Environmental Control of Microbial Growth and Morphogenesis

Walter J. Nickerson, Ph.D.[1]

I have no mycobacteria to talk about, only obscure fungi. In that connection I shall speak on two points. The first is the question how you would recognize *M. leprae* if you did succeed in culturing it. I shall show pictures of fungi distorted experimentally; you would have to know what was done to them to recognize that it is the same fungus. Dimorphic fungi are common as both plant and animal pathogens; dimorphic protozoa are also common. Obligate plant pathogens that cause plant diseases from which the causal organism has never been cultured, are common. This, however, does not "bother" the plant pathologist. The medical microbiologist, on the other hand, has only a few microorganisms that have resisted him, and he wants to conquer them.

In the Dutch elm disease, the causal organism has been called by several names, including *Ceratostomella ulmi*. It is in the yeast form in its host, the tree, but it is a filamentous fungus in culture. I suppose one could say that the Dutch elm organism has never been cultured! Indeed, it has resisted all efforts to make it go back to the yeast form in the laboratory. Many of the dimorphic fungi that cause disease in man or animals are yeasts in the host and filamentous fungi in the laboratory if grown below 37°C. The causal organism of blastomycosis is such a fungus. It has no special nutritional requirement for growth as a yeast or as a filamentous fungus. The nutrient requirements are the same for both forms and are exceedingly simple. It requires a temperature, however, of approximately 37°C to grow as a budding yeast. Now what part does temperature play, e.g., the difference between 34°C, where it grows as a filamentous fungus, and 36°-37°C, where it will grow as a yeast? A great deal of work has gone into this question, but we are almost as much in the dark as when the phenomenon was discovered many years ago.

I shall show you a few bacteria and several fungi that have dimorphic growth habits. And I want to inquire about some of the culture conditions that are responsible for growth of these organisms in one or another form. Our work over the past 15 years or more has been concerned with studies on biochemical bases of morphogenesis. We have been trying to elucidate biochemical mediation of environmental control of form-development. We start with an organism we know is dimorphic, and try to find nutritional control, or some en-

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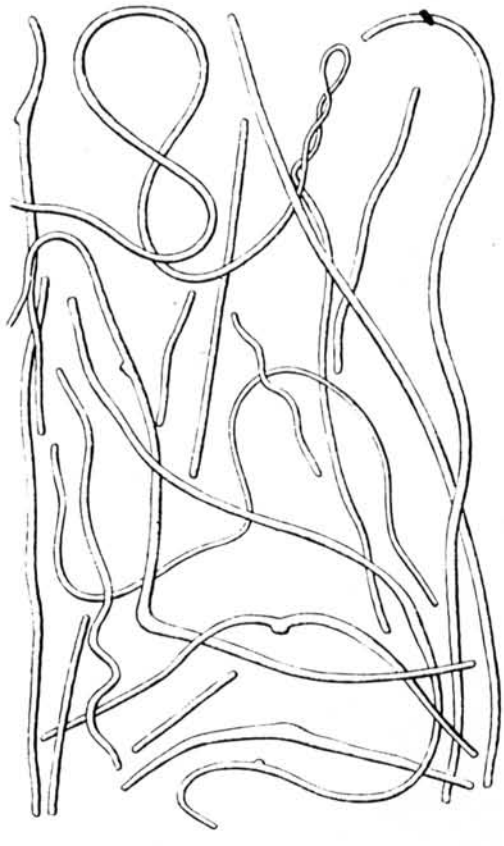

FIG. 1. *Bacterium aceti* grown for 24 hours at 40°-40.5°C; filamentous form; ×1,000. From Hansen (4).

vironmental control that will permit it to have only one or the other form. Then we try to analyze what this nutritional control means. For example, we may find that one form develops with methionine and another with some other source of nitrogen. We try to learn if methionine is working as a nitrogen source or as a methyl-donor. We try to trace methionine through various metabolic pathways into structure that may be responsible for the difference in shape. Hence the question—how would you recognize *M. leprae* if you did culture it?

Now I do have one thing in common with this program. The first figure is from the work of Hansen. But it turns out that this was a different Hansen! Emil Christian Hansen (4) grew *Acetobacter*, the vinegar bacterium, not at 25-37°C (for the incubator overheated), but at 40.5°C. The next morning he found the long snakes shown in Figure 1. The population was all filamentous, and he thought he had a weird contaminant. But he repeated the experiment and within a couple of hours the normal single cell-Gram-negative rod had become a chain of long cells that continued to grow, but not to divide. If these filaments were put at 37°C, however, they divided very rapidly. Thus he appeared to have a nice tool with which to study the division mechanism. Hansen pointed out that if one obtained this result, i.e., filamentous growth, it would mean a different order of bacteria; it could not be an *Acetobacter*.

