THE POLYPHASIC POTENCIES OF THE BACTERIAL CELL; GENERAL BIOLOGIC AND CHEMOTHERAPEUTIC SIGNIFICANCE

RALPH R. MELLON

WITH THE COLLABORATION OF MARION L. HAGAN

Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh, Pa.

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The experimental data of this communication really represent the fourth paper in a series dealing with the effects of the sulfonamide drugs on certain pathogenic bacteria as influenced by variability. The first three papers have already appeared. (McKinney and Mellon, 1941) (Hadley and Hadley, 1941) and (Hadley, Hadley, and Leathen, 1941). The pattern of bacterial variability associated with the action of these drugs *in vivo* has had no clear recognition heretofore; but, by way of anticipation, a clue to its nature is afforded by a certain similarity with what have sometimes been referred to as "environmental modifications."

These latter have never been considered as true bacterial variants, or even as playing any rôle in the variation process, for two rather specious reasons. The first of these concerns their lack of stability; and the second, the misconception that they were merely morphologic alterations and therefore of no importance.

In the first paper of the series we demonstrated that the erroneously styled series of involution forms of the pneumococcus occurring *in vivo* under the impact of sulfanilamide action, were actually phasic entities. This fact was considered of transcendent importance too, because the gradient of disappearing virulence which the several phases represented, made them vulnerable to destruction by the immunologic forces of the host. The proof of our contention as to the essentially phasic nature of the pleomorphic cell-forms, was made possible by their isolation in culture where their biochemical and colonial differences became measurable.

It is our belief that the pattern of variability shown in the pneumococcus, while in itself distinctive, is not in principle a specific effect of the action of the sulfonamide compounds. In fact, the variability phenomena of the organism we are now about to describe have not been evoked by the action of these drugs; and yet the principles involved do not seem to differ and even the phenomena themselves have much in common. On the other hand, the latter are so much more inclusive that a unique opportunity is provided for the integration and clarification of relationships that have long been in dispute. This is due in part to the fact that it is unusual to encounter a single organism possessing such a range of variability as this one did.

Incidentally, the intimate bearing of such considerations on the mode of action of the drugs that have made a new epoch in the history of medicine, should make

timely the re-appraisal of variability phenomena that have been so generally misinterpreted. Curiously, the universally recognized single fact that the action of these drugs is primarily growth-inhibitory (bacteriostatic) rather than bactericidal, seems to have been passed over too lightly; when in point of fact, the importance of growth inhibition for the metamorphosis of living things, large and small, can scarcely be over-estimated.

History of the culture and methods of approach. By virtue of the fact that the culture as originally isolated was morphologically a streptothrix, this paper's title demands a word of explanation. Despite its branching filamentous nature as isolated, the several phases subsequently stemming from it were distinctly bacterial in their morphology; in fact, one of its dissociants was an acid-fast bacillus whose colonies were indistinguishable from our Critical Rough phase of the tubercle bacillus. (Mellon, 1935) (Mellon, Almaden and Richardson, 1936). Moreover, the strain was isolated from a disease commonly regarded as an anomalous manifestation of tuberculosis. Affecting chiefly the skin, lymph glands and bones, this disease is known clinically as Boeck's sarcoid, or Schaumann's Disease, but its pathogenesis has been obscured by the conventional absence of a virulent phase of the tubercle bacillus. In its place are found diphtheroids, cocci, and streptothrices, the latter being of such a low order that they tend to fragment into forms distinctly bacterial in their morphology. Moreover, they frequently possess varying degrees of acid-fastness. On the basis of their dissociative relationship with the tubercle bacillus, our orientation of these organisms into the clinical and pathologic picture has already been presented. (Mellon and Beinhauer, 1937).

The organism under consideration we shall designate as the Johnston streptothrix. It was isolated in 1930 and was for the most part entirely non-acid-fast, although in the course of the long, interlacing filaments, one frequently noted acid-fast bulbous or spindle-shaped enlargements whose staining character often extended along the filament for a considerable distance. After a few transplants however, almost no evidence of the acid-fast character remained. The strain grew rather readily on blood agar at the temperature of the incubator, and somewhat more slowly at room temperature. The colonies were coherent, as well as adherent to the medium. It was transplanted at regular intervals of a month or so on different mediums over the course of four years and during this time showed no special evidence of variability aside from the above-mentioned dissociation to a bacillus indistinguishable from the critical R stage of the tubercle bacillus. (Diagram I).

It was this dissociative relationship of the streptothrix with the critical rough culture-phase of the tubercle bacillus that originally led us to culture the youngest skin lesions of cases of Boeck's sarcoid. In so doing we proceeded on the assumption that the initially infecting organism was perhaps an unrecognized culture-phase of the tubercle bacillus, very low in virulence and so unstable as to dissociate rather early *in vivo* into such a partially acid-fast streptothrix; which in its turn (and somewhat later possibly) dissociated into one or more non-acid-fast, or partially acid-fast stages, usually found in the older lesions of the skin and lymph glands.

Our hope was that, if this *in vivo* dissociative process was apprehended sufficiently early—that is, before the dissociants had attained considerable stability —reversion of the isolated organism to some definitely recognizable stage of the tubercle bacillus might occur more readily. Fortunately this hope was realized, for reversion of the non-acid-fast streptothrix to an acid-fast critical rough culture phase actually occurred. (Stage VI, diagram I.) This change became manifest when numerous secondary, yellowish colonies developed on the gray background of the streptothrix culture. The fact that these were not capable of isolation until they had attained considerable size is a phasic consideration whose bearing and importance will be elaborated further in the discussion. Differences between this critical R phase and what we regard as the intermediate R described by Petroff (1930), Kahn (1933), and others, have been pointed out

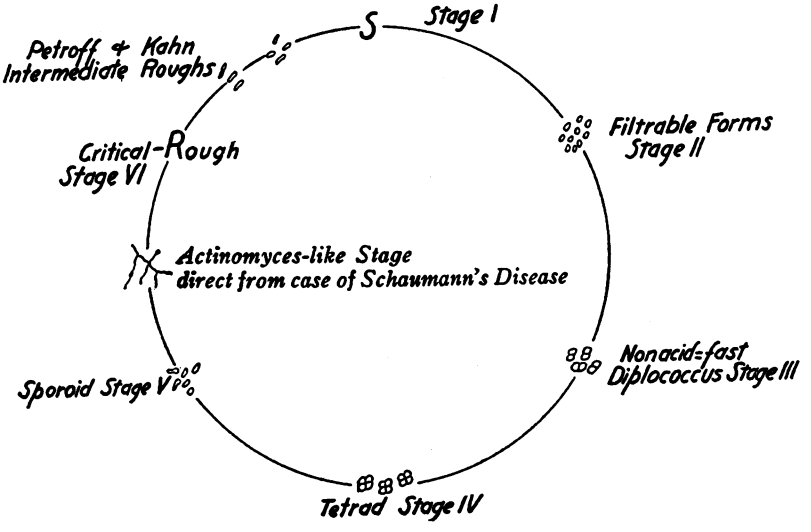

DIAGRAM I. THE DISSOCIATIVE CYCLE OF THE TUBERCLE BACILLUS AS SUGGESTED IN PREVIOUS PAPERS

The Actinomyces-like stage noted above is identical with the Johnston Streptothrix of this paper.

in a previous communication. (Mellon, 1920.) Perhaps the most significant difference lies in a certain residual propensity on the part of their strains to cause a rather typical caseating tuberculosis. This is not true for the critical rough stage.

In June, 1936, the viability of the non-acid-fast streptothrix seemed to change rather abruptly. That is to say, the strain suddenly appeared to be non-viable when transferred from the several sources kept in stock, with the exception of the cultures that, a year previously, had been dried *in vacuo* and kept hermetically sealed. These grew readily enough but, instead of showing exclusively the characteristic rugose, uniformly adherent, tubercle-like growths, there was distinctly more individuality among the colonies of the culture. This manifested itself particularly as a variation in their consistency, some of them being

noticeably softer and more glistening than the conventional dull, dry appearance characteristic of the strain.

Correspondingly, the branching filamentous character of the culture diminished somewhat. In other words, the softer the colony the less branching it exhibited and the more the organisms resembled bacteria. In addition there were numerous small, gray, flat, translucent colonies that were quite nonadherent to the medium. Microscopic examination revealed that in place of the long, branching filaments characteristic of the original streptothrix colonies, these flat translucent ones were composed of small coccoids and of relatively short, curved bacillary forms, many of which were very granular, and diphtheroid-appearing. Transfers to blood agar, Loeffler's and other mediums gradually increased the granular character of this organism so that it could fairly be thought of as belonging to the Corynebacteria.

