Host-Dependent Microbes

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This dissertation against the injunction of Polonius to Laertes: "Neither a borrower nor a lender be" [Hamlet: Act I, scene iii], is not intended to affront the memory of an inspired author. Shakespeare is exonerated. Possibly he did not consider how little Polonius knew about differences between dependencies in men and in microbes.

INTRODUCTION

The origins, character, and possible cultivability of the host-dependent (i.e., noncultivated) microbes have been challenging topics in the minds of many microbiologists. Although speculations regarding origins cannot be substantiated from existing data, they are fascinating. If questions can be raised within an appropriate framework, there is always the possibility that an imaginative experiment can recapitulate one or more of the evolutionary steps. A second possibility is that discussions concerning the nature of problems in host-dependent microbes may suggest principles which are useful to their cultivation.

My own interest in these apparent impracticalities has been enhanced by the fact that this laboratory adopted the mycobactin-requiring Mycobacterium paratuberculosis as a model from which to extract lessons that might be helpful toward the cultivation of M. leprae. The results obtained with this choice of experimental model have produced a series of intellectual shocks. These include the findings that this supposedly fastidious pathogen is but one of a group of free-
living microbes which require microbially synthesized chelators of heavy metals; that the specific growth factor, mycobactin, and other catalysts or substrates are used by bacilli growing within mammalian cells as readily as by those growing in a synthetic medium; that the presumed original physiological requirements of this species have remained so fixed that it has not adapted to major features of host environment, in spite of prolonged passage in cows and sheep; that its major genetic markers are counterparts of those in well-known physiological groups of free-living microbes; and that the major imprint of host adaptation is the sluggishness in saturating in vitro environments with cofactors and metabolites which it synthesizes too slowly from the simplest compounds.

These observations raise several novel questions. One of these questions is whether factor-requiring (i.e., microbe-dependent) microbes, after adaptation to plants or animals, could masquerade as host-dependent, while in fact having dependencies upon the original donors of strictly microbial growth factors and upon physicochemical features and the more universally used nutrients of the host in which the microbe has been discovered. It will be seen that seclusion within hosts or host cells does not isolate host-dependent microbes from microbially synthesized factors. Another question is whether the speciation of host-dependent microbes may have been highly developed prior to their adaptation to plants or animals and whether, given a basis for intelligent search, counterparts of their progenitors might be found in nature even today.

Irrespective of theories, the microbiologist who becomes distrustful of host-oriented concepts will be struck by four straightforward propositions. The notable specializations which distinguish microbes from other forms of life concern membranes which synthesize cell walls. The symptoms common to a broad series of host-dependent microbes are delayed or deficient cell wall formation. Plants and animals are not engaged in this line of business. Whether for the supplementation of membrane systems or wall-forming systems, competent microbes, not hosts, constitute the logical source of specialized cofactors which may be needed by host-dependent microbes, both in vivo and in vitro.

*The Common Feast*

If one wishes to comprehend the many-faceted attributes of host-dependent microbes, it is legitimate to parasitize other investigators, particularly author-dependent authors who have prepared thoughtful reviews. They have repaid at least a portion of our universally intellectual debts by citing highlights and providing references to large areas of pertinent work.

Trager (87) has presented illuminating views of the symbiotic, parasitic, and pathogenic dependencies in an exceptionally broad range of biological forms, including the host-dependent microbes which are indispensable to insects that depend upon microbes. For a general survey of host dependency, or challenging model systems and problems, Trager is an invaluable guide. Moulder (60, 61) has prepared two penetrating analyses of the rickettsiae and the psittacine bacteria. The qualities in these predators are set forth by considering their energetics, their enzymatic deficiencies and "leakiness" as related to adaptation to animal hosts, and the character of their growth and infectiousness as related to cell wall formation. One of the notable by-products was the rescue of these organisms from the humiliation of being mistaken for viruses.

Evidence which suggests that host-dependent states may have existed in many microbes prior to the development of plants and animals will be found in articles cited in Barnett's (3) review of mycoparasitism. Before venturing into this field, the medical microbiologist must resolve not to be dismayed or befuddled by names and terminology. Lest the diet be too rich for many, I have prepared a digest of these matters (see Bedtime Story).

In the present review, several topics will be surveyed to provide background for eventual arguments, speculations or conclusions. In general, the procedure will be to develop the relationships between a pathogen and its physiological counterparts in soil and to determine why such a microbe has appeared to be one of the most fastidious of the microorganisms yet cultivated. Certain of the properties and problems in host-dependent microbes can then be reviewed within new frameworks.

*Microbe-Dependent Microbes*

One step in attaining perspective regarding host-dependent microbes is to recognize that dependent states exist in free-living organisms. This section is concerned with classical examples of dependency upon strictly microbial growth factors which do not have vitaminlike activity for other forms of life. Requirements for L-lysine or wall-forming precursors that are used or supplied by plant and animal hosts would not qualify under this definition. Experimentally induced microbe-dependent states are numerous. They include the streptomycin dependencies in a series of species (see 22), the diaminopimelic acid de-
ficiency in *Escherichia coli* strain W 173-25 (4), and requirements for turbulentostearic acid in tubercle bacilli (49). Curiously enough, the first discovery of a biological growth factor also identified a microbe-dependent microbe (88).

**Chelate-Dependent Microbes**

It is interesting that a natural dependency on microbially synthesized chelators of heavy metals is as critical to several species of free-living bacteria and fungi as to a presumed strongly host-adapted mycobacterial pathogen. The implications of this relationship have not been appreciated for several reasons. First, the growth factor from competent mycobacteria, discovered in 1913 by Twort and Ingram (88) and now known as mycobactin (28), was long thought to be significant only for the fastidious and little-studied *M. paratuberculosis*. Second, the interest in functionally related chelators has centered on studies on antibiotics and soil microbiology. The result has been that all such chelators have been considered “special” rather than “universal” microbial growth factors. The known examples of chelate-dependent microbe have been discovered largely by accident. As will be seen, the deliberate inclusion of microbial chelators in surveys of free-living and host-associated microbes should disclose a variety of additional forms exhibiting this single type of dependency.

In recent years it has been shown that naturally occurring microbial chelators which contain three N-substituted hydroxamic acid groupings (R₁-CO-NOH-R₂) (52) are essential to the growth of microbes. Evidence of the widespread importance of such chelators came simultaneously from three laboratories. Lochhead, Burton, and Theoton (57) demonstrated that the iron-chelating “terregens factor” is produced by growth-competent species of *Arthrobacter* and is required by *A. flavescens* and *A. terregens*. Hesseltine et al. (44) showed that coprogen is produced by a species of *Penicillium* and is necessary for the growth of the dungen-dependent fungus, *Pilobolus kleinii* (45). Neilands (63) isolated ferrichrome from the smut fungus, *Ustilago sphaerogena* and found that it supported the growth of *A. terregens* and *P. kleinii* (64).

New concepts regarding the importance of microbially synthesized iron chelators and their relationships to antibiotics originated from the work at the Federal Institute of Technology in Zurich and the Ciba Research Laboratories in Basle. As a result of these investigations (6, 7), the siderochromes were divided into two major groups: (i) the sideramines, which function as growth factors, and (ii) the sideromycins, which were considered to have antibiotic activity because of being antagonists of the sideramines. A third minor group was proposed to include chelators, such as ferrichrome A, which do not function biologically. Thirteen sideramines have been isolated from *Streptomyces* species and from fungi. The nine sideramines from *Streptomyces* species are closely related. The four from fungi differ and have been called ferrichrysins, ferricrocin, ferrirhodin, and ferrirubin (50, 51).

Although structurally the mycobactin from *M. phlei* is a dihydroxamate, it functions as a hexadentate chelator because of a phenolic OH group and the N atom of the oxazine ring (81). It serves as a growth factor for all the sideramine requiring species. Because of its physical and functional equivalence to sideramines, both Snow (82) and Morrison, Antoine, and Dewbrey (59) have considered it to be a sideramine.

The heterotrophic character of sideramine requirements in free-living dependent species is demonstrated by the readiness with which sideramines, including mycobactin, can be exchanged between diverse species (17, 18, 24, 43, 44, 45, 64, 70, 98). The one example of apparent specificity is the failure of the mycobacterial pathogens to respond to the water-soluble sideramines.

The survival of chelate requiring microbes in nature apparently is due to the universal production of sideramines by growth-competent species, whether they be aerobic or facultative. Hesseltine et al. (45) recovered “coprogen type” stimulators from 10 of 32 species of yeasts, fungi, streptomycetes, and bacteria. Burnham and Neilands (17) demonstrated bound hydroxylamines in the cells of 20 of 25 representative species and stimulation of *A. flavescens* JG-9 by extracts from 37 of 50 species. Failures to demonstrate the production of active chelators by 100% of the growth-competent species probably was due to adequate levels of iron in many of the media employed for the cultivation of such a broad spectrum of microbes. Zahnert et al. (98) found that limitation of iron accentuates the production of large quantities of desferrisideramines by all the streptomycetes and fungi studied and concluded that sideramine-type compounds are produced by all aerobic organisms. Antoine, Morrison, and Hanks (1) observed that iron limitation was fundamental to the production of mycobactin by two synthesizing species. Burnham (personal communication) has reached similar conclusions.