Figure 2 has to do with an old classic problem, the phenomenon of pleomorphism and the question of contamination. At the top is the filamentous form of *Mucor rouxii*. At the bottom is the same organism, grown under a high tension of CO_2. We spent several years working on the biochemistry of this transformation (1, 2) and can only skim over it at the moment. If the yeast form were found in tissue, and the filamentous form were obtained in culture, or *vice versa*, I think you would believe you had a contaminant. The mere absence of oxygen is insufficient to make this organism grow as a budding yeast. It requires approximately 0.3 of an atmosphere of CO_2 in the absence of oxygen. Labeled CO_2 goes into one particular component of the cell wall. The appearance of colonies grown under high CO_2 tension is shown in Figure 3, and the appearance under oxygen also, or under nitrogen for that matter, i.e., nitrogen in the absence of CO_2. These microcolonies were studied in some detail. Figure 4 shows isolated colonies, about 100 cells in each. We grew 50 plates like this and then returned the plates to air; the figure shows that from every one of the yeast cells filamentous growth developed. There was no question of selection from a mixed population; every cell possessed the phenotypic competence to develop in either form.

Ultrathin sections of the two forms are shown in Figure 5. The outer wall is similar in both cases, i.e., similar in thickness, but inside the outer wall in the yeast form is a gigantic layer about nine times thicker than the outer wall—a new element that appears to cause the yeast-like shape. The inner layer of the yeast cell wall is com-

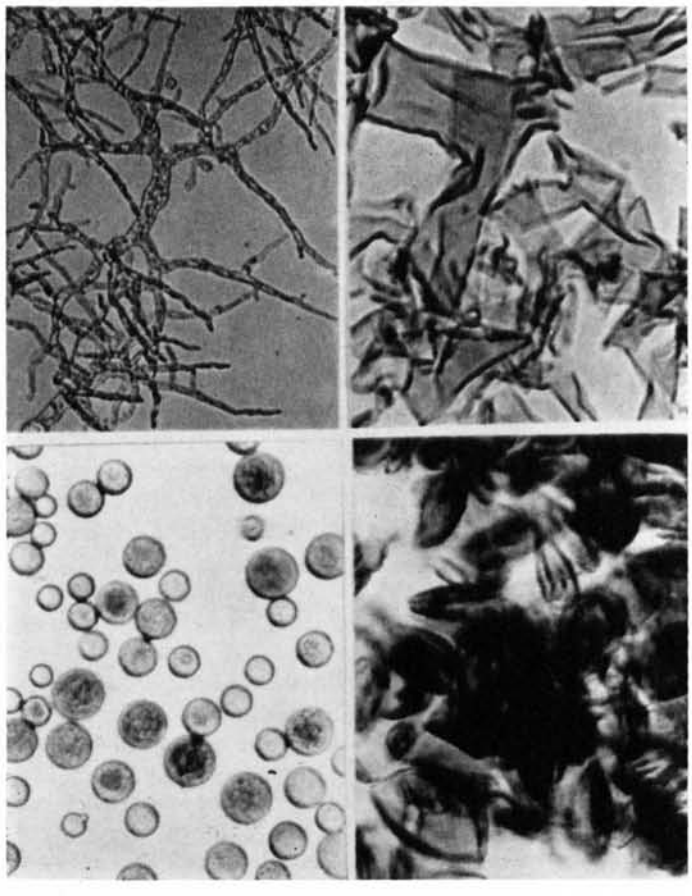

FIG. 2. Whole cells and isolated cell walls of *Mucor rouxii*. Top left, filamentous cells, grown under air; right, filamentous cell walls; lower left, yeast-like cells, grown under CO_2; right yeast cell walls. From Bartnicki-Garcia and Nickerson (2).

prised principally of mannan-protein, which is characteristic of cell walls of many yeasts. We have isolated cell walls from a variety of yeasts and have found mannan-protein to be rich in acidic amino acids. The malic enzyme has its activity increased in parallel with increased CO_2 tension up to a level of about 0.3 of an atmosphere. This enzyme carboxylates pyruvate and leads to the formation of aspartic acid. The trace of mannan-protein present in the wall of the filamentous cell is enough of a primer. I keep insisting that this is environmental control of an operation that is already in existence.

Figure 6 shows another fungus, a yeast (*Trigonopsis variabilis*) that can grow in either a triangular form or in an ellipsoidal form; cultures usually show a mixture of these two cell types. We thought it would be fortunate if we could grow the ellipsoidal form alone, and the triangular form alone, and see what modification of the cell wall could result in this peculiar change in shape. In studying the amino acid nutrition we observed that if methionine were supplied as the nitrogen source, growth was exclusively triangular after an incubation period of 72 hours ([5,6]). Methionine proved to function as a methyl-donor; we could devise systems without methionine, but with other methyl-donors that were equally effective. Methyl-labeled methionine was found to wind up almost exclusively in the cell wall of this organism, which turned out to be rich in phospholipid.

Figure 7 shows still another yeast-like fungus; this is the black yeast, *Aureobasidium* (*Pullularia*) *pullulans*. The organism can be grown in a yeast-like form, or it can be induced to grow almost exclusively as a filamentous fungus. If we feed nitrate or glutamic acid as a source of nitrogen, a crop of yeast cells is obtained; if we feed ammonia or glutamine a crop of filamentous cells results ([3]).