This diphtheroid dissociant and the critical R acid-fast dissociant have both remained stable for five years, thus illustrating a type of variation change that falls into a category wholly different from the fluctuating changes characterizing the variants that form the chief interest of this communication. For the purposes of this discussion the term, "pleobiosis" will be used to cover the former category of variation, variations that transcend the limits of our taxonomic categories such as species, genera, etc., and are exemplified by the fairly well verified transformations between streptococci and diphtheroids. (Mellon, 1920.) While it is well known, this category does not yet have the degree of acceptance accorded the more simply demonstrated M, S, and R pattern.

As has been indicated, the original strain, composed of very long slender, branching filaments, has been replaced by a series of colonies whose filaments were progressively shorter, thicker, and not so conspicuously branching. Serial transfer of the softer, less adherent colonies, resulted finally in frank S and SR types of colony. Of added interest was the fact that on Loeffler's medium a typical mucoid colony developed, albeit very slowly. Yet it is to be emphasized that most of these various colony types were too unstable to be considered true dissociants in accordance with the M, S, and R pattern.[1] In the beginning at least, the colonies readily changed character as the environmental conditions were altered and it was only with difficulty that they remained true to type on a single medium unless transfers were made at frequent intervals.

It is a rather general rule that such marked instability is not associated with the differentiation of biochemical attributes that give the S and R culture phases their distinctive character. Partly for this reason we are introducing the term, "modulation" for these relatively unstable variants. While their lack of stability makes them reminiscent of what have been called "environmental modifications," we prefer not to use this term, chiefly because most bacteriologists

[1] Actually the mucoid culture phase is by no means as stable as the S and R phases, often requiring a special environment to maintain it. For this reason its true phasic status has been questioned by some. However, its virulence connotations have preserved for it a variability status that it would probably have lacked had it received allocation as a mere environmental modification.

have never thought of such fluctuating changes as an expression of true variation. And those who might wish to do so, would be at a loss, because its other implications and usage have also been rather nebulous. Thus, when environmental change induced marked morphologic transformation of a culture, it has meant to some that it is involutionary or pleomorphic, even though the changed culture was actively growing.

Incidentally, the term, "modulation" is borrowed from the realm of musical composition. In the course of transition from one musical theme to another, or even from one chord to another, a re-arrangement of the notes composing the chord may implement the change. The process is spoken of as modulation. Even before one theme is brought to a close, the one that is immediately to follow, can often be anticipated by the emphasis placed on certain key notes, which serves to signalize the re-arrangement that is about to take place.

Again, a later "movement" of a symphony may be anticipated by keynote phrases appearing in an earlier movement. When such phrases are repeated and progressively amplified, they may be thought of as fragmentary *recapitulations* of the theme of the later movement, which indeed, they are *in process of becoming.*

Similarly, aberrant cell-forms in a culture are its keynote phrases which signalize the direction of development which the culture is capable of taking. Thus, when certain centers of change in a smooth streptococcal colony begin a transformation to the rough phase, the first sign is the appearance of rough outbursts of chained forms from the margin of the colony. These chains constitute one sort of pleomorphic form, but they may also be viewed as anticipating the rudimentary out-croppings of the rough phase. This is proved by the fact that their latent potencies, which incidentally are characteristic of all rudimentary structures, must be gradually unfolded, if their development is to culminate in a stable R-phase.

This unfolding is accomplished by several serial transfers of the rough outbursts. As a result, their chained forms attain that degree of differentiation (modulation) that permits them to multiply continuously *as such*; otherwise, they immediately revert on transfer to the smooth-colonied diplococcus phase.[2] Thus the earliest of the chained pleomorphic forms represent the rudimentary, unstable prototypes of another phase in the culture's life history.

Just as the keynote phrases in a musical composition may require repeated amplification at intervals, before progressing to a new movement of which they are to constitute the theme, so do the early chained forms composing the rough outbursts require biologic (biochemical) amplification before they can stabilize in this form, and thereby become the "morphologic theme" of the rough colony. That such pleomorphic formations represent *per se* a re-grouping of biologic

[2] It is exceedingly important to recognize the fact that this reversion is not complete, because each succeeding transfer of a smooth colony develops the peripheral rough outburst earlier than the preceding one did—until finally no reversion occurs, stabilization in the R-phase having been achieved. The conclusion that this process represents a progressive differentiation of a pleomorphic form seems inescapable. In such event it invalidates the conclusions of Rettger and Gillespie (1935) and Wyckoff (1934) who, on the basis of a reversion of coccoidal pleomorphic forms to "normal" bacilli, have denied any truly cyclic potencies (i.e. progressive change) to the pleomorphic forms.

characters was clearly shown in our recent paper (McKinney and Mellon, 1941) dealing with the effect of sulfanilamide on the pneumococcus.

The single-cell approach to the problem. We were fortunate to have as a visiting assistant, Mr. Philip C. Trexler of Dr. Reynier's laboratory in Notre Dame University. We had opportunity to observe first hand the working of the mechanical devices for the single-cell isolation in a microculture developed by Dr. Reyniers. The facility, precision, and ease with which the individual cells were isolated and bred in a microculture by Mr. Trexler bore testimony to the practical value of the instruments for microbiological purposes, to say nothing of their ruling out the ancient criticism, which predicated a *mixture of types* in explanation of the variational changes under consideration. Our observations on these cultures made desirable the isolation of large numbers of single cells. The latter were placed on cover slips under aseptic conditions, and in such fashion as made possible observation of the growing unit until it formed a micro-colony. Such a culture was then transferred to a slant or plate, thus leaving not the slightest doubt that the culture growing under conventional conditions was a descendent of the isolated unit which had been segregated by the pipette method. However, aside from a guarantee of phase purity, this approach, in its present state of development, is not to be recommended for dissociational studies, for reasons that will become clear. Its applicability has been greatly overemphasized by Rettger and Gillespie (1935) by Wyckoff (1934), and others.

EXPERIMENTAL SECTION

1. Experiments on the mucoid phase. (Fig. 1). Microscopic morphology of this phase showed it to be rather pleomorphic, in the sense that although made up dominantly of short curved, gram-negative rods, the latter not infrequently became clubbed, or elongated into short filaments, often showing rudimentary branching. (Fig. 1*a*.) This was particularly true as the culture aged for more than 24 hours. For the most part the rod forms were non-granular, although, as the culture continued to age, a granulation of both rod and filamentous forms appeared. Diphtheroid cell forms were also present. These aging forms were more gram-positive than the young bacilli.

Eight single cells were isolated from a mucoid culture, or rather from its different morphologic components. These were usually of reduced viability. Whether the isolated form was a rod, a branching filament, or a granular diphtheroid form, it usually maintained itself for several divisions in the form in which it was isolated; but as the number of organisms increased, morphologic sampling of the several transplants indicated that all of them soon became morphologically indistinguishable. That is to say, regardless of the morphologic type from which the cultures were started, in 24 hours or so they were all made up of a mixture of rods, some granular and some non-granular, among which filaments of varying lengths were present.

The fact that the aberrant cell-types showed a definite tendency to reproduce as such, if only for a short time, suggested that this tendency could be strength-

ened if the organisms were provided with a more favorable environment than that afforded by the micro-cell. Accordingly, an aging mucoid colony, containing granular diphtheroid-like bacilli showing rudimentary branching and a marked tendency to be gram-positive, was transferred to a filtrate medium[3] having a pH of 8 and not favorable to multiplication of the "normal" bacillary form of the culture. It so happened that the branching pleomorphic forms then promptly germinated *as such*, becoming firmly adherent to the medium as a non-mucoid filamentous growth. They continued to multiply in this phase when re-cultured on this medium. But when transferred to the original medium their prompt reversion to the short, non-adherent form heralded the re-appearance of the mucoid colony.

Thus it seems clear that the continued viability of these pleomorphic forms as such is to no small extent a function of the environment to which they are subjected. Moreover, they appear to be undergoing a progressive change, which must reach a certain degree, before a stability is attained that will guarantee their multiplication in the pleomorphic form on the usual media—*unless* as has just been demonstrated, a changed environment is substituted that will compensate the growth-inhibitory changes that the aging organism has undergone.