The role of the sideramines in the transport of iron and their insertion into respiratory catalysts has been under active study by Burnham (15, 16). The alterations of membrane activities and of
respiratory pathways during iron restriction and mycobactin production are being investigated by Morrison and associates in this laboratory.

In view of the fact that nearly 40 years were required to establish relationships between a growth factor for a fastidious pathogen and a general mode of microbial dependency, it is likely that yet additional examples of chelate requirers await discovery.

Within the genus *Bacillus* an example has recently come to light. This involves the response of *B. megaterium* to schizokinen, which has been recently characterized as a sideramine (66). Schizokinen promotes the growth of the *Arthrobacter* JG-9 strain (Lankford, personal communication). Similar effects on *A. terrengens* and *A. flavescens* have been confirmed by Morrison in this laboratory.

**Circumvention of mycobactin requirements.** In spite of the many billions of sideramine-requiring cells from four genera of microbes which have been planted on a variety of media in the absence of sideramines, there are no reports of the emergence of competent mutants.

Insight into the circumvention of mycobactin requirements (58) arose through Morrison's desire to examine the differences between strain Mj68 of *M. paratuberculosis*, which strictly requires mycobactin, and two mycobactin-"independent" strains (Teps and III-V) which had been grown for 33 years on the Watson-Reid (WR) synthetic medium. The findings regarding the development and the causes of "independence" were quite unexpected. Although pellicles, i.e., organelles, of Mj68 required 1 μg of mycobactin per ml to make a successful transition from complex media to the WR medium, subsequent transfers demonstrated that they had become "independent" during the first transfer.

Further investigation of the three strains showed that the "independent" growth does not alter the response to submicrogram concentrations of mycobactin. Even in low iron media growth is not associated with the production of detectable mycobactin. The initiation of growth depends in part upon low pH and in part upon complexes which are formed during the autoclaving of the glucose-containing medium at pH 5.5. Autoclaving of key ingredients of the medium results in the formation of compound(s) which show characteristic absorption maxima at 230 and at 290 μm (Fig. 1).

At this point, the evolution of knowledge regarding the "hosts" for a fastidious pathogen had developed through three stages, the successive requirements being: cows or sheep, mycobactin,

![Absorption spectrum produced by 1% glucose in 0.015 M phosphate after autoclaving at pH 5.5 for 15 min at 121 °C (Morrison, unpublished data).](image)
and, if pellicles were to be used, the autoclaving of an appropriate medium.

Curiously enough, pellicles grown in the absence of mycobactin have become our standard sources of physiologically active, but mycobactin-requiring, cells. Although the transfer of strain Mj68 from complex, mycobactin-containing media to the WR medium may involve the selection of clones adapted to the new physical environment, it will be shown later that declumped, diluted suspensions of all three "independent" strains exhibit a strict requirement for mycobactin and that the minor adaptations have not selected cells with adequate capacity to saturate environments with cofactors and metabolites.

The circumventions described illustrate two basic points regarding the character of M. paratuberculosis. In the first place, aside from the mycobactin requirement, this "fastidious" pathogen has retained the capacity for a chemosynthetic existence. Second, the original genetic deficiencies which are related to mycobactin have persisted in strains Teps and III-V during some 33 years of growth in the absence of mycobactin. This stability of requirement is typical of sideramine-requiring microbes from soil. It seems pointless to argue that intracellular environments selected this property while having no similar influence on bovine-type tubercle bacilli.

Replacement of chelate requirements. In the case of the heterotrophic sideramine requireurs, hemin has been found to produce growth stimulations, provided it is used in narrow ranges of concentration. Burton, Sowden, and Lochhead (18) reported the growth of A. terrengens in the presence of heme at 0.1 μg/ml but not at concentrations one log higher or lower. Burnham and Neilands (17) reported a 50% maximal response by A. flavescens JG-9 to 0.08 μg/ml. Demain and Hendlin (24) reported that Microbacterium lactium responds to hemin below concentrations of 50 μg/ml.

Subsequently, Morrison conceived that growth stimulation by sideramines might not depend upon special structures but upon the formation of relatively nonpolar metal chelates. Experiments with A. terrengens and A. flavescens demonstrated that the naturally occurring iron chelators can be replaced by synthetic bidentate chelators, such as 8-hydroxyquinoline, salicylaldehyde, and acetylacetone (59). At appropriate concentrations the chemical chelators produce typical dose-response curves. They are toxic in higher concentrations. With respect to the narrow range of useful concentrations they are reminiscent of hemin. In the choice of biologically active chemical chelators, two properties appear essential: the compound must chelate ferric ions and the resulting chelate must be lipophilic.

In respect to dependent microbes in general, it now is evident that presumed specific physiological requirements can be met in several ways: by microbial growth factors from related or unrelated species, by empirical discovery of media or conditions which tend to circumvent the requirement, or by selecting suitable compounds from a chemical catalogue.

**Other Microbial Dependencies**

The frequent, often quantitative, occurrence of unanalyzed dependencies is illustrated by the following. The catalogue of the American Type Culture Collection (ATCC) notes that the growth rates of B. subtilis 12695 and E. coli 12696 are increased 10 times by a growth factor from two strains of Aspergillus. A personal communication from Mrs. Daley of the ATCC states that the B. subtilis strain was isolated from the intestines of 12-day-old chicks which had been fed on mash enriched with the Aspergillus growth factor. Anaerobes seem not to have been tested for the production or requirement of sideramines. Nevertheless, an example of critical requirements for unknown microbial factors in anaerobes is found in the report of Hardy, Lee, and Nell (42) on the isolation of new types of oral spirochetes. These appeared as satellite colonies near contaminants on a plating medium which had been developed as optimal for the known types of spirochetes. The new types have since been propagated by means of unknown factors in culture filtrates from a microaerophilic diphtheroid. It will be noted that these oral spirochetes could not have acquired their apparent host dependencies because of prolonged enclosure within host tissues or cells. Although they depend upon the human host for warmth, moisture, and certain nutrients, they evidently rely upon adjacent competent microbes to meet certain additional requirements.

**Utilization of Microbial Growth Factors During Intracellular Growth**

Before inquiring whether host-adapted microbes could rely upon plants or animals to convey microbial growth factors to intracellular agents of disease, the critical question of the possible impenetrability of host cell membranes must be considered.

The intermediate position of M. paratuberculosis and the wood pigeon mycobacteria in the spectrum of mycobacterial species and the combination of microbe dependency with host adaptation enabled Wheeler and Hanks (93) to explore
two problems. First, it seemed desirable to explain the fact that the intracellular growth of mycobacteria had been proportional to their capacities for growth in vitro. The growth-competent pathogens and tubercle bacilli were known to grow readily in the three major cell types under a variety of conditions. M. paratuberculosis and related types had not been studied. M. leprae-murium grew very slowly (19, 69, 90). M. lepra had not yet been propagated in vitro. Second, in the case of the noncultivated mycobacteria, there is the question whether the host cells or extraneous compounds are the likely sources of special factors which might be useful during intracellular growth.

The primary issue has been resolved (93). When securely phagocytized within cells which have been washed free from external bacteria and then surrounded with streptomycin to prevent extracellular growth, M. paratuberculosis and the wood pigeon mycobacteria do not depend solely upon the metabolites or constituents of the host cells. External supplies of factors which promote the growth of declumped bacilli in synthetic media stimulate the intracellular growth of these microbes just as effectively as though they were in test tubes. The factors studied were mycobactin, iron, CO\textsubscript{2}, and glycerol. Mycobactin could not be shown to modify the appearance or overall metabolism of the host cells. The dramatic influence of external supplies of mycobactin on intracellular growth represents a new phenomenon, the intraphagosomal availability of strictly microbial growth factors. Elberg's (27) review contains equally clear evidence that externally supplied macromolecules, such as lysozyme and antibody globulins, exert their typical effects on securely phagocytized brucellae. Finally, Chang (18) and Garbutt (30) have reported the slow in vitro growth of M. leprae in tissue cells. Both groups emphasize that multiplication does not depend upon items which are required to maintain healthy cells, but upon unknown factors in serum or other components of the medium.

Taken together, this series of observations revolutionizes earlier views regarding the role of tissue cells as hosts for intracellular agents. Whether one is interested in nutrition or inhibition, it is evident that the situation for intraphagosomal microbes is not fundamentally different from that of the extracellular types, that the different biochemical environments in various tissues can be important determinants of intracellular growth, and that genetically controlled levels of metabolites or inhibitors in different individuals can have a marked influence on susceptibility or resistance.

