, karyogamy, meiosis, gene switching.

Problems of a higher order of complexity may be worked out by dissecting the more simple. It is in the best tradition of microbiology, that of pointing the way to seeing the simple in the complex, the unity in the diversity. For this reason alone, if for no other, the study of microbial aggregation may contribute to the clarification of the conundrums not only of cell aggregation in higher plants and animals but also of human society. Who knows, the study of microbial aggregation for its own sake may ultimately lead to the proper study of mankind.

Chapter 3

IDENTIFYING THE PROBLEMS: THEORETICAL AND EXPERIMENTAL APPROACHES

I. GROSS PHENOMENOLOGY

To get started on the experimental study of microbial aggregation, one has to simply observe an organism that exhibits the phenomenon or to look for it in an organism in which it has not been observed before, or, as in certain cases, to stumble upon it while looking for other things. Any of the three approaches pertains, because the phenomenon is grossly observable, at least in most of the systems.

A number of microbial aggregation systems are old phenomena. The aggregative nature of Acrasiales was known to Brefeld[42] in 1869 and to van Tieghem[43] in 1880, and that of the myxobacteria to Thaxter[44] in 1892. Algal aggregation was described as early as 1881 by Berthold.[45] Pasteur reported yeast flocculation in 1876 in his classical studies on beer.[46] At the unfolding of this century, the literature on yeast flocculation had begun to accumulate.[47-54] Mycologists of the last century, Brefeld,[55] de Bary,[56] and Reinhardt[57] among them, were well aware of hyphal aggregation in filamentous fungi. Although the impetus to its study came after the discovery of mating types in *Paramecium* by Sonneborn[58] in 1937, conjugation in ciliates was known to Bütschli[59] in 1876 and to Maupas[60] and Hertwig[61] in 1880. The rest of the better studied systems were identified fairly recently.

Some microbial aggregation systems are so obvious there is no question as to their being observable at all. A good example is the sexual agglutination of the yeast *Hansenula wingei* (Figure 1), but even such an impressive reaction had to await demonstration because the complementary mating types had to be discovered at the same time.[62] The reaction is observable only upon mixing of mating types, even as the mating types are ascertained experimentally by the agglutination reaction: like types being unable to bring it about, complementary types being able to do so. The resulting diplont is normally not agglutinative.

Wickerham's discovery in 1954 of sexual agglutination in *Hansenula* species prepared the way for the discovery of sexual agglutination reactions in other yeasts. Very vigorous reactions have been shown for *Saccharomyces kluyveri* (Figure 3, Chapter 2), *Citeromyces matritensis* (Figure 2), and its haploid form *Torulopsis globosa*,[35,36,63] but until recently, only these four genera were represented in a short list of sexual agglutination systems in yeasts.

So long as the fission yeast *Schizosaccharomyces pombe* was grown on agar, its ability to flocculate remained unreported, although the mating types had been known for some time.[64] Homothallic strains grown in malt-extract broth flocculate during stationary phase.[65] Flocculation is sex directed, being a prerequisite to conjugation.[66] The heterothallic strains, unlike those of *Hansenula*, do not flocculate immediately when mixed together. They require at least a round of cell division together.[67] Sex-directed flocculation is found also in other species of fission yeast.[68]

Aggregation in other sexual systems came to be recognized with hesitation. The demonstration of aggregate formation during the mating reactions in *Blepharisma*[32] and in *Euplotes*[33] has already been mentioned in the previous chapter. Sexual agglutination in *Saccharomyces cerevisiae* was noticed[69] only many years after the identification of mating types.[70] Cells of **a** and **α** mating types agglutinate when mixed under appropriate conditions, but this reaction had been regarded as not so worthy of study as the pheromonal control of the mating phenomenon.[71,72] Still other systems had to be proved with difficulty. The formation of mating aggregates during conjugation in *E. coli* is now convincingly demonstrated,[28] but

the hard evidence came long after the initial demonstration of bacterial sex.[73] The occurrence of clumps during bacterial conjugation had been known in the literature but only as a minor inconvenience to be avoided or ignored by those who aspired to the rewards of molecular biology.

Other than in the well known or in the very obvious, where does one look for microbial aggregation? How does one go about looking for it, what is there to look for, and how does one know when he has found it? Because agglutination and sex tend to go together, one might look for aggregation in conjugation. One suspects that a good many sexual systems are indeed aggregation systems if only the right conditions are found.

In systems where mating types are concerned, the demonstration of agglutination calls for the mixing of cultures of complementary mating types. Where the complementary mating types are descended from a clone, as in homothallic strains of yeasts, the mating types are already present in one culture and it becomes a matter of inducing the culture to agglutinate under appropriate conditions. Potential aggregation systems might have been missed because the right conditions for induction or the opportune time when the cells are ready for mixing has not been found. Quite often, because it is a developmental system, cell aggregation is found at the end of or outside the cell cycle. In liquid culture, it is often found during stationary phase. It may be grossly visible in the form of clumps or flocs of cells. The primary flocs may coalesce to form even larger flocs. They may separate from the uninduced cells, fall out of suspension, and settle to the bottom of the culture vessel (Figures 1 and 2).

One has to show that aggregation is not merely pairing but the formation of an aggregate of many cells. Where it is glaringly a multicomponent association of cells, one has still to show that the aggregated structure is not the consequence of failure of cells to physically separate after cell division and that there are fairly stable contacts among them. Indeed, where aggregation is suspected, one has to show that it is not excluded by the definition and that it meets the criteria for inclusion among microbial aggregation systems.

One can screen for agglutinative strains of yeasts by exploiting sex and the events that occur after aggregation. An example is the heat-treatment method worked out by Wickerham and Burton.[74] Yeast spores are slightly more heat resistant than vegetative cells. A sporulated culture then may be subjected to high temperatures, say 58°C, and the survivors plated. Presuming that spores are the products of cell complementation of mating types, one may test the agglutinative properties of spore clones when mixed together in various combinations. The agglutinative strains of *Hansenula* were found this way. In this connection, the alcohol-treatment method of Leupold for the recovery of fission yeast spores is also worthy of mention.[75] Indeed, spores are a good starting material for the isolation of mating types.

Gross observations may reveal right away whether an aggregation is obviously heterotypic or not. If it is not obviously so, it is far from easy to prove it is homotypic. Initial observations may yield valuable information, such as when aggregation occurs with respect to the life cycle of the organism or with respect to the stages of growth of a cell population, cultural conditions of its occurrence, hydrodynamic properties of the aggregates, certain environmental factors, including temperature, ion concentration, various chemical effectors, etc. The primary objective of a gross phenomenological study is simply to demonstrate the phenomenon in such a way as to allow others, if they so desire, to demonstrate it to themselves with reliability and to their satisfaction.

II. QUANTITATION OF MICROBIAL AGGREGATION

Far too many reports concerning microbial aggregation have relied upon subjective judgment by eye. Yet the description of any phenomenon must eventually become quantitative,