One of the pleomorphic micro-colonies above referred to was plated on Difco beef-brain-heart agar preliminary to a subculturing of the individual colonies. The only difference noted among the latter had to do with the formation of secondary colonies on aging. Of 40 colonies isolated, 4 failed to show secondary colonies on aging, either in the original isolation or after repeated transfers; 8 colonies showed only mucoid secondary colonies on aging, while 2 of the colonies showed secondary outgrowths that were rough in character. In Experiment No. 3 the relation of secondary colony development to a culture's pleomorphic phases will be shown, while in the following experiments the influence of even slight changes in environment in overcoming their lowered viability and permitting active multiplication, will be demonstrated.

Experiment No. 2. Influence of environment on fluctuating changes of phase. A mucoid colony started from a single cell 48 hours previously was transferred to a long slope of Difco brain-heart infusion agar containing an appreciable amount of water of condensation in the bottom of the tube. This relatively young colony already contained such pleomorphic forms as granular bacilli and occasional short filaments showing rudimentary branching. In the condensation water and on the adjacent agar, covering about the lower one-sixth of the slanted surface, a typical soft mucoid growth, composed of short bacillary forms, was reproduced. The upper five-sixths of the slant was covered by conical rough colonies, composed of branching filaments. They were dry and lustreless toward the top of the slant, grading into softer, less adherent colonies in its middle and lower parts.

On the other hand, when this same mucoid colony was transplanted to a blood-

[3] This medium represented a filtrate of an old broth culture of a coccus dissociant of the streptothrix. Two per cent agar was added to this broth filtrate.

agar slant, a pure mucoid growth was obtained over the entire surface. When plates of Difco brain-heart medium, with a noticeable amount of condensation water present, were incubated under anaerobic conditions, no rough colonies appeared, but only the pure mucoid. Yet when these colonies were transplanted aerobically to long slopes of brain-heart agar, the conical adherent rough colony again appeared in the upper portions of the slant. Thus it appears that blood-agar of an alkaline reaction tended to preserve the culture in its mucoid phase, while the brain-heart medium tended to permit the culture to assume its rough growth phase—except under conditions where sufficient moisture, or anaerobiosis, or both, appeared to neutralize this tendency.

The mycologists have frequently referred to this phenomenon as "pleomorphism," while the bacteriologists would be more likely to speak of it as an "environmental modification"! Trexler's direct observations on micro-colonies started from the branching pleomorphic filaments of pure mucoid colonies, and implicate them as the obvious origin of such environmental modifications. But monomorphic bacteriology still regards them as "pleomorphic" or "involution" forms, which interpretation would scarcely be reconcilable with its conception of an environmental modification. Indeed, generally speaking, these closely related phenomena have not been thought of in the same connection.

Their integration as different aspects of the same developmental process helps to dispel this sort of confusion in the use of terms; and the characterization of both of them as modulations is in the same direction. It seems obvious that they are undergoing a rearrangement of their biologic characters after a fashion that is reminiscent of the rearrangement of the notes in a musical chord. The purpose in either case is to implement a transition to a new pattern of development. That phase transitions, similar to that just described, will occur without resorting to special conditions for their isolation, other than the time factor as described in Experiment No. 1, will be recorded in the following experiment.

Experiment No. 3. Phase transformations within the mucoid colony (secondary colonies). It was not uncommon to find that, after the mucoid colonies had aged from 3 to 6 weeks, secondary or daughter colonies made their appearance. The earliest ones were smooth and glistening and in consistency resembled drops of balsam in its natural state. We shall refer to them as globular. Those secondaries appearing later were rough, being either conical or flat-rough. The flat-rough variety could also form as a secondary colony on the surface of the globular colonies.

Microscopically the flat and conical roughs were indistinguishable, being composed of rather coarse branching filaments. In stability they differed greatly, the flat being of the same order as the rough colonies of the M, S, and R pattern. The stability of the conical-rough and the globular colonies was not noteworthy, although if transfers were made at intervals of a few days or a week and on media which tended to favor one phase or the other, the colonies maintained their purity more or less indefinitely.

The chief point to be stressed here is the cytologic origin of the rough colonies as they develop within the mucoid. They undoubtedly represent a multiplica-

tion of occasional rudimentary branching filaments that constitute one of several pleomorphic cell types, developing within the mucoid colony as it ages. They will be considered collectively in the discussion. The older the secondary colonies, the more likely they were to grow when transplanted. This supports our view that the modulation change is progressive, or maturing in its nature.[4] In other words, we do not appear to be dealing with a discontinuous or sudden form of variation, as has been assumed by some. (Winslow, 1932.)

The microscopic prototype of the globular colony, as it appears within the mucoid is a fairly long rod, coarser than those developing into rough colonies. They may occur as filaments and appear to be enveloped by an achromatic capsule-like substance. It is this substance that probably accounts for the extreme coherence and toughness of the colonies and which prevents their being indented by a platinum wire of heavy gauge. (Figs. 2 and 3.) This phase is often highly pleomorphic (fig. 2*a*) and usually requires single cell isolation to guarantee its purity. The gum-like nature of the colonies suggests some sort of polymerization of the polysaccharides that are the characteristic component of the mucoid colonies.

Viewed as a whole, Experiments 1, 2, and 3, indicate rather strikingly that in a "pure" mucoid colony only 48 hours old the few rudimentary filamentous structures represent "key-note" cell-forms, whose rôle is to "modulate"—that is, effect the transition of—the mucoid phase into a rough phase of varying stability. Thus, in the 48-hour culture the aberrant cell-forms established their status as antecedents, or prototypes, of the stable "R" phase, when relatively insignificant changes of oxygen tension and moisture permit the latter's isolation.

The same progressive modulation is effected in the aging mucoid cultures, these filamentous structures multiplying to such an extent that they are able to segregate themselves as secondary colonies. When transplanted, these structures yield conical R-cultures of an appreciable stability, which in the flat-rough variants become comparable to those of the M, S, and R pattern. This is taken to mean that the modulating influences have impressed themselves to such an extent that the new, or "R" pattern, has become dominant.

This sort of observation should, it seems to us, place beyond question the essential phasic status of such aberrant cell types. Instead of being regarded as aberrant, they should be thought of as *specialized cell-forms* because of their modulation capacities. They may, of course, be viewed as involutionary in the sense that they usually have a restricted growth rate and a capricious viability; nevertheless this represents a superficial interpretation of their underlying

[4] It will be recalled that the secondary colonies (critical rough and acid-fast) developing from the original streptothrix were also non-viable until they had attained a certain size and age. This despite the fact that, once "matured," they grew with ease. This observation quite accords with the interpretation that the acid-fast segments and conidia appearing in certain filaments composing the original streptothrix colony were pleomorphic modulants whose rudimentary or undifferentiated nature rendered them non-viable; and yet in maturing with age, their function was to modulate the transition of the streptothrix to its acid-fast stage.

potencies. Otherwise, they would not be capable of modulation into other phases possessed of strikingly different characters and with a growth-rate and viability fully the equal of the parent phase. The incongruity of an involutionary interpretation that is based on poor viability, or a restricted growth-rate, will be considered in the discussion.

Thus it appears that the mucoid modulant, or growth-phase, represents a virtual stabilization of the culture in its short rod form, in which it is able to secrete mucoid material profusely. The conical rough phase appears as a quasi-stabilization of the culture in its filamentous branching phase, with a small amount of mucoid material being secreted, which is more noticeable in the older cultures. (Figs. 4 and 4*a*.) The flat-rough phase is a quasi-permanent stabilization of the culture which is removed sufficiently far from the mucoid that the secretion of this component is not recapitulated, even in old cultures.

Although there can be no question of the purity of these two clearly distinguishable growth-phases, it is obvious that one of them soon recapitulates the other to a certain extent, and therefore may not remain pure, phasically speaking. Thus culture purity may be said to exist only in a relative sense, both the mucoid and the rough being essentially bi-phasic cultures. Indeed, each of these cultures is not only biphasic, but potentially polyphasic, because each is capable of elaborating several other phases, some of which may be thought of as occupying an intermediate position between the mucoid at one extreme and the rough at the other. That the diversity of the modulation process is not exhausted by the mucoid-rough pattern, becomes clear from the observations that follow.