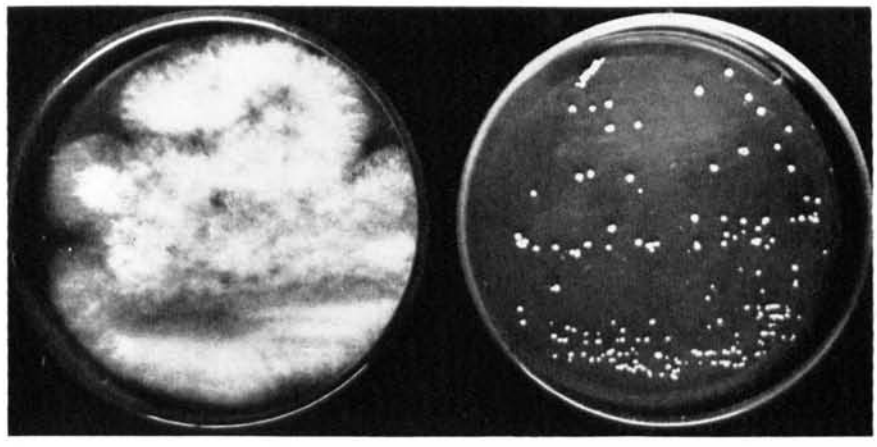

FIG. 3. Growth of *Mucor rouxii* on agar medium with incubation for 24 hours under air (left); under CO_2 for 72 hours (right). From Bartnicki-Garcia and Nickerson (1).

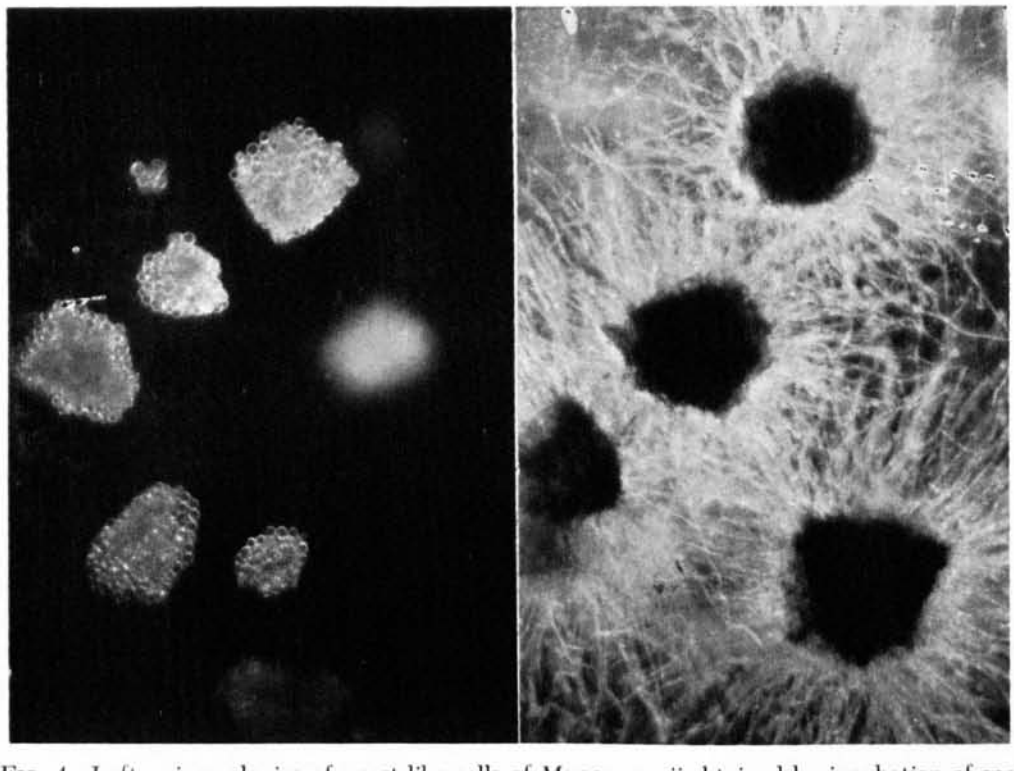

FIG. 4. Left, microcolonies of yeast-like cells of *Mucor rouxii* obtained by incubation of spores under CO_2 for 24 hours. Right, microcolonies as shown at left after exposure to air for 8 hours. From Bartnicki-Garcia and Nickerson (1).