**Plants and Animals as Conveyors of Microbial Growth Factors**

There is no novelty in the fact that the very existence of plants and animals depends upon chemical transactions and vitamin syntheses by microbes. Since the uptake and distribution of strictly microbial growth factors within plants and animals has not been examined experimentally, the evidence in favor of this possibility must be circumstantial. The considerations fall into two categories: the diversity of compounds normally taken in through rootlets and the gut, and the question whether compounds of the same order of size but unrelated to host economy could be excluded.

The sap which is piped upward in plants contains minerals, nitrates, vitamins, etc., and will nourish other plants. The sap flowing downward fails to do so. To be brief, White (94) has concluded that the sap which flows upward in plants is "in most important regards a good soil solution." However, for want of more precise information on what can be excluded, we shall leave open the question whether plants could convey strictly microbial factors to the noncultivated microbes which they support.

In the case of animals the probabilities are high. To secure an adequate intake of vitamins, proteins, lipids, etc., animals are not content with consuming all manner of natural products. They have wrapped their hollow bodies around a digestive tube which houses an astonishing variety of microbes that engage in chemical transactions and syntheses. The ruminants have elaborated on this arrangement by installing way-stations for highly diversified fermentations. While absorbing the long list of items essential to host economy, animals are known to take up dyes, drugs, antibiotics, etc., which are unrelated to host economy. As shown by many studies on atopic sensitization (26, 56), even adults absorb low proportions of the antigenic proteins which they ingest.

In view of the total evidence, it seems likely that animals could provide small but continuous supplies of metabolites, membrane supplements, or cell wall precursors which are useful solely to apparently host-dependent microbes.

**Imprints of Genetic Character and of Host Adaptation in Chelate-Requiring Pathogens**

*A. terregens* has been used as the "*E. coli*" for studies on sideramine requirements. Similarly, the
once noncultivable *M. paratuberculosis* and the wood pigeon mycobacteria have been employed as models for examining the ways in which host adaptation may have increased the fastidiousness of the incompetent mycobacteria. The choice of the chelate requirers as models for the host-dependent species rests upon the following considerations. (i) For 17 years the cultivation of *M. paratuberculosis* from lesions in the intestinal walls of cows and sheep had been impossible. There was a lag of 5 years in learning that the mycobacteria in wild wood pigeons can be cultivated by incorporating killed mycobacteria in diagnostic media (see 93). Were it not for the original inspiration of Twort and Ingram (88), both types might remain uncultivated even today. (ii) As in the case of other chelate requirers, the major deficiency has not been circumvented by rare mutations to competency or by extracts, complexes, and compounds from plants and animals (28, 80, 88). (iii) If deprived of mycobactin, the resultant noncultivable cells, at least in some respects, should be counterparts of those from noncultivated species. (iv) They are the most fastidious mycobacteria which can be studied by laboratory disciplines such as nutrition, metabolism, cell culture, etc.

Efforts to evaluate the peculiarities in chelate-requiring mycobacteria thus far have been restricted to the physiology of growth. Wheeler and Hanks (93) used suspensions of declumped cells from three strains of *M. paratuberculosis* and two of wood pigeon bacilli to study parallelisms between growth in vitro and within host cells. The sources of these strains, a description of *M. paratuberculosis*, and the formula of the modified WR medium have been given by Morrison (58). The wood pigeon mycobacteria have been characterized by Wheeler and Hanks (93). The preparation of bacterial suspensions for inocula, methods for measuring growth, the sources of mycobactin, and general specifications regarding experimental conditions have been described (93). The growth requirements defined to date are those for cell suspensions diluted only five times below the threshold of visible growth, not those for diagnostic inocula.

**Major Peculiarities**

In general, the pertinent findings in the presence of mycobactin have been as follows. Growth at neutral pH, whenever obtained, was poor. At any pH, growth is readily inhibited by complex nutrients from plants or animals. Growth occurs most readily in simplified media at pH 5.5-4.5. The NH₄⁺ ion is an optimal source of nitrogen. Dilute peptones stimulate the onset of growth without increasing cell yields. The lag prior to the onset of growth has been eliminated by mycobacterial metabolites, but not by the host components tested. These six points will be considered in some detail, because they become the basis for a series of arguments, speculations, and conclusions.

**Inhibitions at neutral pH and by complex nutrients.** After the classical fashion of the medical microbiologist, both Reich and Hanks explored their assumptions that the growth of *M. paratuberculosis* MJ68 could be improved by combining mycobactin with complex supplements or host components at the pH values (6.5 to 7.2) which are conventional for other mycobacteria. The overall results were: more inhibitions than stimulations, irregular or unsatisfactory growths, and failures to produce cell crops which were suitable for physiological studies.

Hanks found that strain MJ68 differed from tubercle bacilli in fundamental respects. Even in the presence of mycobactin at 1 μg/ml, it failed to grow in a synthetic medium containing red blood cells at 2.5% (v/v), a medium known to initiate growth from minimal numbers of saprophytes and the three types of tubercle bacilli (38). It grew slowly and irregularly in the synthetic medium plus 10% human ascitic fluid fortified with 0.3% purified serum albumin, a supplement known to be superior to serum for other mycobacteria. In the absence of the ascitic fluid, poor growth occurred in the synthetic base containing 0.3% Trypticase (BBL). Supplementation with unknown factors in 0.15% Phytone (BBL) increased growth four times. Complete inhibition occurred with 1% Trypticase. This inhibition was not relieved by the ascitic fluid or by any of six separate additives. It was overcome only during prolonged incubations in the presence of ascitic fluid plus 20 μg of mycobactin per ml or by decreasing the oxygen tensions. Irrespective of nutrients, slow growth and poor yields of cells indicated that growth was not proportional to the nutrients provided and suggested that the favorable combinations were merely counteracting inhibitions.

In more detailed studies, Hanks and Wheeler employed all five strains of chelate requirers in the WR medium with various supplements and at pH values of 4.5, 5.0, 5.5, 6.5, and 7.5. No strain behaved as though adapted to grow in the presence of the pH values and the complex nutrients which occur in the cytoplasmic compartment of host cells. For example, diluted inocula of strain WP9, which grows the most readily of all strains and is the least subject to inhibition, grew in the WR medium without added myco-
bactin. Nevertheless, it has a strict requirement for mycobactin on Loewenstein-Jensen and other complex media. Omission of the L-asparagine and the diethyl glutamic acid from the 8a medium of Reich and Hanks (70) doubled its rates of growth.

Preference for simplified media and low pH. Aside from the requirements for mycobactin and low pH, the five strains possess synthetic capacities which, though slow, are the equivalent of those in tubercle bacilli. By adding fumarate to the WR medium, Morrison has shown that they obtain their total nitrogen from the NH₄ ion as readily as from L-asparagine.

The apparently fixed requirements for simple nutrients and for low pH were of special interest to Wheeler and Hanks during their studies on factors which promote intracellular growth of the chelate-requiring mycobacteria (93). In further efforts to define the role of pH, it was first confirmed that autoclaving the WR medium at pH 6.5 and 7.5 was disadvantageous, even when such media were adjusted to pH 5.5 before being inoculated. The experiments which are most instructive for present purposes were conducted in lots of the WR medium which had been autoclaved at pH 5.5 to standardize their content of "autoclave factors," and then adjusted to provide a series of pH values: 7.5, 6.5, 5.5, and 5.0 or 4.5.

All five strains initiated growth most successfully in media adjusted to the pH range of 5.5 to 4.5. Results with strain MJIII-V are cited as an example, since this strain had been grown on the WR medium without mycobactin for 30 years. After 10 weeks (mycobactin, 1 μg/ml), cell yields at pH 6.5 were 12 and 25% of those at pH 5.5 and 5.0. At pH 7.5 no growth appeared. Strain MJ68 multiplied at pH 4.5 as readily as at 5.5. Its poor responses at pH 6.5 and 7.5 will be illustrated later. The most competent strain, WP9, was the only one which grew with or without mycobactin and in all segments of such experiments within 10 weeks. Data obtained with this strain (Fig. 2) probably illustrate a pattern which would emerge with larger inocula or with pellicles of the other four strains. Since strain WP9 was an exception because of its ability to initiate growth at pH 7.5 (even without mycobactin), its distinct preference for pH 5.5 over 6.5 and 7.5 emphasizes the importance of low pH for the growth of such organisms.

Response to peptones. The initiation of growth and rates of growth of all five strains were stimulated by tolerated levels (0.2%) of a peptone (Trypticase-Soy) from combined animal and plant sources. The peptone, however, did not increase cell crops. Strain WP9, the only strain which attained full growth within 10 weeks, has been used to illustrate the acceleration of growth without increase in cell crops (see Fig. 2).