Experiment No. 4. The rough growth-phase. As a result of repeated single-cell isolations followed by colony selection and plating, it has been possible to separate several rough colony types. As originally isolated the streptothrix filaments gave no intimation of the elaboration of a mucoid component, although such may have been present without our attention having been called to it. But as a result of colony selection following single-cell isolation there was obtained first, the conical rough colony (fig. 4) which on aging secretes at its base a progressively increasing amount of a perfectly clear mucoid substance; so much so in fact that the colony appears to be bathed in it. (Fig. 5.)

There is in addition a conical rough colony, rather lustreless in appearance and without mucoid material as far as the naked eye can detect. Nevertheless it was observed that in the microscopic manipulation of these filaments, an intercellular mucoid substance was present. It seems likely that environmental conditions largely determine whether this substance will be elaborated in a degree sufficient to be observed with the naked eye. Finally, there is the very stable, flat, non-adherent, rough colony which we have mentioned before and which is characterized by a marked tendency to spread over the surface of the plate. It has given no indication of the elaboration of mucoid substance, either macroscopically or microscopically.

Experiment No. 5. The adherent diphtheroid-like phase. (Fig. 6, small colonies.) This phase, both colonially and microscopically, resembled the diptheroid

that dissociated spontaneously from the streptothrix R colony, when the latter originally developed the instability that threw it into its polyphasic state. In its remarkable adherence to the medium it retained a dominant character of the mucoid and the R, but its granular morphology it had in common with the original diphtheroid, thus suggesting its biphasic nature (fig. 6*a*). Even more significant in this respect was the fact that pure cultures contained many non-granular filaments of medium length, (fig. 6*b*), whose granular clubbed ends are to be viewed as forecasting their fragmentation into more or less typical diphtheroids.[5]

Recalling again the musical modulation analogy, these diphtheroidal clubbed ends of the filaments are comparable with the emphasis placed on certain key-notes which signalize the introduction of a new theme that is to give character to the succeeding movement of a symphony. That is to say, the adherent diphtheroid appears as a stage in the progressive modulation of the "clubs" to true non-adherent clubbed diphtheroids.

Thus morphologically and colonially, the adherent diphtheroid appeared as a biphasic intermediate bridging the gap between the streptothrix and the original, typical diphtheroid. Its adherent character was probably referable to its mucoid or gum-like secretion. Moreover, its anti-serum agglutinated almost to full titre the smooth, non-adherent suspensions of the original diptheroid, as well as exhibiting rather striking cross-reactions with such other modulations of the streptothrix as were amenable to this test. This cosmopolitan character of its anti-serum, together with its antigenic community with the original diphtheroid, even when the absorption test was employed, left no reasonable doubt that the latter was not a contaminant.

This adherent diphtheroid-like phase arose after 6 serial transfers from the original rough culture which had been single-celled from the rough phase of the streptothrix. It was noted simultaneously in 3 plates, each of which, as it happened, was 6 transfers away from the original single-celled culture. Two of the transfers of the 6 generations showed a mixture of very adherent colonies, together with a soft non-adherent type which however, was similar in all other respects.

Again, we see exemplified the principle that the pleomorphic forms of a colony are recapitulating its phasic potencies; and therefore may be expected to modulate new growth phases or dissociants from time to time. This evidence supports a similar study made in 1919 (Mellon, 1919) where we observed the multiplication under special conditions of the club-like forms of a branching streptothrix. The latter were essentially a modulation, inasmuch as they had almost no stability under ordinary environmental conditions.

[5] The situation here is reminiscent of the views held by Vicentini (1897), Billet (1890), and others, who in the nineties employed the term, "dissociation" as applying to the pleomorphic fragmentation of filamentous structures. At that early date technical methods were not developed to the point that would enable them to give these "phasic beginnings" the interpretation that we can, with confidence, place on them today. Through the years other writers have recognized frank diptheroids as a stage in the life history of streptothrices. (Gay, 1935).

The fact above-mentioned, that the adherent diphtheroid-like phase is so similar antigenically to the original (and typical) diphtheroid, supports the view that the nature of the variation process in all of its categories is probably similar, whether we are dealing with the readily reversible modulations, the rather stable M, S, and R dissociants, or the exceedingly stable pleobiotic stages—using the latter term to characterize changes that transcend species or generic limits. Viewed as a whole it suggests that the various categories correspond to different degrees of differentiation that are capable of taking place in certain of the pleomorphic forms,—at least, phases of very similar morphology, such as the adherent diphtheroid and the true, original diphtheroid.

The antigenic relationships of the various growth phases. The fact that bacterial pleomorphic and involution forms can be isolated in pure cultures which are morphologically and biochemically distinct from the "normal" form of the culture, is sufficient to identify them as distinct phasic entities. It is not unlikely that the conditional stabilization that such cultural isolation entails, implies that a greater degree of modulation has taken place than that possessed by the un-isolated pleomorphic phases.

Inasmuch as continued modulation of these cell forms eventuates in stable dissociants, comparable with the M, S, and R pattern, it is conceivable that some degree of antigenic reorganization (expressed as "group" antigens) may make its appearance, even in advance of such a dissociation end-point. Thus antigenic mixtures would to an extent take on the same significance as phasic mixtures. While the several pleobiotic stages of the tubercle group are further removed from each other antigenically, than the S and R culture phase of any single pleobiotic stage, nevertheless the possibility of cross reactions, implemented by group agglutinins, was subjected to test.

Agglutination experiments. According to diagram I, the position that we have allocated to the several streptothrix modulants lies between the frankly acid-fast, but avirulent Critical Rough stage on one hand, and on the other, the non-acid-fast stages of the tubercle bacillus such as the Sporoid (diphtheroid) and Diplococcus.

It will be recalled that the streptothrix in its partially acid-fast form transformed into the acid-fast Critical Rough stage of the tubercle bacillus. From this Rough an S-culture was dissociated, and characteristically its acid-fast bacilli formed large, terminal, spore-like coccoidal bodies. (Figs. 8 and 9.) On special media these were cultivated and observed to dissociate step-by-step into enlarged acid-fast cocci, then into partially acid-fast cocci, (fig. 10), and finally into normal-sized non-acid-fast cocci.[6] (Unpublished work.)

[6] This far-reaching transition, implemented by some of the most typical of the so-called "involution forms," indicates that it is not necessary for them to revert to the bacillary form in order that their progressive development be effected; this, it will be recalled, was the case when the pleomorphic chains of smooth streptococci were dissociated into the Rough (see footnote 2). Indeed, it appears that these distinctly coccoidal or spore-like structures are capable of undergoing modulating changes that are much more far reaching than the latter. That is to say, they fall into the pleobiotic category.

In an attempt to effect a serologic bridging of this very considerable gap existing between acid-fast bacilli (Strain 257-S) and the non-acid-fast cocci (Strain 257-C) dissociated from them, rabbit antisera were prepared against the Mucoid, Conical, Rough, Globular, and Adherent Diphtheroid strains, as well as 257-S and 257-C. The latter two strains were considered as representing the extremes, and the modulants of the streptothrix the intermediates, between the two extremes. It was as if the acid-fast bacilli and the non-acid-fast cocci were bridgeheads that required spanning by the streptothrix modulants.

TABLE 1

ANTIGENS	ANTISERA					
	257-S	Mucoid	Globular	Coccus	Adherent diphtheroid	257-C
257-S	100%	++	−	50%	++	
257-Coccus	−	±	−	−	−	100%
Mucoid	±%	100	−	100	50%	±
Adherent diphtheroid	75	50	50	50	100%	50%
Globular	25	25	100	5	50%	±
Coccus	50	±	50	100		
Original diphtheroid					100%	

Explanation of table: The figures represent the approximate agglutination of antigen in antiserum. The ++ is approximately 10% agglutination; ± is a trace and is not significant. All tests were made in a serum dilution of 1:1280.

Analysis of agglutination table. As was anticipated, there was a complete absence of cross agglutination between the acid-fast bacillus and the non-acid-fast coccus dissociated from it, despite an homologous agglutination of each antigen of 3^+ to 4^+, in a serum dilution of 1:1280. The Adherent Diphtheroid antigen showed definite indication of bridging this gap, as is shown by a 50 per cent agglutination of its antigen in the anti-coccal serum and 75 per cent in the anti-bacillary serum. These results were checked with the absorption test.