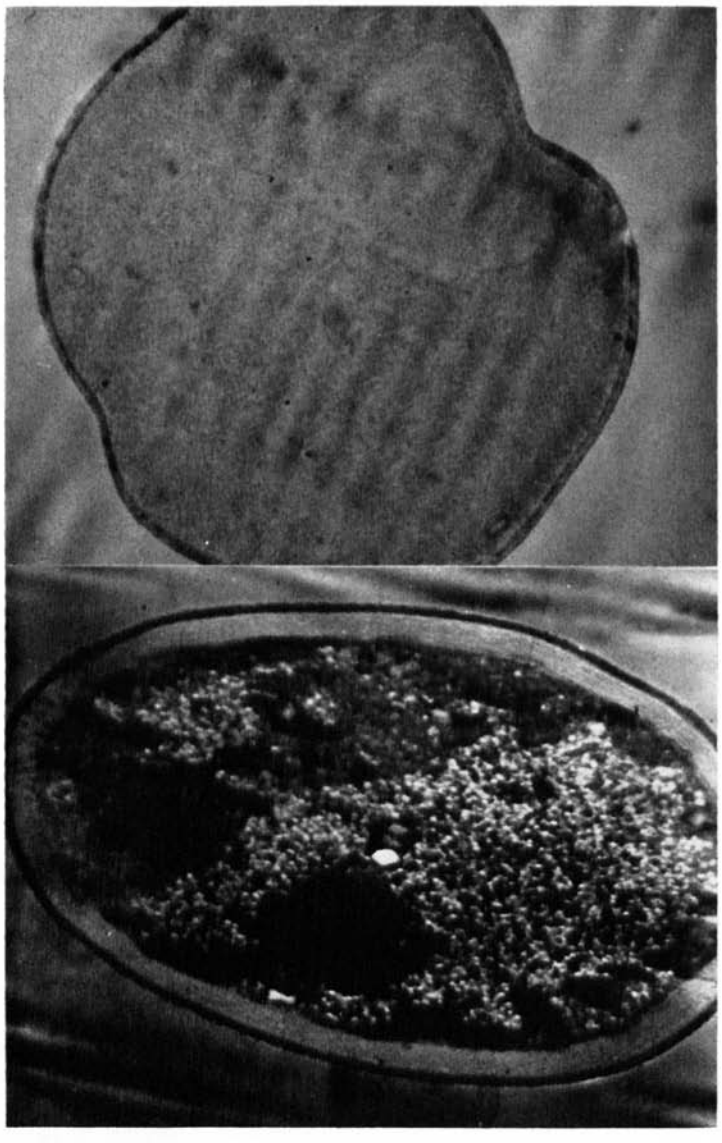

FIG. 5. Electron micrographs of ultrathin sections of filamentous form (top) and of yeast-like form (bottom) of *Mucor rouxii*. From Bartnicki-Garcia and Nickerson (2).

My introduction to the study of morphogenesis in fungi was through the pathogenic yeast-like fungus *Candida albicans*. A wild type strain and a filamentous mutant derived from the wild type are shown in Figure 8. The mutant grows as well as the parent, giving the same dry weight, and you will notice that the wild type is characterized by the production of filaments at the free borders of growth. There is never any filamentation between the parallel streaks. Explanation of the difference between this mutant and the wild type involved many years of work. No difference was found between the cell walls in chemical composition or by electron microscopy, and yet one consisted of tubular filaments, while the other was a budding yeast. The difference we finally distinguished was a change in the composition of the wall at the point of budding. The filamentous mutant was not able to bring about the localized weakening of the wall that is required for budding (6).

These are just some of the organisms we have examined in our study of environmental control of the morphologic development, and in our attempt to elucidate biochemical mediation of the phenomena. We have examined temperature, CO_2 tension,

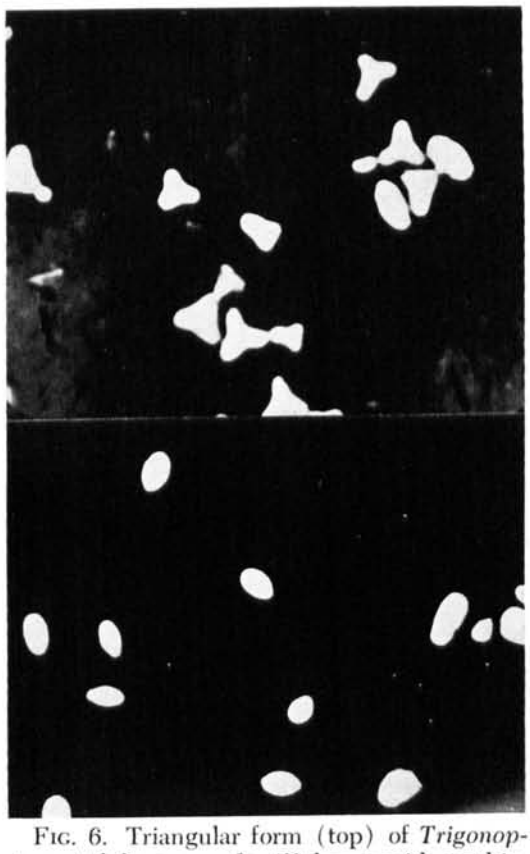

FIG. 6. Triangular form (top) of *Trigonopsis variabilis* grown for 72 hours with methionine as source of nitrogen; ellipsoidal form (bottom) grown with ammonium nitrogen. From Sentheshanmuganathan and Nickerson (8).