Although the more fastidious strain MJ68 began to grow in full-strength WR medium at pH 5.5 within 6 weeks (see Fig. 3), this strain exhibited osmotic sensitivity when the medium was used at 50% of the usual concentration. In the diluted medium, a combination of low pH, mycobactin, and 0.2% peptone was required. During the development of the growths shown in Fig. 3, traces of growth occurred at pH 5.5 and later at pH 6.5 in the absence of peptone. When it was suspected that these growths might not succeed, microscopic examinations revealed swollen cells and rods with central or terminal enlargements. These observations suggest that the dilute peptones may provide unknown factors that facilitate cell wall formation.

When inhibitions by conventional concentrations of peptones are coupled with osmotic protection by dilute peptones, two types of explanations seem to be required. First, since peptones facilitate the synthesis of N and deoxyribonucleic acid (DNA) in growth-competent mycobacteria (85), they may contribute to certain biosynthetic pathways without exerting corresponding effects upon the chelate-dependent systems. Second, dilution of peptones may prevent this state of imbalance and at the same time provide critically useful precursors for rate-limiting steps. As an example, Work (96) has demonstrated that the dianminopimelic acid in bacteriological peptones is derived from microbial contamination. It will be shown below that only microbial derivatives

![Figure 2. Effect of pH and of 0.2% peptone on the growth of strain WP9 in the absence of mycobactin (Wheeler, unpublished data). Solid lines: WR medium at 50% of the original concentration. Three lots were autoclaved at pH 5.5, then one was adjusted to pH 6.5 and one to pH 7.5. Broken lines: as above, but supplemented with 0.2% Trypticase-Soy peptone (TS).](http://mmbr.asm.org/)

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have been found capable of eliminating the lag periods in the chelate-requiring mycobacteria.

Having gained experience in certain conditions which are optimal for the chelate-requiring mycobacteria, the question of utilization of host components was resurveyed in collaboration with D. A. Power. The WR medium was employed at optimal pH (5.0), with asparagine replaced by the NH₄ ion plus malate or fumarate. Inhibitions again were demonstrated when the concentrations of Trypticase or Phytone were increased from 0.2 to 0.6% and also when sera which stimulate tubercle bacilli were added at 20% (v/v). When organic acids and Tween 80 were present in the NH₄-type WR medium, the addition of L-asparagine proved to be inhibitory. It seems apparent that the stimulation of growth has not been obtained by the application of host-oriented concepts, and that new approaches are in order.

Elimination of lag. *A. terreusens*, the chelate re-quirer from soil, is regarded as having multifactorial requirements, since it can achieve practical rates of growth only when supplemented with vitamins or yeast extract in addition to sideramines (18). If judged by the criterion of growth without excessive lag period, the chelate-requiring mycobacteria also have multifactorial requirements. The usual statements regarding slow growth are inaccurate. When data are plotted as in Fig. 2 (97), it is evident that essentially one-half of the experimental periods have been due to lag prior to the onset of growth, and that subsequently growth proceeds at reasonable rates.

The long periods of lag indicate a sluggishness in saturating media and cells with essential cofactors and metabolites. The ease with which these impediments can be relieved has been demonstrated in two different ways: by “lend-lease” of factors synthesized by more competent mycobacteria and by “do-it-yourself” through the use of impounded cofactors. As shown in Fig. 4, the lags in onset of growth have been eliminated and rates of growth improved by adding to the WR medium equal volumes of autoclaved supernatant fluids in which *M. phlei* had grown and also by merely adding 20% gelatin to the WR medium. Both methods reduced lag periods by approximately 5 weeks.
The absence of lag periods within tissue cells and the stimulating effects of CO$_2$ have been documented elsewhere (93). Highly viscous solutions of gelatin enclose the bacilli within a multitude of artificial acidic vacuoles, perhaps producing situations which are analogous to those in the phagocytic vacuoles of tissue cells. The tests in a viscid menstrum of gelatin were suggested by the work of Necas (62) and Landman and Halle (54) on the regeneration of complete yeasts and bacteria from protoplasts and L forms. The latter investigators suggested, inter alia, that mass conversions of these labile forms to complete rods might be due in part to the retention of wall precursors in close proximity to cell surfaces.

**Discussion**

Bearing in mind that the foregoing information has been obtained from factor-requiring models, not from host-dependent species, analysis of the following sequence of points may assist in clarifying prevailing views regarding the origins of host-dependent microbes in general. (i) The factor-requiring mycobacteria are adapted to grow within the phagocytic vacuoles, but not in the cytoplasm of host cells or in body fluids. This disadvantageous property could not have been selected or imposed by prolonged existence within host cells or body fluids. (ii) This and other properties in no way resemble those of tubercle bacilli and brucellae, which may have passed through animal hosts for an equivalent period of time. (iii) The interesting peculiarities are simply counterparts of those found in well-known groups of nonpathogenic microbes. (iv) The imprint of host adaptation is manifest by sluggishness in saturating environments with cofactors.

**Adaptation to phagocytic vacuoles.** Discussions on intracellular parasitism frequently are meaningless because of failures to relate the growth requirements of infectious agents to the fact that animal cells provide two very different physicochemical environments: that within phagocytic vacuoles and that within cytoplasm.

The evidence that the chelate-requiring mycobacteria differ decisively from tubercle bacilli and brucellae and that their genetically determined qualities should prevent the initiation of growth within the cytoplasmic compartment of host cells seems adequate. The internal milieu of cytoplasm has an overall pH of 7.2 and is rich in particulates, enzymes, proteins, nucleoproteins, and the trade goods of intermediary metabolism. At corresponding pH values in vitro mycobactin is always required. When growth occurs, it is poor and readily inhibited by complex nutrients derived from cells or body fluids.

Phagocytic vacuoles, formed by invaginations of cell membranes, are compartments providing lipids, organic acids, protein hydrolysates, etc. As judged by color indicators (71) and the pH optima of the hydrolytic enzymes (46, 94), the pH ranges from 4.5 to 6.0. Within these phagosomal compartments, the pH, the nutritional background, and the impounding of bacterial cofactors should promote the growth of chelate-requiring mycobacteria.

**Selective pressures.** The significant point regarding a fixed adaptation to phagocytic vacuoles is that this quality could not have been favored or selected by prolonged existence within animal cells or by intermittent exposures to body fluids. An analysis of the well-known events in the natural history of such infections will demonstrate that "intracellular" existence could favor the heterotrophic properties observed in tubercle bacilli and brucellae, but not the properties found in chelate-requiring mycobacteria.

In the first place, whether because of toxicity or sensitization, the phagosomal membranes which enclose infectious agents are susceptible to lysis. The resultant exposures to cell cytoplasm would be acceptable to tubercle bacilli and brucellae, but unfavorable to chelate-requiring mycobacteria. Second, the intracellular pathogens cannot find a continuous haven within tissue cells. Even the least toxic of the mycobacteria, rat and human leprosy bacilli, damage tissue cells (35). During the interludes before the mycobacteria can be ingested by a new host cell, exposures to the unfavorable pH of 7.4 and to the complexity and the inhibitions of body fluids are inevitable. Inability to adapt to these universal features of host environment cannot be attributed to prolonged existence within animal hosts.

Under this point, known properties in two noncultivated mycobacteria may be included. Metabolic and other studies by Hanks and Gray have shown that serum and body fluids at their normal pH cause a rapid disruption of the metabolism (33, 39) and of the infectiousness (34) of *M. lepraemurium*. Although there are no similar data on *M. leprae*, it will be seen that its limited multiplication in bacteriological media occurs at the low pH values and under conditions suitable for the chelate-requiring mycobacteria. Thus, the evidence from both the chelate requirers and the noncultivated species indicates that their major peculiarities have been determined, not by host cells and body fluids, but by original genetic straitjackets.

**Imprints of host adaptation.** The foregoing facts and conclusions do not oppose classical evidence that microbes become modified during their
adaptation to plants and animals. Even with inocula which need to multiply only five times to yield visible growth, the mycobacterial models are inept in saturating in vitro environments with cofactors. The relief of these impediments solely by bacterial derivatives suggests that the multifactorial deficiencies in these types involve systems which are unique to microbes and unrelated to host economy.

Evolutionary Implications of Genetic Markers

Aside from the imprints of host adaptation, the major peculiarities in chelate-requiring mycobacteria may best be interpreted as an interesting combination of properties of free-living and non-pathogenic microbes. The requirement for mycobactin is an authentic counterpart of the sideramine requirements in free-living microbes. Requirements for low pH are common in non-pathogenic yeasts and fungi and mandatory in *Ferrobacillus ferrooxidans* (78), where pH values as low as 3.0 assist in the uptake of large amounts of iron. Lichstein (55) has suggested that low pH facilitates the penetration of dicarboxylic acids into the propioni bacteria. Organic acids have been shown to be of prime importance in stimulating the onset and smoothness of growth of saprophytic mycobacteria, a tubercle bacillus, and chelate-requiring mycobacteria (67). Other properties, such as the requirement for an iron-bearing growth factor (in this instance heme), a preference for the NH₄⁺ ion and simplified media, and the stimulation of growth by dilute peptones without increase in cell crop, are classical markers in cultivable ruminocola (13). Furthermore, given a synthetic medium autoclaved at proper pH and either mycobactin or a sufficient inoculum, the complete capacities for chemosynthesis from NH₄⁺, glucose, glycerol, and organic acids has been retained in these supposedly fastidious pathogens as faithfully as in the saprophytic mycobacteria.