The Mucoid antigen showed no cross-agglutination in sera of either of the 257 strains, while the Globular and Conical Rough antigens precipitated to the extent of 25 per cent and 50 per cent respectively in the anti-bacillary serum. In the anti-coccal serum, however, these antigens did not react. When it is recalled that the tubercle bacillus is dissociable into a mucoid phase (McKinney and Mellon, 1939), the mucoid phase of the streptothrix might have been expected to react with its serum. The Coccus, Rough, and Globular strains react moderately with the other streptothrix modulants, but the adherent diphtheroid is the most cosmopolitan, since it reacts to a certain extent with all the others. Particularly significant is the complete precipitation of the original Diphtheroid

Incidentally, these coccoidal structures which dissociated step-by-step on the special egg medium employed, failed completely to do so in microcells, under any conditions employed by us. But they readily reverted to the bacillary form, which however, would have been wholly misleading as to the potencies of these forms for progressive development.

antigen in the serum of the Adherent Diphtheroid, since the former is a very typical and a very stable diphtheroid.

Because some of the data are difficult to interpret in light of our knowledge of the agglutination reaction, and because of the difficulty of obtaining high titre antisera with these organisms, further work is indicated before the bridging-over principle is established with the acid-fast—non-acid-fast group. However, the position is supported by a similar study made several years ago where the antigenic gap between a mucoid hemolytic streptococcus and a non-hemolytic diphtheroid was clearly bridged over by the S and R dissociants of the streptococcus (Hadley, unpublished work). Here the antigenic relationships are so well worked out as to leave no reasonable doubt that the interpretation given is correct. (See diagram II.)

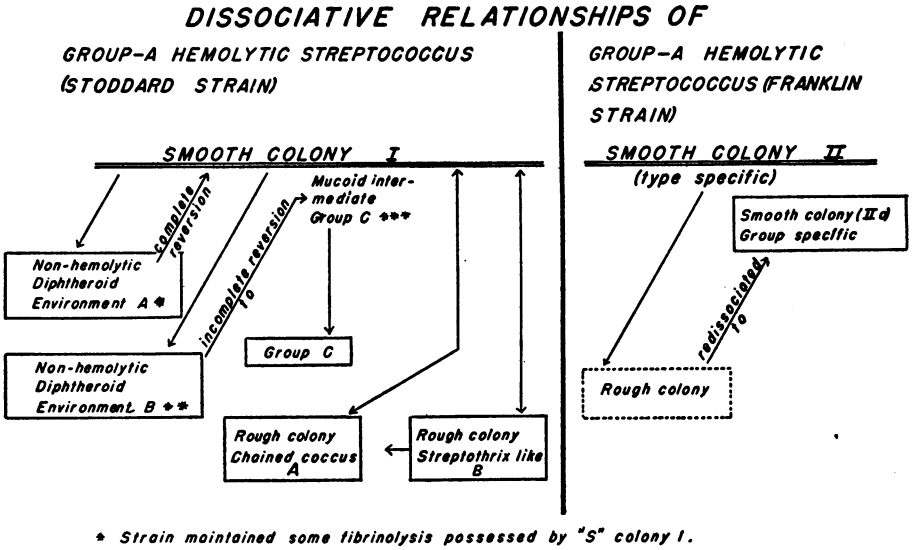

DIAGRAM II

The left hand side of the diagram shows chiefly that a non-hemolytic diphtheroid dissociated from a hemolytic streptococcus was capable either of reverting to the original Group A streptococcus or to a Group C, depending on the modulations induced in the diphtheroid by the environment under which it was held.[7]

The right hand side of the Diagram shows that a rough dissociant of a smooth colony (II) became the seat of a re-grouping of its characters to the effect that a smooth colony (II-a) re-dissociated from it elaborated group agglutinins for smooth strains representing other serologic types of Group A. In addition,

[7] The fact that Group A is found in human streptococcal infections and Group C in small animals, is of possible epidemiologic interest in connection with the rôle of variation mechanisms in the adaptation of microorganisms from one species of animal to another.

smooth colony II-a showed cross reactions with sera of Group C strains.[8] In view of the transformation shown from Group A to Group C such cross reactions may be viewed as signalizing such a possibility.[9] Apparently, such "recapitulation" of antigenic characters has the same significance as the pleomorphic recapitulations, namely a modulation toward the elaboration of a new growth phase.

The bearing of these facts on the antigenic bridge-over between the diphtheroid and the hemolytic streptococcus focuses significantly on the extent to which the antigenic characters are unfolded in the two Rough culture phases A and B. Thus, sera developed in rabbits against the several strains represented in diagram II, showed that the streptothrix rough (B) agglutinates to the full titre of the diphtheroid antiserum (5120), while the chained coccus rough (A) agglutinates completely in only a 1:640 dilution of the serum. Apparently, the streptothrix-like rough signifies that the modulation from coccus to diphtheroid has been carried one step further than is represented by the conventional rough phase. which is composed of chained forms.

The inter-relation between the streptococcus and diphtheroidal groups is made particularly striking by the fact that this particular diphtheroid is not wholly lacking in antigenic community with the streptococcus—a rare situation in our experience. This is shown by the agglutination of the diphtheroid in the Smooth antiserum to a titre of 1:640. The reaction was confirmed by absorption. No reaction of the Smooth antigen in the anti-diphtheroid serum occurred. This sort of asymmetrical reaction was rather characteristic here, as it was found to be with the streptothrix modulants of the tubercle group.

These antigenic correlations support the variational experiments of many former authors whose studies have indicated a certain community between acid-fast bacilli, streptothrices and diphtheroids. This situation, particularly with reference to leprosy, has been recently reviewed by Gay (1935). Microbiologically, it is virtually the same as occurs in Boeck's sarcoid, which afforded us the Johnston streptothrix culture. Gay regards the several genera (?) of organisms isolated from leprous lesions as growth-phases of the classical acid-fast organism of this disease. He refers to the earlier study of Claypool (1913) in his laboratory. Using the complement fixation test, she found a gradient of reactions from non-acid-fast streptothrices to acid-fast tubercle bacilli. Gay says that she even found cross immunity reactions between the divergent morphologic

[8] This dissociative unfolding of the antigenic complex of such phases that results in a broadening of the antigenic coverage may have a practical bearing. Flosdorf and associates (1941) in their immunologic work with pertussis vaccines emphasize the desirability of such a coverage in strains that are to be used prophylactically. They also emphasize the ease with which phase transformations in the pertussis organisms may occur unless they are suitably stabilized. A multiphasic serum was accidentally produced as the result of such a change occurring in the interval between inoculations.

[9] Otherwise, they might be open to the interpretation that they were purely a nonspecific chemical flocculation, such as Bliss (1938) has recently shown to be due to peptone, when heterologous streptococcal groups were tested against Group C antisera. However, such nonspecific reactions emphasize the necessity for caution in drawing conclusions without supporting variational evidence.

forms observed in single strains. These include acid-fast and non-acid-fast culture forms and are reminiscent of our "modulations."

The point of view that group agglutinins are to be correlated with "mixed phases" of a culture is not new. Certain of our dissociation experiments upwards of 15 years ago point in this direction rather clearly. (Mellon and Jost, 1926.) Even more striking was the difference in agglutinative response between two reversible phases which appeared in sequence in the same culture. This situation rather closely paralleled the streptothrix one, more particularly in its pleomorphic aspects.

The organism under study was an alkaligenes bacillus, growing in coccoidal form. During the first 24 hours of its growth in broth, its phasic status was one of a spontaneously agglutinated sediment, which would not absorb antibodies from its homologous serum. After 24 hours the broth culture grew diffusely so that at 48 to 72 hours the supernatant was very turbid. In this state it absorbed and agglutinated completely! As a result of aging apparently, two distinct growth phases occurred in the one tube of broth but they were not stabilized; for when the supernatant phase was transferred to a tube of fresh broth, the above sequence of events recurred.

That the cells in the supernatant represented a phase of growth distinct from those in the sediment is also indicated by the fact that they were morphologically almost identical with the diffusely growing form in which the culture was originally isolated. In other words, the spontaneously agglutinating strain *in its diffusely growing state*, recapitulated the morphology and tinctorial characters of the strain as originally isolated; and before it had dissociated into a spontaneously agglutinating phase, which was morphologically distinct. The biologic significance of such biologic recapitulation will be discussed presently.

DISCUSSION

The foregoing experiments demonstrate anew that the subsequent course of development of certain pleomorphic cell-types depends on the nature of the environmental change brought to bear on them. Thus, under one environment, the so-called "branching involution forms" of the bacillary mucoid culture-phase may reproduce *as such*, but in colonies resembling those of the mother colony; under another environment, they may germinate as well-developed branching structures, whose colonies are adherent, rugose or tubercle-like, and quite different from the mother colony; or under other conditions, they yield non-adherent, rough, spreading colonies of branching filaments, whose noteworthy stability implies a still greater separation from the mucoid type of colony.