and nutritional variables in the course of this work. I know nothing about what has been done in attempts to grow the leprosy organism. I suppose that negative results are hardly ever published. I imagine everybody is waiting for a positive result to be published. I do not know if anyone ever tried lowered oxygen tension. Now you know that in shake flasks or in the test tube you have no control over oxygen tension. This is possible only with fairly large scale cultivation. In a 5 liter flask we can control oxygen tension to any point specified. Also we can control pH. Now you know perfectly well that you cannot control pH in a test tube, or in a shake flask. This is possible only in large scale equipment. But I suppose that if one could not induce the organism to grow in a test tube, he would not dare inoculate a 5 liter flask, where you do have environmental control. It is conceivable that this organism needs a CO_2 tension of perhaps 1 per cent, not 0.03 per cent, as in air. Try to get 1 per cent CO_2 tension in anything but large scale equipment. In the same way, if you want lower oxygen tension, not 20 per cent, as in air, but 14 per cent, for example, it will be difficult. And if you want pH control, not brought about through buffers, this again is a large scale proposal.

In closing I wish to mention one special requirement. There was talk of it earlier. The organism might require something that does not occur naturally. Some years ago we published studies on the role of biotin in multiplication of yeasts. In the course of an attempt to define a replacement for biotin, the only thing we found that would replace it was a synthetic compound known as glycerol monooleate (5). Glycerol monooleate does not occur naturally. We have tried several yeasts; those that required biotin grew very well with glycerol monooleate at a level of about 40 mgm. per liter. This was somewhat higher than the required concentration of biotin, of course, but essentially the same population resulted as with biotin. And the surprising fact is that the organisms grown in glycerol monooleate contain biotin. They make it. Biotin is very easy to extract because it is so resistant. If biotin were not known, I would have discovered a new growth factor for yeast, viz., glycerol monooleate. Again, not knowing what media have been tried for the cultivation of *M. leprae*, I wonder what constituents are put in. Perhaps blood agar has been tried, and probably sugar was autoclaved with the medium. I could not grow *Streptomyces* if I did that. I don't think these contributions will help with *M. leprae*, but that is the end of the story.

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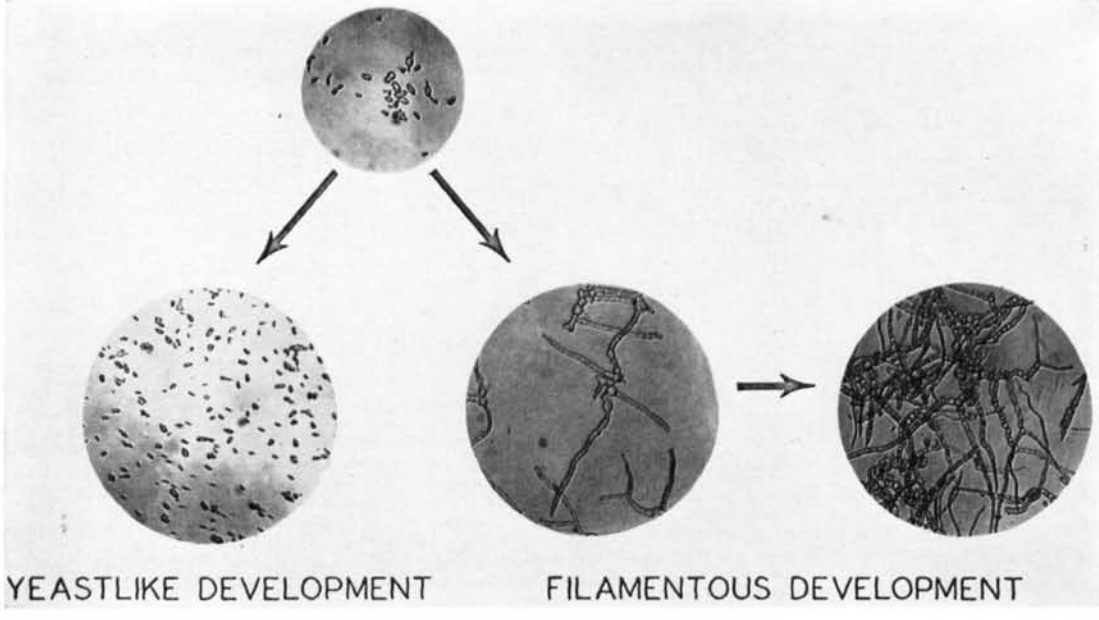

FIG. 7. Development of *Aureobasidium* (*Pullularia*) *pullulans* either in a yeast-like form (when grown on nitrate or glutamate as source of nitrogen) or in a filamentous form (when supplied with ammonium or glutamine as a source of nitrogen). From Brown and Nickerson (previously unpublished)