In brief, the genetic markers of soil and free-living microbes have not been deleted from factor-requiring mycobacteria by a presumed prolonged intracellular existence. Because of original genetic straitjackets, they have not become "intracellular" parasites at all. Being merely "intraphagosomal" parasites, it seems that the major role of the cells in cows and sheep has been in part to reduce natural hazards and in part similar to that of gelatin, to provide small acidic pockets which have impounded mycobacterial cofactors while permitting the original rates of synthesis to decline.

Given this much insight, and having demonstrated that certain similar properties and problems occur in the noncultivated species, one should be consistent in the pursuit of logic. If one suspects that evolution has not terminated, he should speculate that even today the progenitors of such microbes exist in favorable ecological niches and on occasion give rise to the famous, noncultivated mycobacteria in men, mice, and rats; in water buffalo in the Dutch East Indies; in the green parrots of Mexico, in Pacific salmon, and even in Bolivian bullfrogs (see 36). He also should suggest that those interested in the cultivation of the host-dependent mycobacteria may have been betrayed by having first discovered such types of mycobacteria in animal hosts.

Origins of Host-Dependent Microbes

The lessons learned from competent, requiring, and dependent mycobacteria may not be relevant to all types of host-dependent microbes. It is of interest, therefore, to consider whether host-dependent species can be imposed upon growth-competent pathogens by, or within, their hosts and to examine several alternative possibilities.

Regressions

A theory of regression from growth-competent pathogens to host-dependent microbes, and even to viral particles, fascinated earlier scholars (32, 53). Such views encounter insurmountable obstacles. A major difficulty is the lack of interrelated forms with single, double, or triple deletions of synthetic capacity. Even the family of mycobacteria, which is so large and so diverse that new species and types are being discovered continually, fails to provide the required relationships. No matter how long bovine-type tubercle bacilli and Johne's bacilli have lived side by side in the lymph nodes of the cow, the antigenic and physiological differences are so great that they indicate separate origins. It is simplest to suppose that evolutionary and speciating factors exerted their influence upon the progenitors of these two types before they learned to live in the cow.

The properties of competent pathogens argue against the possibility that they could give rise to dependent types. The classical pathogens have retained competency in spite of prolonged and continuous exposures to the interiors of plants and animals. Although these types undergo profound metabolic (8) and biochemical differentiations (79, 83) in vivo, they defend their capacities to regenerate all systems which are useful in vitro. Selection tends to bring the more competent and virulent clones into predominance. Those with the greatest ability to synthesize cell walls and capsules in vivo have superior capabilities to initiate growth prior to the onset of immune
response and altered physiology in host tissues. They also are most capable of withstanding the hazards of transmission. It matters not if they yield less competent clones. These pathogens presumably have not emerged as separate species of infectious agents because of lying in the wake of forms which kill the more susceptible hosts and excite the defenses of the more resistant individuals. Finally, although immunological mechanisms in animal hosts may impair systems and structures in competent pathogens, such mechanisms could not explain the occurrence of host-dependent microbes in plants.

We conclude, therefore, that growth competency in pathogenic microbes establishes a definite trend against host dependency.

Antiquity of Disease

Uncritical audiences are delighted to learn that host-dependent states arise in microbes because of prolonged existence in plant and animal hosts. But who will demonstrate that the staphylococci, typhoid bacilli, brucellae, and tubercle bacilli are more recent, whereas rickettsia, psittacine bacteria, and leprosy bacilli are more ancient? As students of infectious disease most of us have focused too narrowly on plants and animals. We have not perceived that every class of dependency known in medical microbiology is exhibited in endless variety among the free-living mycoparasitic fungi (3). These dependencies include the necessity for proximity in order to acquire unidentified growth factors, intracellular dependency upon living cells, insidious syplastic growth as wall-deficient forms (transitional L forms), and even the phage-viral trick of extruding genetic units and cytoplasm into new host cells. If it is a question of antiquity, we may assume that many dependent microbes lived among and upon the earliest forms of life for eons prior to the emergence of plants and animals.

Possibilities

Having eliminated the influence of hosts upon growth-competent pathogens and questioned the theory of antiquity, we may examine clues obtained from experimental studies on microbes themselves. The mutagenic effects of ultraviolet light cause specific deletions of competency in vitro and probably in nature. Certain deletions, e.g., the synthesis of diaminopimelic acid and tuberculostearic acids, create microbe-dependent microbes. Whether mutagenic deletions could induce requirements for metal chelates and thus explain a well-known, widespread natural deficiency could be examined experimentally. The question is whether microbes thus impaired could survive in nature by borrowing from adjacent microbes and also have the fortune to find and tolerate the havens offered by plants or animals. During the evolutionary process, it matters little how many millions of experiments fail. There is always the possibility that one form of bad luck can be compensated within an accidentally favorable niche.

Given more time and experience, recent tamperings with evolution by exposing microbes to drugs and antibiotics, both in vitro and in vivo, may yield instructive insights. A succinct summary of the effects of antibiotics on microbial membranes and of resultant problems in cell division, wall formation, and cultivability will be found in Landman's discussion of the psittacine bacteria (see 31).

The likelihood of producing ecologically successful alterations in growth-competent pathogens in vivo seems remote. The complete deletion of competent forms from many individuals leaves the impaired forms confronted with the problem of emerging in experienced populations while the more readily transmitted forms are immunizing all in whom they gain a foothold.

It is known, however, from the observations of Wittler et al. (95) that antibiotics can induce wall-deficient, tissue cell-dependent states in labile and potentially pathogenic strains of diphtheroids. A similar range of sub-bacterial forms and the attendant difficulties in the regeneration of complete cells can be induced in vitro (2, 21).

To summarize, I would offer the view that growth-competent microbes are not easily assembled and that, having circumvented the endless vicissitudes of creation and of becoming pathogenic, they are not likely to be degraded to consistently host-dependent states. The best suppositions appear to be: (i) that innumerable defective creations failed until competent microorganisms became established, (ii) that thenceforth they have been sustained by borrowing and by more intimate dependencies, and (iii) that some of these labile forms [e.g., those with problems in cell wall formation (see Problems Shared by Host-Dependent Microbes)] have benefitted from the favorable environments later afforded by plants and animals. Since such types should kill hosts less readily and alter immune response less dramatically, there might be left open a wider pathway of evolution toward additional dependencies.

Problems Shared by Host-Dependent Microbes

The great variety of problems in energetics, synthesis, cell wall formation, and "leakiness" in the plasmodia, the rickettsiae, and the
psittacine bacteria have been summarized by Moulder (60). Among the difficulties which have prevented the study of noncultivated mycobacteria on a similar basis, the foremost has been a misconception concerning the role of rugged and relatively impenetrable cell walls and of the way in which new cells are generated in vivo. Parallelisms between the growth cycle of the psittacine bacteria, or bovine-type tubercle bacilli, and of *M. leprae* are instructive.

An in vivo dissociation of spheroplast generation from cell wall formation and the role of wall formation in the pathogenesis of disease are clearly illustrated by Moulder's (61) description of insidious onset of disease during the time when rapid replications of protoplasts outstrip cell wall formation, and phenomenal increase in toxicity and transmissability as growth slows down and the replicated units become enclosed within analyzable cell walls. Perhaps the term "transitional L" forms could be used to interpret the rapid replications of genetic units and cytoplasm within flexible and slowly dividing membranes and "complete cells" for the "dense bodies" which possess mature cell walls.

While studying the infection of rabbits with bovine-type tubercle bacilli, Brieger, Fell, and Smith (9) were intrigued by the virtual disappearance of stainable bacilli and the insidious development of a fulminating infection before complete bacilli could be found again. Though spleen homogenates were devoid of stainable or cultivable mycobacteria, they were highly infectious when passaged to guinea pigs. Electron micrographs of sediments obtained by high-speed centrifugation of spleen and lung homogenates revealed an abundance of 5- to 8-μ spheroplasts (10). Brieger and Glauert (11) stated: "Ultrathin sections of tissues from infected rabbits did not contain any structures which could be identified directly as bacilli. However, very dense, irregularly shaped bodies (0.2-0.8 μ) were seen in a number of cells and were clearly distinguishable from the mitochondria and other cell inclusions of normal cells." Cell cultures of spleen fragments explanted during the "eclipse" contained few, if any, demonstrable acid-fast bacilli during the first 6 to 7 days, but then became loaded with mycobacteria at unexplainable rates (9). In 1954, I assisted these workers in demonstrating that during the latent period many cells were already richly loaded with gram-positive nucleoid bodies, both individually and in short rows.