We can only infer that something more than the process of natural selection of fully developed hereditary biotypes ("pure lines") is at work here; for it is apparent that certain environments not only select culturally the rudimentary branching forms from the mucoid culture, but they also modulate them progressively[10] into fully developed rough, or streptothrix-like colonies. In other words,

[10] It is precisely this failure to recognize the progressive nature of this process that forms the crux of the endless confusion over the matter of "contaminations," "mixtures of hereditary biotypes," etc. We know that appropriate environments make possible the segrega-

they are in process of *becoming*, and the kind of environment to which they are subjected largely determines *what* they shall become. This modulation processs we regard as akin to that of differentiation of specialized tissues among the higher forms, but without its implications as to organization. That is to say, we do not regard the "differentiated" bacterial phases as the equivalent of the organs and tissues of the higher forms as do Henrici (1928) and Winslow (1939). To this point we shall presently return.

The progressive character of this modulation change is evidenced in other pleomorphic cell-forms. Thus the clubbed, diphtheroid-like structures forming on the ends of the branching filaments appear to be recapitulating a phase in the development of the true diphtheroid, originally dissociating from the streptothrix. This suggestion is clearly supported by their antigenic community.

This rather characteristic ability of any single culture phase to repeat in the course of its development those changes of form that give character to other phases of the culture's development is reminiscent of the biogenetic law as formulated for the higher forms of animal life. Condensed into a biologic aphorism by Haeckel, it states that "ontogeny recapitulates phylogeny." That is to say, the higher animal species, in its individual development, runs through in its morphologic details the more or less "fixed" or permanent stages below it. (Haeckel, 1821.)

What might be considered a *sort* of ontogeny for the individual of any bacterial species would comprise progressive development through the culture phases G, S, and R.[11] Morphologically, they begin as the tiniest of coccus forms (the G) and progress to the coccus, chained, and filamentous structures; the latter being characteristic of the rough phase culture in all families above the Coccaceae. An interesting parallel exists with reference to the great so-called family groups of bacteria. The progression of the micrococci to the Coccaceae, to the Bacillaceae, to the Bacteriaceae—the Mycobacteriaceae—the Actinomycetaceae —etc., exemplifies it.

Accordingly, when a pneumococcus for example, exhibits in aging cultures such pleomorphic forms as chains, micrococci, bacillary elements resembling

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tion of biotypes as well as phasic entities of a culture; but according to definition (Cole and Wright, 1916) they cannot effect progressive changes in one biotype to the extent that it takes on the characteristics of another biotype—even a closely related one.

Therefore biotypes should not be confused with culture phases. Indeed, in view of the lack not only of evidence, but also of criteria, for the existence of bacterial biotypes, their present status may be regarded as almost mythical; and with it, the use to which the principle of "natural selection" has been put in the interpretation of bacterial variability. (Cole and Wright, 1916).

The probability that all bacterial cultures are essentially polyphasic means that they are "potential" mixtures—not of hereditary biotypes to be sure, but of phases whose stability may be sufficient to cause them to be mistaken for biotypes. *Accordingly, a potentially mixed culture may become an actually mixed one whenever the modulation changes reflected by its specialized cell-forms have proceeded far enough to guarantee their cultural isolation (as a new phase) under the environment to which the culture has been subjected.*

[11] The position of the *mucoid phase* in ontogenetic development is, at present, difficult to assign.

bacilli, and branching filaments, it would mean that we have a morphologic gradient that is recapitulating the taxonomic organization of bacteria, a system which begins with the more simple coccus structures and proceeds to the more highly organized chained and filamentous ones—that is, to those structures much more prone to develop conidia, branching, and those interesting reproductive structures formerly described by us that so strikingly resembled zygospores. (Mellon, 1925.)

In line with the significance of the recapitulation principle, these rudimentary or undeveloped zygospore forms would be regarded as signalizing the existence of a true bisexual stage somewhere in the life history of bacteria. It represents possibly a further differentiation of the rough phase than has ever been attained experimentally. That such pleomorphic forms are symbolic of a progressive change of some sort is evidenced by their dissociation into such patterns of variability as the G, S, and R.

Perhaps the most striking confirmatory aspect of the existence of a principle of recapitulation is the antigenic one. Thus the appearance of Group C hemolytic streptococcal agglutinins in a Group A antiserum means that the Group A strain is recapitulating its inherent potencies for conversion to a Group C strain (diagram II). The cytologic and cyclogenic origin of such antigenic recombinations was by no means as clear when our earlier studies (Mellon, 1926a) first suggested the existence of a recapitulation principle for the bacteria.[12]

However clear the case for the principle *per se*, the biogenetic law as formulated for the higher animals is scarcely more than an adumbration when applied to the bacteria. This for the reason that in several respects the cyclic phenomena in the two orders differ. Thus, the progression from stage to stage among the bacteria is by no means inevitable, being influenced, as the process is, vastly more by environment than is the case for the higher forms. Again, the progressive modulation is reversible at almost any of its stages and these are capable of stabilization and of rather unlimited multiplication.

Failure to recognize clearly these disparities between what we may speak of as a dissociation cycle for bacteria and the conventional life cycle of the higher forms, is responsible for much misunderstanding. This has shown itself particularly in some of the technical approaches that have been used to test the cyclic potencies of the so-called involution forms.

Concrete dissociational data cited above (footnotes 2, 4, and 7) exemplify some of the reasons why the micro-cell cannot of itself be expected to provide a categorical answer on the point. This technique is more applicable to higher

[12] Actually, our first glimpse of a developmental principle of some sort occurred when we observed clear-cut diphtheroid intercalations in long chains of streptococci. The fact that the latter had been dissociated from a pure diphtheroid culture gave the phenomenon its real significance. It has been the pole-star that has guided many of our subsequent experimental inquiries into the variability field.

The idea, naturally embryonic in the beginning, was destined to undergo several *permutations* throughout the years, in our attempts to interpret its meaning. It seems likely that its formulation as given in this communication is as far as we shall be able to carry it. Our initial study was published in full in the *Journal of Bacteriology*, **2**, Nos. 2, 3, 4, in 1917.

forms because the cyclic tendency in them is so much more inherent and therefore less subject to environmental influence. In any case, its employment in bacterial variation must be highly discriminating in respect to a number of variables.

Accordingly, the almost inevitable failure of warm stage techniques to yield a type of cyclogenic information limited to the higher forms, led bacteriologists to think of bacterial dissociation as a phenomenon wholly detached from any sort of cyclogenic mechanism. Thus Winslow (1932) distinguished rather sharply between cyclogenic and dissociative variability. This somewhat artificial distinction was contributed to by the fact that dissociation was regarded primarily as a colonial phenomenon. Therefore, the obvious integration (footnote 2) between its cyclogenic and its dissociative aspects was overlooked. Had this relationship been properly assessed, the bacterial life cycle would not have been thought of as a "must progression" from stage to state. (Rettger and Gillespie, 1935.)

This integration of bacterial variation with pleomorphic cell-forms is strengthened when we consider the cytologic origin of those forms which often involves other modes of germination than binary fission. Branching, budding and conidial formation are the commonest. (Hort, 1917) (Bergstrand, 1918) (Gardner, 1924) (Mellon, 1926b). But even a successive snaring off of coccoidal forms from the end of a branching Shiga bacillus was shown in a motion picture by Wyckoff (Personal Communication). The latter mode of germination is particularly characteristic of the fungi. These initial indications testifying against the involutionary nature of such structures assume a categorical import when the latter can be shown to modulate—that is, implement the transition of—the several categories of bacterial variation. Thus, their classical designation as special "growth forms" is particularly appropriate, implementing as they do new "growth phases" in the culture's life history.

By way of integration of all these phenomena into a pattern which resembles even more that of the higher forms, we now come to the paradoxical character that led early bacteriologists to designate these specialized cell-forms as involutionary. It had to do with their viability, which at best was capricious, and at worst amounted to a virtual dormancy—a phenomenon which incidentally, is characteristic of similar specialized cell-structures of plant organisms from the yeasts on up the scale.