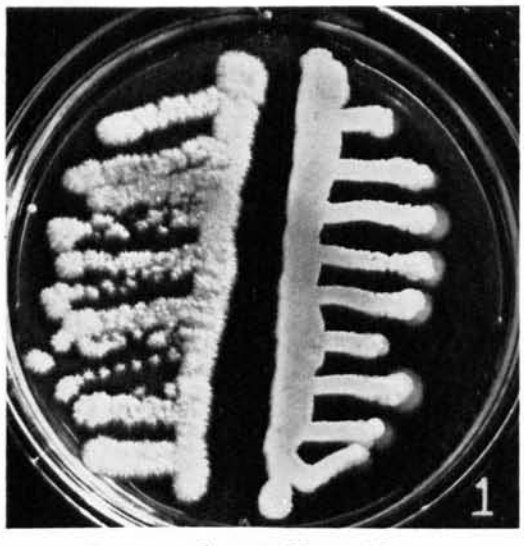

FIG. 8. Normal yeast-like wild type strain of *Candida albicans* (left) and filamentous mutant derived therefrom (right). From Nickerson and Chung[7]

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DISCUSSION

Dr. Emmons. We will proceed now with the discussion of Dr. Nickerson's interesting and stimulating paper. It will be opened by Dr. W. F. Kirchheimer, Chief of the Laboratory Branch of the U. S. Public Health Service Hospital, Carville, Louisiana.

Dr. Kirchheimer. Dr. Nickerson made a very wise choice when he selected for his studies microorganisms that grow and multiply well in simple culture media and which, in addition, readily display morphologic changes. In particular, he has shown that certain phenotypes make their appearance when certain physical and/or chemical factors prevail in the environment. He identified such selective conditions and, in addition, elucidated the relevant chemical changes produced in the cells, which in turn make the observed morphologic changes possible. Compounds providing methyl and sulfhydril groups, and the absence or presence of oxygen and/or increased tension of carbon dioxide, all influence cell-wall composition in some organisms, thus giving free play to the effects of physical forces or to the action of enzymes. Other agents are capable of blocking cell division, but do not interfere with growth in general.

We are assembled here to determine how knowledge gained from the effects of environment on some microorganisms can benefit the seemingly thorny prospects for culturing leprosy bacilli. As far as its culture is concerned, the latter microbe has the distinction not of doing something needing explanation, but of not doing what every living cell must do lest it perish. But there is proof that it has not entirely lost its growth capabilities outside of the body of its natural host. There is multiplication at selected sites of intact animals. In addition, elongation has been observed in cultured human histiocytes.

There is evidence that some of the parameters that Dr. Nickerson found to motivate certain activities of some microbes probably are of great importance for the leprosy bacillus to assert itself. It can be assumed that growth and multiplication of the Hansen bacillus in its natural habitat depend on suitability of temperature, oxygen and carbon dioxide tensions, and hydrogen ion concentration. Apropos of temperature requirements, *M. leprae* possibly might resemble *Mycobacterium balnei* and *Mycobacterium ulcerans*. Ordinary oxygen tensions probably are unsuitable for growth, multiplication, and survival of an organism adapted to intracellular existence. Increased tension of carbon dioxide seems to promote multiplication of *M. lepraemurium* in monocytes. Elongation in culture media, without multiplication of *M. lepraemurium*, is facilitated by a high hydrogen ion concentration. Furthermore, high hydrogen ion concentration in conjunction with high carbon dioxide concentration, is needed to circumvent the mycobactin requirement of some factor-requiring mycobacteria.

All these observations suggest conditions that might have to be satisfied in attempts at culture, regardless of whether or not we know at this time their *modus operandi*. It is possible that the composition of the cell-structures and surface adherents of *M. leprae* impedes diffusion of nutrilites. This, of course, would account for the low metabolic activity and low rate of multiplication that prevails even in its natural host. Here the question would arise if it might not be possible to facilitate diffusion by means of lipid-soluble carriers. Might we not find enzymatic or other ways to remove interfering surface layers without impairing viability?

Cells, failing to divide, might be viewed as being blocked at some point in their preparation for division. Might we not gain something from the use of prospective "unblocking" agents? Ribonucleic acids, glutathione, gibberellic acid, or active substances that could be present in tissues of persons with lepromatous leprosy and during reactive states of the disease, might be among the candidates.

Dr. Emmons. Thank you, Dr. Kirchheimer. Dr. Nickerson's paper is now open for general discussion.

Dr. Morrison. Dr. Nickerson, I would like to ask about the question of replacement of biotin with glyceryl monooleate in the yeast system. Lichstein has recently shown that biotin replacement by oleate in some bacterial systems is a question of surface-active effects on the initial inoculum. Hence the trace biotin levels of the medium are better utilized. Do you think this is possible in your case?

Dr. Nickerson. The trace levels of biotin in our media were so small that there was no growth in the absence of added biotin or glyceryl monooleate. The inoculum was so small that the amount of biotin contained would not support growth even if it were all liberated. Furthermore, we used for inoculum cells that had been grown on glyceryl monooleate. As I said, they contain biotin, as much as they would if given optimum nutritional levels of that substance. The surface-active effect of glyceryl monooleate is not sufficiently specific to explain the results with the yeast system. Of all the monoesters of oleic acid only the glyceryl ester works. Glyceryl monostearate does not. I might point out that it has been known from the very early days of work with biotin that oleic acid is not suitable as a replacement with yeast. It is suitable with mycobacteria, and claims that oleic acid has supported the growth of yeast in the absence of biotin, really revolved around the turbidity measurements, which were probably spurious. Here we are working with a specific effect in which only one ester of oleate has this activity.