Although *M. leprae* must be studied as recovered from lesions, i.e., without control of growth cycles, its propensity for growth within weak walls resembles that of the psittacine bacteria. Because of the large spherical masses illustrated by Danielsen and Boeck (23) in 1847 and the descriptions used by Hansen (41) from 1868 to 1873, we must conclude that transitional L forms were the first evidence that microbes cause disease in humans. The failure to recognize the significance of L forms in lepromatous leprosy until recently is an interesting example of a celebrated invention causing a total eclipse of learning. It is ironic that Neisser (65) in 1879 and throughout the remainder of his life took such controversial pride in his staining of "Bacillus leprae" by dyes. (The spheroplasts and L forms are not acid-fast, and their delicate membranes are ruptured during the drying of films.) As late as 1910, Unna (89) and others were fascinated by the undiscerned meaning of the unique "globus" form of growth. However, the staining of rugged rods outlined these earlier workers and interesting truths were neglected by microbiologists until 1963, when studies on the cultivation of *M. leprae* fell into the hands of two men with sufficient training in cytology to use wet mounts routinely.

In early 1963, B. R. Chatterjee in this laboratory observed that transitional L forms were proliferating from single rods placed in a spheroplasting medium, and he stated that similar forms were abundant in lepromas. He enlarged upon the significance of such forms with respect to the insidious, then often explosive, onset of lepromatous leprosy and upon possible relationships between wall-deficient forms and latency of the disease. In August, C. V. Reich, formerly of this laboratory, reported from the LWM Laboratory in Cebu, Philippines, that *M. leprae* showed approximately a 10-fold increase in the numbers of globi in vitro. He soon reported that this occurred only when the pus from reaction blisters was placed in cultures of a coccodiptheroid bacillus "X"; that the membrane-bound forms frequently resembled a "bag full of marbles"; and also that the globi, when ruptured, release complete bacilli and round bodies of about 1 μ in size.

In retrospect, these two series of observations clarified the actual implications of Hansen's earliest statements and of isolated observations by others. Denney's (25) "wet-mount" study in 1934 contained descriptions, demonstrations, and micrographs which prove that flexible membranes surround the L forms of *M. leprae* in vivo. Friere (29) observed the development of transitional L forms in vitro, but his attention apparently was diverted to the study of yeastslike contaminants in his material. Micrographs shown by W. M. Meyers in 1963 convinced Chatterjee and me that C. K. Becker, a missionary in the depths of
the Congo, also had seen proliferations of transitional L forms of *M. leprae* in organ-type cultures of lepromata before 1960 (5).

Further work by Chatterjee with water-washed rods has demonstrated that spheroplast inducers, such as penicillin, are neither necessary nor desirable. Low pH, high CO₂, and other conditions useful to *M. paratuberculosis*, and tested thus far, are advantageous. Because of the limited material available and the variety of developing forms, the extent of replication of genetic units and cytoplasm has not been estimated. As measured by the less successful conversions to yugger rods, the net increases in bacilli have risen from approximately 10 to 30 to 40 times the inoculated numbers before growth ceases. This means that operational knowledge has been improved but that the key factors or conditions required for full and transplantable growth have not been found.

A survey of a spectrum of mycobacteria shows that the problem of wall formation becomes more complicated as one progresses from saprophytes to noncultivated species. During maximal rates of growth in synthetic media of approximately 1 iso-osmol and containing 1 to 3% of Tween 80, the saprophytic *M. phlei* produces flexible or swollen cells. In the same medium the attenuated tubercle bacillus RIRv develops an osmotic sensitivity that interrupts growth for some hours (67). In similar circumstance, *M. paratuberculosis* requires 3.5 iso-osmols and grows as spheroplasts. In the absence of Tween 80, *M. paratuberculosis* is handicapped in the WR medium at 1.8 iso-osmols, but thrives at 3.5 iso-osmols. In similar media the limited multiplication of *M. leprae* occurs in the form of spheres and transitional L forms.

Although these observations suggest declining capacities for wall synthesis, there is also evidence that the walls constructed by saprophytes and the tubercle bacilli are less complicated than those on the noncultivated species (37). As judged by impenetrability, the differences are at least one order of magnitude. During incubation in physiological solutions, the intervals required to stain 50% of cells by 0.04 m crystal violet were: for *M. phlei*, 0.15 hr; for BCG, 0.45 hr; for *M. lepraemurium*, 4 hr. The periods required for *M. leprae* from untreated and sulfone-treated patients ranged from more than 2 hr to 0.4 hr, respectively. Differences in the periods required to stain 100% of rods were much greater, 30 min for *M. phlei* and 14 days for *M. lepraemurium*. For some time, the extreme impenetrability of the noncultivated species was regarded as a potential impediment to the transport of nutrients. It is exciting to discover that these impenetrabilities are altered during outbursts of new growth. It now appears that supplementation of wall formation has become the more challenging problem.

The sluggishness of cell wall formation by certain host-dependantt microbes will be recognized as extreme modulations of phenomena which occur during differentiations in yeast and fungi and during the rapid growth of microbial cultures. The vulnerability of young as compared with mature cells was described many years ago (74, 75). This has since been explained by the fact that rates of synthesizing genetic units and cytoplasm exceed the rates of cell wall formation, and by the subsequent gains in wall components while the synthesis of internal components is tapering off (76). In view of the handicaps imposed upon highly competent microbes by cell wall impairments, it may be that one of the major vulnerabilities in consistently intracellular microbes has been recognized.

**Introspections on Circumventing Certain Impediments to In Vitro Study**

The problems in host-dependent microbes are so diverse and in some instances so serious that to discuss their solution would be presumptuous. Nevertheless, I would emphasize that, before proposing to deal with truly deep-seated problems, a first consideration is to protect, activate, and supplement all operable systems. To do so requires attention to four principles.

**Physicochemical environments.** The present investigations illustrate the necessity of relating the definable properties of an infectious agent to the very different conditions which prevail in the phagocytic vacuoles and in the cytoplasm of host cells. Though this compartmentalization is destroyed in sensitive or heavily infected cells, it is likely that one compartment or the other will favor the growth of highly specialized microbes to critical levels. Larger spaces can then be saturated with cofactors that permit more extensive growth.

With regard to intracellular microbes in general, the following considerations are fundamental. Mammalian cells provide physiologically balanced electrolytes, the ratios of major cations being inverse to those in extracellular fluids (77). Internal protein concentrations of 28% increase osmotic pressures by approximately 1 iso-osmole (77). It will be noted that important osmotic effects are produced by amphoteric macromolecules. For 60 years it has been known that sucrose and other nonelectrolytes displace cations from cell surfaces (47). One or two cations and an excess of sucrose are merely crutches that prevent
osmotic rupture and delay cell death. Experiments by Power and Hanks with *M. paratuberculosis* in a synthetic medium providing approximately 3.5 mammalian iso-osmols demonstrated that both spheroplasted forms and whole cells are inhibited when the osmotic pressures of the WR medium were further increased by means of NaCl, KCl, NH₄Cl, or more concentrated medium. Nevertheless, the increased osmotic pressures due to adding 20% gelatin permitted prompt growth. In view of the importance of cations for the generation of complete cells from spheroplasts (54), and the differing ionic exchange capacities of various macromolecules, their influence on osmotic strengths and ratios cannot be ignored.

**Protection of labile systems.** Soft agar mashes impede convection currents, trap CO₂, and establish oxygen gradients suitable for aerobic, microaerophilic, or anaerobic growth. Dense solutions of viscid molecules, such as gelatin, are superior in several respects: greater osmotic pressures, higher impedance of diffusion, and the series of marked stratifications which develop during incubation in containers that prevent evaporation of moisture. This column stability undoubtedly establishes gradient concentrations of oxygen. Such conditions may protect labile microbes, supplements, or supplementing systems which do not tolerate adverse or unstable oxidation-reduction equilibriums in vitro.

**Impounding of metabolites.** A hungry boy, while passing a banana stand, consoled himself with an ancient adage: “The Lord helps those who help themselves.”