In this connection it seems particularly significant that the equivalent of the dormant state has been induced in yeast cells and the fibroblasts of the chick heart embryo by radiation with appropriate dosages of X-ray. (Quoted by Needham, 1932.) Moreover, by employing low temperatures which kill the chick embryo, its heart fibroblasts may have their growth totally inhibited, but without causing their death. In the X-ray experiments, the authors showed clearly that respiration and glycolysis were scarcely affected, despite the total growth inhibition. According to Needham (1932), this phenomenon is a clear example of "dissociation of some of the biologic functions occurring in the

ontogenetic cycle." That is to say, a dissociation of growth from the functions of respiration and glycolysis.

In any case, cells that are still respiring are not dead, despite their growth inhibition. In our wound-healing experiments (unpublished) we have discovered that certain chemical compounds very dramatically cause the cells of these dormant tissues to recover their ability to multiply.[13] In view of the evolutionary (variational) potencies shown for certain so-called "involution forms," is it not a fair presumption that their traditionally poor viability affords one more example of a dissociation of the functions of respiration and glycolysis from growth?

In such event there is no longer so much mystery behind the failure of the early bacteriologists to induce multiplication of these structures. And this applies to such modern supporters of the monomorphic point of view as Holman and Carson (1935), Henrici (1928), Rettger and Gillespie (1935) and Wyckoff (1934), to name but a few. The necessity of environmental adjustment in order to compensate those modulations undergone by the pleomorphic cells was not adequately considered in their experimental conditions. Not only has it been traditionally assumed that the poorly viable forms should be expected to multiply under conditions appropriate for the "normal" culture phase (that is, the phase most commonly recognized by bacteriologists); but experimental conditions were employed that were well known to be prejudicial, even for the actively growing phase.

While it is not possible to clarify here all of the confusion incidental to a misapprehension of the implications growing out of polyphasic potencies of the bacterial culture, the oft-repeated criticism of cell injury in its relation to cell-variability requires consideration. Briefly, it not only considered aberrant cell-types as degenerate beyond reclamation, but even actively growing dissociants were viewed by some as a pathologic expression, especially if they had been implemented by growth-inhibiting substances, as indeed they characteristically were!

Now this point of view is in flagrant disregard of a principle in biology so appropriate and so well established that it has found expression in the axiom that "dividing cells are not working (differentiating) cells." This is another way of saying that active cell multiplication is not only inconsistent with, but also often prejudicial to, cell-differentiation; and *vice versa*, cell differentiation is conditioned by growth-inhibition (bacteriostasis), or cell injury, if one pleases.

Nor has there been any sensing of these relationships in the chemotherapeutic field on the part of bacteriologists. Even one as traditionally reasonable in his attitude toward bacterial variation as Gay (1937), misinterpreted the phasic significance of the chained pleomorphic forms of the hemolytic streptococci as

[13] By inducing growth inhibition of actively growing fibroblasts with special chemical compounds, we are also able to modulate (differentiate) them into enlarged terminally-developing "coccoidal" structures, almost indistinguishable from macrophages. (Mellon and McKinney, 1941.) Yet with almost as much reason with the bacteria, they could be designated as "involution forms"!

they occur *in vivo* under sulfanilamide action. Their decrease in virulence and invasiveness escaped him because, under the conditions of his experiments, these pleomorphic modulants were given opportunity for quick reversion to their original virulence. Together with others who have been on the alert for signs of sulfanilamide's dissociative action, Gay apparently anticipated something as dramatic as the S and R pattern; because its failure to emerge resulted in a denial of any sort of variability. This was natural in view of the general failure to recognize that purely environmental modifications are indeed among the earliest beginnings of the S and R culture phases. In fact, when sulfanilamide is permitted to act long enough, as can be expedited *in vitro*, the S-culture phase readily results from the mucoid. (Hadley and Hadley, 1941.)

Despite the parallelism in several important respects between the phasic organization of the bacterial culture and that of the higher forms, as indicated above, we cannot go so far in this direction as Henrici (1928) and Winslow (1939). If we interpret their "culture cycle" correctly, they view the "differentiations" occurring in bacterial cultures incident to their age, as resulting in an organization *literally* the equivalent of a multicellular organism.

Thus Henrici says that ". . . there can be drawn no hard and fast line between populations of one-celled organisms and multicellular individuals; that a higher plant or animal is but a population of more highly differentiated cells." In thus drawing the most far-reaching conclusion that has yet been advanced in these matters, two considerations seem to have been overlooked. In the first place such a view implies an interdependence of function on the part of the differentiated cells that makes possible the function of the organism as a whole. The lack of experimental evidence for any such interdependence on the part of the phasic age-components of bacterial cultures is therefore highly unfortunate. Reinforcing it is Henrici's interpretation that, since the cell structures in question occur in the culture's death-phase, the only fate possible for them is death—quite the antithesis of their demonstrated rejuvenation as new culture phases.

Another sort of incongruity is presented when certain of these so-called involution forms multiply as such within colonies, until they result in secondary colonies which, as we have seen, often have the status of a new culture phase. Logically, it seems, the question is raised as to whether the new phase is to be regarded as a neoplasm (cancer) of the Henrici composite culture. Indeed, comparisons by cytologists (Carrel, 1931) have not been lacking that the process of bacterial dissociation was reminiscent of a neoplasm, particularly in respect to its interruption of cellular differentiation by cell multiplication.

Despite certain parallelisms between the processes *per se*, their relationships are oriented when we recall their antithetical effects within their respective biologic organizations. Thus, for the higher forms of organization, a malignant neoplasm means the death of the whole organism; for the bacterial culture on the contrary it is often the only guarantee of its life. That is to say, the phasic changes, being of the nature of an adaptation to environment, obviously have a high survival value.

Until these dissonances are reconciled, what Henrici refers to as a "perfect

analogy" between his bacterial cytomorphosis and the metamorphosis of a multicellular organism, must remain for the present, merely an analogy—nothing more. And by the same token, we cannot agree with Winslow (1939) that, "acceptance of the validity of this analogy . . . very greatly clarifies the long conflict between pleomorphists and monomorphists."

Finally, the writer does not wish to be understood as believing that all bizarre morphology in a culture has some hidden meaning. He believes that autolysis and death of cells is commoner perhaps than their survival; and yet, if some of the cells were not capable of developing resistance to environmental inclemencies, chemotherapeutic work would be less of a problem than it has proved to be.

SUMMARY AND CONCLUSIONS

Isolated from an anomalous clinical variety of tuberculosis, the streptothrix culture described is considered unique in that its variability range has been shown to include all the known categories of this phenomenon. Graded according to their stability and functional distinctness, they include first, that category where its several phases are stable in only a very narrow range of environment—our newly christened *modulation* category; second, those with a stability approaching that of the M, S, and R category of dissociative variation; and third, those that transcend species and generic limits—our *pleobiotic* category. In view of the highly unsatisfactory state of the criteria for bacterial mutation, we are not considering this phenomenon.

The earliest morphologic beginnings of the modulation category are the several kinds of specialized cell-types, traditionally known as pleomorphic or involution forms. Appropriate environmental selection favors sufficient differentiation of these cell-forms as to result in their active multiplication as distinct phasic entities. Accordingly, they can be, and have been, isolated culturally. They resemble the well-known environmental modification, being readily reversible to the "normal" phase of the culture when its environment is restored. The several different orders of pleomorphic cell-forms are viewed as modulants because the changes occurring in them can implement transitions between, and throughout, the variation categories. Thus, dissociative or variability phenomena are predicted on the inherently polyphasic nature of the bacterial culture.

The Johnson streptothrix was shown to have several modulant phases whose colonies were respectively rough, smooth, mucoid, globular, etc. Collectively, they are regarded as occupying a position in the dissociation cycle of the tubercle bacillus intermediate between its acid-fast critical rough stage and its non-acid-fast coccus stage. In addition to the variation experiments effecting such transformations, are the results of the serologic studies. The indications suggest that each modulant phase of the streptothrix has elaborated its own fragment of an antigenic mosaic that, when all of them are pieced together, serves to bridge the antigenic gap normally existing between the Critical Rough acid-fast stage and the non-acid-fast stages of the tubercle bacillus. A dissociative cycle among bacteria has certain characteristic differences distinguishing it from the life cycles of the higher forms, particularly in the animal kingdom.

The modulation category of variability has been closely integrated with the mode of action of the sulfonamide compounds, which does not appear to be so true for the other variation categories.