Dr. Middlebrook. With regard to CO_2 and biotin requirements of certain strains of mycobacteria, and the relevance of the problem of temperature for the multiplication of *M. leprae,* I would like to say something that may not be generally known. Those strains of *M. tuberculosis* found in the sputum of some cases which require CO_2 at higher concentrations than .03 or .05 per cent or require added biotin, will grow at 33-35°C without any increased concentration of CO_2 or biotin. They will not grow, however, at 37°C without these additives. This is relevant to the problem of temperature requirements of other mycobacteria, the probable maximum temperature for growth of *M. leprae* being somewhat less than 37°C.

Dr. Ulrich. I would like to know if the sulfhydryl effect on the change of phase could be brought about also by using the disulfide under reduced conditions.

Dr. Nickerson. Dr. Goldman asked me earlier if I wanted to answer this question, and I said no—it is much too long. There are about twenty papers from our laboratory on this problem. The application of external sulfhydryl compounds affecting the form of cells is known from studies of microorganisms, tissue culture, plant root tips—any number of areas—and in general it has been found to promote cellular division. This observation goes back to Rapkine and to Voegtlin in the late 1930's for sure. Early in our work we found that addition of glucose had the same effect as addition of cysteine, to a stock medium. So, being young and naive at the time, I said there must be some metabolic connection between carbohydrate metabolism and disulfide reduction. As it worked out, glutathione reductase was found first. Cystine reductase, which we found, was second. Now both these enzymatic activities are present in the filamentous mutant of *C. albicans.* Therefore, they cannot explain why the addition of external SH still causes the cells to become yeast-like. In Candida there is an enzymatic system that I say plasticizes the cell wall. You know the story; it gets highly involved with the geometry of the cell, etc., and I do not wish to go into it further here.

Dr. Reich. The sole classification criterion that we have for the isolation of *M. leprae* is acid-fastness. It is generally accepted that this characteristic must be adhered to in the isolation of the organism. Now, can you, off-hand, or from any information that you have available, give us any

information of related characteristics in the dimorphic fungi?

Dr. Nickerson. Let me answer by going to the Gram stain, which may have some relevance. All yeasts are Gram-positive. Of course there are many bacteria that are Gram-positive. No chemical similarity is known between yeasts and Gram-positive bacteria. Why yeasts are Gram-positive is not clear. Why is anything acid-fast? I do not know if it is known in complete detail, or if the question is in the stage that the Gram stain is in. There are many theories. There may be ten reasons why anything is Gram-positive and perhaps ten why anything is acid-fast. I would put them on a par. I fear that does not answer your question.

Dr. Ulrich. In relation to the acid-fastness in *Nocardia*, you will find that the degree of acid-fastness is much less than that in mycobacteria, and you will find also that, even though you destain for relatively short periods of time with some strains, there is a tremendous amount of variability among the bacillary forms. For example, you can destain serially, i.e., for 1 minute, 2 minutes, 3 minutes, etc., and even at the very short and the long periods of destaining you may find essentially the same kind of patterning in the percentage of acid-fastness, within a range of course. There is a great deal of variability among individual organisms and in their acid-fastness.

Dr. Reich. Would either Dr. Nickerson or Dr. Ulrich care to make a statement as to whether the mycologist would accept acid-fastness as a classification criterion?

Dr. Nickerson. I do not believe it would be a criterion, because fungi are classified on the same basis as higher plants, on the basis of sexual structure. There is a nice waste-basket called the "fungi imperfecti." If you cannot get them to fruit, you throw them there, and even dermatophytes have been found to have sexual stages now. We have this nice wastebasket; you probably have an equally good one for acid-fast microorganisms.

Dr. Emmons. Some streptomycetes are partially acid-fast. The conidia formed by streptomyces are often acid-fast, and the acid-fastness of *Nocardia* varies notoriously.

Dr. Ulrich. One of the most consistent attributes in the fungi is their inconsistency with respect to any particular characteristic. They have a very broad range of variation, and as a result, in making identifications, you very rarely, if ever, hold to one characteristic in making an identification. You put together all the macroscopic and microscopic characteristics observed and see how they fit a pattern or picture. It is rather dangerous to rely upon just one characteristic as a means of identification.

Dr. Nickerson. During the course of my thesis work I isolated a yeast that had the property of making a lot of acid. It took me about a year to identify it. I could not get it to sporulate and I was reluctant to put it in a nonsporeforming genus. After much work I finally got it contaminated, and the contamination caused it to form spores. Then it was easy to identify. It turned out to be one of the conjugating yeasts, a *Zygosaccharomyces*.