Many of the universal shortcomings in host-adapted microbes might be overcome to a remarkable degree by impounding near cell surfaces those cofactors and metabolites which are leaked too rapidly or synthesized too slowly. Saturation of systems and cells with soluble factors is influenced by inoculum size, by rates of synthesis, by the impounding effects of cell walls or host cell membranes, etc. Competent microbes synthesize at high rates and are corsetted within cell walls. Nevertheless, the beginner in microbiology is told that a single microbe can grow on agar, or even better in poured agar or agar mashes, but that it may be lost in even a modest sea of the same composition. Being devoid of cell walls, delicate spirochetes, mycoplasmas, and L forms are propagated in soft agars more readily than in liquids. The cells of higher forms of life are accustomed to environments which entrap cofactors. Though plant cells have rigid cell walls, they are cultivated as tissue cubes. Mammalian cells are habituated to pockets within a gelatinous matrix containing hyaluronic acid and mucopolysaccharides. In vitro such cells require large inocula, diffusion barriers, or metabolic assistance. In primary explantations, lack of the usual metabolites and the original gelatinous matrix is compensated by inocula of 10⁴ or more cells per milliliter of liquid (73). The cloning of single cells from fairly competent cell lines depends upon “feeder layers” of living cells (68) or upon implantation in microdroplets or capillary tubes (73). The student of apparently obligate intracellular microbes will note that what is fair for the host-cell “goose” might be even more important to the intracellular “gander.” The student of phagocytosis will recognize that properly devised impounding systems can be the equivalent of artificial phagocytic vacuoles. It will be evident that the efficient impounding of single microbial cells substitutes for the inoculation of a critical mass of cells, yet avoids the rapid depletion of factors or nutrients which may be present in limited amounts.

Agar gels impede diffusion measurably but inefficiently (54). The earliest discovered demonstration of markedly superior impounding was provided by Necas (62). Employing yeast protoplasts which could not grow in a liquid medium, but which multiplied as L forms in soft agar (about 1% of cells regenerating after 5 days of colony growth), he found that essentially 100% of the protoplasts generated walls during 24 hr of incubation in gelatin, provided the concentrations were in the range of 20 to 40%. This result was not obtained on gelatin surfaces or by adding hydrolysates of 30% gelatin. It depended upon actually enveloping the protoplasts with gelatin solutions which gelled at the incubation temperatures. Landman and Halle (54), employing protoplasts from a nonsporulating strain of *B. subtilis* with strong propensities for regenerating cell walls, showed that 2.5% agar is superior to 0.9%. However, only 5 to 15% of the protoplasts planted on 2.5% agar succeeded in regenerating walls, and then only after the “L” forms had multiplied into masses. After demonstrating that gelatin solutions of 15 to 35% permitted regeneration of walls of 100% of the protoplasts, they cited evidence that the impedance of diffusion in agar is minimal. From this work and a subsequent study by Ryter and Landman (72), it appears that the inefficiency of protoplast growth as L forms is related to the “ballooning” of protoplasts in the usual osmotic environments and to the loss of mesosomal invaginations. It was not determined whether reversions in gelatin depend primarily upon the impounding of wall-forming factors or upon osmotic shrinkage and re-establishment of the
mesosomal crypts that are vital to septum formation and cell division.

In using dense solutions of gelatin to eliminate the 5-week lag periods in the chelate-requiring mycobacteria, Power and Hanks sought merely to impound slowly synthesized cofactors. The benefits were attributed to the viscosity and osmotic effects of high concentrations of gelatin for three reasons: (i) the chelate-requiring mycobacteria are chemoautotrophic, (ii) 20% gelatin was five times more effective than 10% solutions, and (iii) peptone-type fragments of gelatin would be expected to be inhibitory if exceeding 0.2%. As noted earlier, it cannot be assumed that a commercial product such as gelatin is free from wall-forming supplements.

In many instances synthetic macromolecules might provide the necessary critical effects without complicating nutritional backgrounds. The beneficial effects of methyl cellulose in shake cultures of mammalian cells were first attributed to high viscosity. However, dilute solutions of low viscosity grades have since been found to be equally effective (12). Bryant (personal communication) has suggested that adsorption on cell surfaces may be an additional mode of action for such macromolecules.

Even though not tested on an adequate basis, the principles discussed above have been shown to be useful to host-dependent microorganisms. Trager (86) observed that 6% gelatin plus 0.7% bovine serum albumin fraction V improved the maintenance of Plasmodium lophurae. The presence of these two proteins enabled the malarial parasites to utilize contributed adenine triphosphate (ATP) and pyruvate and to develop beyond the merozoite stage.

A practical advantage of dense solutions of macromolecules over agar is that the physiological state of cells can be studied after the cells have been recovered by dilution and washing.

Lend-lease of critical cofactors. Borrowing has been emphasized as a universal expedient among heterotrophs. The remarkable effects of factors other than mycobactin on chelate-requiring mycobacteria have been illustrated in Fig. 4. The rapid formation of satellite colonies of Mycobacterium paratuberculosis near unrelated contaminants on diagnostic media containing autoclaved cells of competent mycobacteria has been described several times (see 84). Similar effects on hitherto host-dependent microbes are exemplified by Reich's discovery that a coccobacillus assists M. leprae in vitro and by the unknown factors in culture filtrates which permit the growth of new types of oral spirochetes (42). It might be wise to explore more widely the principle that competent microorganisms may produce metabolites and precursors which are uniquely useful to noncultivated microorganisms.

The borrowing of supplements for energy-capturing systems is a fascinating and no longer controversial topic. The dictum that phosphorylated intermediates cannot enter whole cells can be demonstrated conveniently, provided one uses competent cells and completes his observations within 30 to 60 min. During the early adjudication of such questions it was not known that membranes in intracellular microbes are "leaky" or "open." Moulder (60, 61) has cited some 16 instances of the usefulness of adenine diphosphate (ADP), ATP, nicotinamide adenine dinucleotide (NAD), acetyl CoA, etc., to rickettsiae, the psittacine bacteria, and malarial parasites. Chatterjee and Williams (21), while studying the various types of incomplete bacteria which arise from the chromatinic bodies of B. megaterium, demonstrated that ATP and yeast extract increase the regeneration of complete cells.

Imponderables. Microbes with limitations in systems which normally are used for independent growth are likely to be extremely susceptible to inhibitors, unbalanced growth, or the accumulation of end products which competent microbes utilize or excrete in harmless forms. The associations between limitations and limitations in growth capacity within the spectrum of mycobacteria have been reviewed by Hanks and Gray (40) and further illustrated in this review. In the case of the chelate-requiring mycobacteria, presumptive evidence of limitations by volatile end products of metabolism has not been analyzed. The lepromatous patient has a distinctive odor, not unlike that of an incubator full of mycobacterial cultures. This indicates both the production and the disposal of volatile end products from M. lepra. The various mechanisms by which plant and animal hosts may oxidize, utilize, excrete, or respire microbial end products are lacking in vitro. Feedback inhibitions may be more inhibitory to host-dependent types than to competent species.

Given conditions or factors which promote competent performance, it may be hoped that certain of these imponderables will become unimportant. If not, replacement of one aspect of host activity may require considerable ingenuity.

SUMMARY AND APPRAISAL

Comprehension of the fascinating problems presented by host-dependent microbes lags far behind existing information regarding the interdependencies between heterotrophic forms of life.
Therefore, it has seemed of interest to test whether specialized knowledge of given groups of host-dependent microbes, if examined in terms of broader precepts, might afford at least rudimentary insight into the origin and character of host-dependent states. The results of this examination have led me to challenge the view that host-dependent microbes have been derived from growth-competent types, and to suggest that they more likely arose from types which were defective prior to their adaptation to plants or animals.

One point of departure is to recognize that a community of symptoms in the intracellular types of host-dependent microbes is related to membranes that yield delayed or defective cell wall formation, and that plant and animal hosts are not engaged in that line of business. It, therefore, may be useful to look to growth-competent microbes for cofactors or supplements which may be lacking in, or supplied to, host-dependent microbes, both in vivo and in vitro.

A further step toward clearer understanding of the origin and character of host-dependent microbes depends upon the fact that loaning and borrowing of strictly microbial growth factors is as natural as the more familiar transactions in factors which are used by all forms of life. Genetically immutable dependencies upon microbial growth factors, such as iron chelators, occur in a series of free-living microorganisms as well as in the most fastidious of the mycobacteria which can be maintained on bacteriological media. Similar requirements for unknown, strictly microbial, factors occur among soil microbes and in delicate oral spirochetes. Some of the ruminal bacteria are cultivable and others are not. Among the fungi that live in soil, or stumps, or any old place, many exhibit dependencies upon strictly microbial factors or upon living fungi. Given proper study, the number of noncultivated microbes in nature could well exceed the number known to students of infectious disease. In brief, although dependent states should be fostered by prolonged existence within plants or within the parenteral compartments of animals, there is little reason to assume that they originated by that mechanism.

The deliberate offering of strictly microbial growth factors and components during surveys of soil, plant rhizospheres, and the miscellaneous nooks and crannies in animals should lead to the discovery of additional microbe-dependent forms. Considering the potential multitude and the manifold potentialities of such microbes, it would be expected that some have learned to live in intimate association with plants and animals. As long as the strictly microbial character of certain deficiencies is not suspected, any such microbe can masquerade as host-dependent while depending upon microbes for special factors and upon the host for those used more universally.