ADDENDUM

Long after this paper had been submitted for publication the writer listened to a Round Table discussion on genetics at the 1941 meeting of the Society of American Bacteriologists in Baltimore. Certain of the considerations brought forward by us are so interwoven with any discussion bearing on genetic variations among bacteria, that the Editor of the JOURNAL OF BACTERIOLOGY generously assented to their re-emphasis. This with the idea of "clearing the decks"—so to speak—for an unconfused consideration of genetic changes.

As fundamental to this objective is the necessity for the recognition of *criteria* that would serve to distinguish genetic variations from other kinds. Had this Round Table, consisting of ten papers, devoted mostly to the genetics of fungi, permitted discussion at its conclusion, the writer would have advanced this single basic consideration which, incidentally, was virtually untouched by the papers.

Particularly is this imperative when, after two or three decades of intensive study, the evidence is so formidable that we are dealing with phasic changes of the order of those encountered in differentiating mesoblastic tissues, for example, of the higher animals. Certainly there is no good reason for viewing these as of genetic implementation.

In view of this situation, those who would advocate the genetic nature of bacterial variations will be required to prove their contention in accord with truly genetic criteria. Since the latter rest largely on an organism's capacity to inter-breed, it is not exactly clear to the writer how such criteria are to be obtained with bacteria. But that is the problem of those who would make the claims. At present, it is clear that their only support rests on analogy with changes in characters that can be brought about in fungi by radiation, for example. Conceivably, these may in time grow in number to a point where they become impressive. Even so, conclusive proof will, of course, require evidence that is more direct than analogy.

In 1916, Cole and Wright demanded direct proof that bacterial variants were not merely the result of natural selection acting on mixtures of genetically determined biotypes, which they considered bacterial cultures to be. In short, single cell isolation was deemed necessary by them as a satisfactory criterion for the recognition of a "pure line," genetically speaking. An abundance of such isolations by many students of bacterial dissociation, has yielded overwhelming evidence that bacterial cultures are not after all mixtures of distinct genetic lines; but, on the other hand, consist of easily reversible phases, a point the present paper has sought to crystallize. Nor is the postulate of "reversible genes" so well established from a frequency standpoint that it can be invoked satisfactorily to explain our phase reversions.

In other words, the proponents of the genetic nature of bacterial variants

are in the same position as were those of us in 1916 who claimed that "bacterial variation of any sort involved something more than a "natural selection" of pre-existing variants (biotypes). We shall expect them to furnish us positive proof that what we regard as differentiated phases resulting presumably from nutritional changes, are genetically motivated, if indeed that is the claim.

While the writer has no doubt that true mutations and other genetic changes do occur among bacteria, we ourselves, have no criteria at the moment for distinguishing them from the environmentally implemented "dissociations"; nor are we aware of the existence of such. Nevertheless, we may anticipate that future experimental progress may develop them. Only after this has been done will bacteriologists be prepared to assess finally any variation as genetic, or otherwise.

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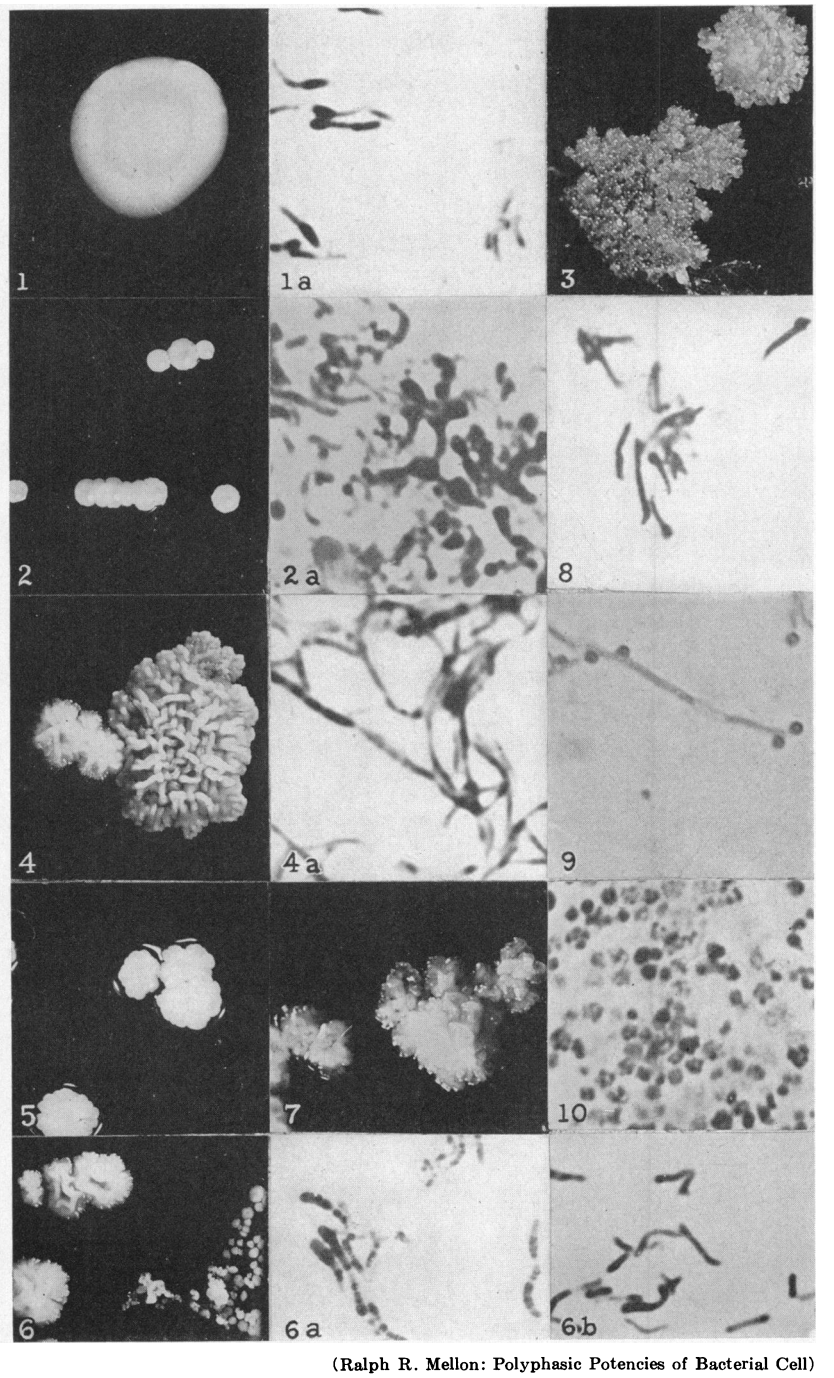
EXPLANATION OF PLATE 1

FIG. 1. THE MUCOID GROWTH-PHASE.
FIG. 1*a*. MICROSCOPIC VIEW OF COLONY 1 × 2000.
FIG. 2. GLOBULAR GROWTH-PHASE.
FIG. 2*a*. "PLEOMORPHIC FORMS OF GLOBULAR COLONY × 2000.
FIG. 3. GLOBULAR GROWTH-PHASE, COALESCENT COLONIES.
FIG. 4. ROUGH GROWTH-PHASE.
FIG. 4*a*. FILAMENTS OF ROUGH GROWTH-PHASE × 2000.
FIG 5. CONICAL ROUGH GROWTH-PHASE BATHED IN MUCUS AS INDICATED BY DETACHED MARGINAL AREAS—AGING COLONY.
FIG. 6. ADHERENT DIPHTHEROID-LIKE PHASE SHOWN IN SMALLER COLONIES.
FIG. 6*a*. ITS DIPHTHEROID-LIKE PHASE × 2000.
FIG. 6*b*. ITS SHORT FILAMENTOUS PHASE × 2000.
FIG. 7. CONICAL ROUGH PHASE IN TRANSITION TO THE GLOBULAR PHASE, AS INDICATED BY PROGRESSIVE TRANSLUCENCE DEVELOPING AT THE MARGIN OF THE COLONY.
FIG. 8. A LONG BACILLARY FORM OF THE CRITICAL SR PHASE OF THE TUBERCLE BACILLUS PRODUCING TERMINAL CONIDIA. × 1500.
FIG. 9. A FILAMENTOUS STRUCTURE FROM THE SAME CULTURE AS FIG. 8. × 1500.
FIG. 10. MULTIPLICATION OF THESE ACID-FAST CONIDIAL FORMS OF FIG. 9 IN RELATIVE PURITY.



(Ralph R. Mellon: Polyphasic Potencies of Bacterial Cell)