Dr. Hanks. Since the topic of acid-fastness is under discussion, let me offer what is perhaps an oversimplified view. To comprehend the vagaries and the character of acid-fastness, it seems best to recall that many microbes, given adequate nutriment and good circumstance, can synthesize their genetic units and cytoplasm at much higher rates than cell walls. As this aspect of growth tapers off, the relative rates of cell wall formation catch up, possibly because of increases in the net rates of wall synthesis. To apply this to the mycobacteria, we need not theorize that the carbolfuchsin stained complexes are specific for intracellular components or, indeed, for walls. We need only to recognize that with increasingly rugged and impenetrable walls, the trapping of the dyed complexes during decolorization or differentiation becomes more efficient. If we pursue this thought a bit further, it will be evident that

cells grown as spheroplasts, as weak-walled forms, or at very high rates during early logarithmic growth, will be largely or entirely nonacid-fast.

Dr. Goldman. In support of that, Dr. Hanks, we have been growing some of our *M. tuberculosis* under forced aeration conditions in a medium that will give very high yields; it is almost impossible to make an acid-fast stain on these. They are completely atypical in the amount of stain they will retain, whereas the inoculating culture taken from a pellicle is made up of perfectly good acid-fast organisms. I would like to ask a question, which I suspect should be directed to Dr. Hanks. We have been talking about environmental factors and many of us are not familiar with the negative experiments that have been done with *M. leprae*. Would it be at all feasible to have a brief summary of what has been tried with negative results in the cultivation of *M. leprae* in terms of temperature, atmosphere, and carbon-nitrogen sources? Is it possible to have a brief run-down on heaven knows how many man years of work on this topic?

Dr. Hanks. If we start with the discovery of *M. leprae* as being 10 years before the discovery of the tubercle bacillus, I believe it may be said that no decade has passed without some microbiologist being convinced that if he put his hand to it, he could cultivate *M. leprae*. I assure you, therefore, that essentially everything that may be extracted or derived from plants or from animals that walk, crawl, or fly has been incorporated in conventional media for incubation under many circumstances. To epitomize the problem as of a year ago, we may ask a simple question: "Why has *M. leprae* not been grown in cells removed from susceptible persons having lepromatous leprosy?" I believe we shall learn more about this question later during the conference.

In respect to temperature, much work has been done at 32°C as a matter of policy. This temperature may be at the precarious upper limit. As you know, in biology there are many examples of organisms or systems that are competent at a given temperature but require additional amino acids, vitamins or supplements at higher temperatures. Possibly the fairly universal work on *M. leprae* at 32°C has been shortsighted.

As to pH, the work by Morrison and by Wheeler and myself on the mycobacteria-requiring models systems has defined the necessity of autoclaving the Watson-Reid synthetic medium at pH 5.5 and the marked preference of these types for initiating growth at pH 5.5 to 4.5. CO_2 also is important. Drs. Chatterjee and Reich report that these conditions favor limited multiplication of *M. leprae in vitro*.

The importance of oxygen tension is suggested by the fact that the mycobactin-requiring and the noncultivated mycobacteria do not cause pulmonary diseases. This fact and the low cytochrome content of *in vivo*-grown mycobacteria have not yet been taken properly into account. Lest we think that *M. leprae* and *M. lepraemurium* afford the sole examples of noncultivable states in mycobacteria, I may say that there are at least six mycobacterial diseases of nonpulmonary tissues from which the causative agents have not been cultivated.

Dr. Emmons. Dr. Nickerson, do you have anything to say in closing?

Dr. Nickerson. I want to ask Dr. Hanks a question. At Atlantic City we talked about the mode of action of copper oxine as an antifungal agent, and this is a copper chelate. Of course, chelates are one way to get metals into a lipid barrier. Copper oxine is intensely soluble in nonpolar solvents, in chloroform, for example. Anything you do to it to decrease its solubility in lipid solvents abolishes its antifungal and antibacterial activity. Is the mode of action of chelates in the connection you just mentioned known?

Dr. Hanks. The solubilities of the microbially synthesized chelators of iron are consistent with your suggestion; also with their universally presumed role in the transport of heavy metals. Having considered the physical importance of nonpolar chelates of

iron, Dr. Morrison conceived that appropriate chelates of iron and synthetic chemicals might meet the growth factor requirements in the soil microbes that we use for the assay of mycobactin and other sideramines. Perhaps I could call upon him to tell you what kinds of "poisons" serve as growth factors in that system.

Dr. Morrison. Briefly, there are species of factor-dependent arthrobacters that will respond to mycobactin. It is possible to replace mycobactin as a growth factor with oxine and several other synthetic chelators such as acetylacetone, salicylaldehyde, or cupferron. The mode of action of the synthetic metal chelators, as Dr. Nickerson has just mentioned, may be related to the transport of metals into or across a lipid membrane.

Dr. Emmons. Our next speaker is a very versatile microbiologist from the illustrious group of microbiologists at the Mayo Clinic. Dr. John A. Ulrich will speak on the subject of "Observations of fungal growth *in vitro* and *in vivo*." This just about covers the water front but I am sure that Dr. Ulrich's comments will not be as diffuse as his title.