For microorganisms to utilize strictly microbial growth factors while growing in plants and animals seems to present no fundamental problem. For example, the rootlets of plants do not exclude other soil extractives while taking up minerals, nitrates, and vitamins. The digestive tract in animals cannot exclude microbial growth factors while absorbing microbiologically synthesized vitamins and antibiotics and even proteins from milk and egg white. In the case of microbial factors which are not metabolized rapidly by the host, the exclusion of a microbe within mammalian cells presents no impediment to their utilization. The intraphagosomal utilization of external supplies of iron, glycerol, or mycobacterin, a microbial growth factor with a molecular weight of 870, has been clearly demonstrated. Other studies with securely phagocytosed microbes have shown that proteins, such as lysozyme and plasma globulins, are not excluded from this compartment of mammalian cells. Intraphagosomal microbes, therefore, do not depend solely upon the components or systems of animal cells. Aside from the universal benefits of osmotic protection and the impounding of cofactors which are synthesized very slowly, the nutritional situation for intracellular parasites does not differ from that for the extracellular types as fundamentally as has been suspected.

Specific clues regarding the general relationships outlined above have been obtained by comparing chelate-requiring microbes from soil with analogous mycobacterial pathogens, which were noncultivable for some years and have since been regarded as highly fastidious, and by analyzing the conditions which permit the latter to grow in vitro and within mammalian cells. Requirements for pH and simple nutrients demonstrated that these pathogens are adapted to grow in phagocytic vacuoles, but not in body fluids or in the cytoplasmic compartment of tissue cells. This indicates that original genetic straightjackets, though highly disadvantageous, have not been modified by prolonged existence in mammalian cells or by repeated exposures to cytoplasm and body fluids. Furthermore, in spite of selective forces in vivo, the lag periods in growth could not be shortened by means of host components or derivatives from eggs. These caused more inhibitions than stimulations. Meanwhile, aside from the chelate requirement, these pathogens have retained the synthetic capacities.
of saprophytic mycobacteria. In fact, the major markers and requirements proved to be those commonly found in free-living and nonpathogenic microbes.

After attempts to reduce the protracted lag phase in the absence of tissue cells, it was concluded that the only imprint of prolonged exposure to intracellular habitat was sluggishness in saturating environments with cofactors and metabolites. These effects of host adaptation were relieved completely by two principles which are applicable to microbes in general: the impounding of slowly synthesized factors near cell surfaces by means of gelatin, or the lending of factors in the supernatant fluids of growth-competent mycobacteria.

Such observations indicate clearly that "adaptation to an animal host" has played no crucial role in molding the character of these once apparently noncultivable microbes.

The question whether growth-competent pathogens could give rise to host-dependent types within host environments was raised by the striking contrasts between the behavior of the chelate-requiring mycobacteria and tubercle bacilli or brucelle bacilli, both in vivo and in vitro. In the case of growth-competent forms, rapid passages or prolonged infections promote the selection of increasingly virulent clones. Being more readily transmissible, these clones kill off the susceptible and immunize the more resistant members of host populations. Since this process should block the emergence of less competent sublines, it was concluded that competency, once acquired, cannot be reversed. An effort was made to comprehend the possible ways in which fundamental impairments might be induced in competent microbes either before or during their sojourn in hosts. To include both plant and animal pathogens, it was necessary to disregard immunological impairments of cell wall formation in animal hosts. The considerations which seemed pertinent led to the suggestion that, once successful forms of life existed, less perfect creations could have been sustained in favorable ecological niches and later have found havens for further evolution in plants and animals.

Irrespective of theories, universal symptoms in dependent microbes indicate different types and degrees of deficiency in membrane physiology and cell wall formation. During introspections on the further investigation of dependent microbes, primary consideration was given to protective and supplementing measures. The four cornerstones contributed by a variety of physiological investigations were: (i) knowledge of the compartments, electrolyte balances, amphoteric osmotic agents, and components within host cells; (ii) methods for establishing regional gradients of oxygen tension and for the protection of labile microbes and supplements; (iii) development of systems for impounding near cell surfaces the soluble components which sluggish microbes may synthesize too slowly; and (iv) lend-lease of supplements and systems from growth-competent microbes. Nearly all these principles can be applied simultaneously. When this has been done as well as possible, the truly deep-seated problems in synthesis or energetics at last may emerge to be analyzed.

This discourse represents an effort to open wider the shutters and foggy windows of host-oriented concepts regarding the origins and the needs of host-dependent microbes. Many of our prevailing views are inadequate to progressive study of the interesting and delicate organisms which we understand and manage so ineptly. It is my hope that a mutual re-examination of new perspectives may enable us to see this poorly illuminated terrain more clearly.

**Bedtime Story**

*Myriobiologicus* var. *medicus* had been taught to focus on expensive diseases in values hosts. Although he labored mightily, his tunnel vision created the liability of spending a lifetime without noting that the musty odors from the dewy grass, the green-scummed pond, the rusty stumps, and the copse of trees whisper of substances liberated for the continuance of life and of humble agents which might teach the lessons he strives to learn. Once upon a time, having struggled long with the complexities and arguments of his own world, he layed aside his telescope to listen to the tall stories of *Mycologicus naturalis*.

*Naturalis*, being steeped in mythology, sacrificed to *medicus* first a generous flagon containing an intermediate from the goddess *cerevisiae*. He then considered whether his own experiences could afford a moral or model to persons as innocent as *medicus*. Finally, he proceeded as follows, quoting always from good authority (3).

Although many writings describe the orderly processes and useful services of the fungi, favorite legends dwell on departures from the norm. In ancient days, such stories were introduced by stating that fungal communities are happy about living in an industrious manner and not paying taxes. However, they may be subject to tribute at any time and in such a way that "few groups are free from parasites, some of which may even be their close relatives."

*Naturalis* hesitated about whether to enlarge upon the growth-competent bandits, lest tales of
stupid cupidity might destroy the fascination he hoped to create in medicus. But, being a realist, he could not resist calling by name the characters that scarcely pause to say "Jack Robinson" before launching their mortiferous antibiotics from a great distance, trumpeting against the walls of Jericho until the tremor of a touch will crumble them, or sacking the city after direct assault. Once inside a residence, they plan to stay only overnight. They keep on their virulent overcoats and boots while burning the furniture. Each knows that by dawn his host will be gone toward heaven and that he will be going for the door. What reckless fellows. They can be kept in custody for years, often becoming less noxious, but always disgracefully greedy to reproduce in glass houses on the slightest pretext and minimal encouragement.

Naturalis took the nodding of medicus to mean that medicus appreciated a retelling of the folklore which he could understand. He next thought it timely to quietly offer medicus a mouse of a story designed to keep the mountain of the latter from falling flat on its prairie.

The interesting dependencies are more subtle. In some instances, Gamma depends upon Beta, who in turn must rely on Alpha. Some sly ones find it sufficient to live intimately, side by side with a provident neighbor. Gonatobotrys, the simplex, and Calcarisporum, the parasiticum, being perhaps added by ultraviolet, are known to be dependent on a growth factor which is made and also required for the growth of many fungi (92). The elixir to which they are addicted is not known, but it is true that they must have it, even if the amount be only one needle amidst a million haystalks. [Extracts having "mycrotrophine" activity (92) have since been shown to contain bound hydroxylamine and to supply the sideramine requirement of A. terregens (Morrison, unpublished data).]

Jonesii of the Chaetocladium clan, being devoid of some essential, lays a finger on the more industrious Mucorales. Contrary to some reports, this Good Samaritan does not set jonesii on his ass, but in a shady nook helps him to build a picnic table of wonderous design (14).

With others it becomes to a question of breaking and entering, not from boldness, but from vital necessity. Among the Chytrids there are many legends concerning the five Rozella brothers (48). They intrude into a new domicile by drilling a hole through the wall and squeezing through it with the finesses of a phage, leaving their hats and overcoats outside. They insinuate themselves into the good graces of the host before taking a seat at his table. During reproduction, they are modestly invisible. The children grow and multiply without wailing off their genes and cytoplasm in visible integuments. During their youth they are harmless. As growth slows down they clad themselves in nylons, hoods, and skates and prepare to make their way into the world. If there is trouble in opening the door, they tear round and round within the hollow shell of host remnants. If the Rozella brothers and their heirs understood righteous indignation, they could sue the psittacine bacteria and M. leprae for infringement of patents.

Medicus, being weary of keeping his mind in constant focus, could only wonder whether his folks had sent him to the wrong kindergarten. He loaded his syringe with mysterious yet powerful mixtures of drugs and antibiotics. He prepared to contemplate these fanciful tales while composing himself for rest.

Sleep well, Microbiologicus medicus, perhaps some later night you can invent a better story. Meanwhile, there is much work for the morrow.

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