SECONDARY COLONIES OF BACTERIA WITH SPECIAL REFERENCE TO BACILLUS MYCOIDES

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Received for publication June 22, 1932

INTRODUCTION

Although the phases of growth which occur in a primary colony of bacteria are well known, our knowledge of the origin and significance of secondary colonies is incomplete and in many respects unsatisfactory. It has long been known that, under certain conditions, the colonies of many species of bacteria are capable of resuming growth after a more or less extended period of senescence. This secondary phase of growth may be of short duration, resulting in the formation of minute papillae, or it may be so prolonged as to result in the formation of well developed daughter colonies.

Various explanations have been offered to account for this phenomenon, but there are still differences of opinion both as to its cause and its significance. The literature of the subject has been reviewed by other writers, especially Dobell (1912), Penfold (1911), Hadley (1927), Arkwright (1930) and Stewart (1927). Some brief references must be made at times to the experiments and conclusions of others, but no necessity exists for a full review of the rather extensive literature.

Secondary colonies have been reported for many species belonging to various genera, but the coliform bacteria have received more thorough study by experimental methods than other groups. With a single exception, the spore-forming species do not appear to have been very thoroughly studied.

The first experimental study of this troublesome problem appears to have been that of Preisz (1904). He noted that old cultures of some, but not all, strains of B. anthracis became so
studded with papillae and secondary growth as to resemble a mixed culture. Since secondary colonies were never observed in asporogenic races but developed in cultures of spore forming strains heated to 65°C for one hour, he concluded that they owed their origin to germination and subsequent growth of spores within the primary colony. He noted the occurrence of similar colonies in cultures of *Vib. cholerae* and *B. diphtheriae*, which he attributed to the growth of cells with greater vitality and power of resistance to unfavorable environment. This early work of Preisz has been frequently quoted and appears to have been generally accepted, but evidence of confirmation for *B. anthracis* or other spore-forming species is lacking. On the other hand, there is much evidence that important factors are involved which he failed to recognize. The conclusions which he reached are, therefore, subject to doubt until fully confirmed.

Other spore-forming species which have been studied briefly are *B. subtilis* by Soule (1928) and *B. mycoides* by Lewis (1932). No attempt was made in either of these cases to determine the factors involved in the origin of such colonies, but experiments were performed in each case to determine whether or not the secondary colonies were due to dissociation. Subcultures from the smooth variety of *B. subtilis* were about equally divided between the two forms, while all strains derived from the rough colonies were of the rough variety. Subcultures from *B. mycoides* could not be distinguished from the parent strain.

The most important contribution to our knowledge of this subject was made by Neisser (1906) who first discovered the now well-known type which, because of its behavior on lactose media, is known as *B. coli-mutabile*. This organism does not ferment lactose, but gives rise to a subrace which has this capacity. This characteristic may be acquired in broth containing lactose or by papillation of colonies on lactose agar. The subrace ferments lactose and does not again produce secondary colonies. Reversion to the non-fermenting type occurs, in some instances, while in others very minute papillae have been observed on agar containing lactose. Since these fail to develop into true secondary colonies, their origin is doubtless due to some other cause.
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This important pioneer discovery has been completely confirmed by Massini (1907), Burk (1908), Reiner Müller (1909), Burri (1910), Baerthlein (1911, 1912), Mellon (1925), Stewart (1927) and Kennedy, Cummings and Morrow (1932). The same principle has been shown to apply to many species besides B. coli-mutabile, and various sugars or alcohols may cause the phenomenon.

Penfold (1911) showed that B. typhi forms secondary colonies on agar containing dulcitol and that subcultures are enhanced in power to ferment this compound. Similar results were obtained by Bernhardt and Markoff (1912) for a strain of B. dysenteriae Flexner when cultivated on maltose. Reiner Müller (1912), worked with three strains of coliform bacteria which could not ferment arabinose but which formed papillated colonies on arabinose agar. Subcultures from the papillae still failed to ferment the compound, but grew more vigorously in its presence. He reported similar results with B. typhi on agar containing rhamnose. When cultivated on raffinose agar, B. paratyphosus B produced secondary colonies with enhanced capacity for fermentation.

Additional examples might be cited, but could add nothing of value, since the principle appears to be the same in all cases. Variation in presence of a specific sugar or alcohol with formation of secondary colonies which show newly acquired or enhanced capacity for fermentation of the specific compound and failure to produce secondary colonies has been shown in each case. The secondary phase of growth appears to be due, therefore, to beneficial variation.

Various other theories have been advanced to account for the development of this secondary phase of growth in colonies but, aside from the work of Preisz and that of the authors previously mentioned, there is not much evidence of experimental investigation. In most, if not all, of these cases the explanations are rather vague and with no foundation other than theoretical reasoning based on uncertain hypotheses.

Because B. mycoides was previously known to form secondary colonies under some conditions, it appeared to be a suitable
species for investigation. The present study is concerned specifically with an attempt to discover whether or not the secondary colonies arise in this species from spores or from vegetative cells, and whether the principle of variation in response to carbon compounds applies.

EXPERIMENTAL

The strains of B. mycoides employed in the experiments included the four strains (A, B, C and American Type Culture Number 80) previously studied as to dissociation and life cycle, Lewis (1932). Two variants of strain C and fourteen additional strains isolated from soil, air and water were also included.

The first experiment was performed to determine the extent of secondary colony formation on standard beef-extract peptone agar. Cultures consisting of giant colonies on the surface of agar in petri plates were held at room temperature for a period of ten days, protected against desiccation. At the end of this period strain C, previously known to form such colonies, its two variants numbers 22 and 94 and one additional strain, A2, indistinguishable from C, were positive, while all of the remainder were negative. The cultures were held for a total period of sixty days, but with no change in the results. It thus appears that this medium is unsatisfactory for the phenomenon or that it does not occur in a great majority of the strains.

Attention was next directed at the effect of specific sugars and alcohols. B. mycoides is generally recorded as producing acid from glucose, maltose, glycerol and sucrose, but not from lactose or mannitol. The several strains were cultivated as giant colonies on the surface of standard nutrient agar containing 2.0 per cent. respectively, of each of the above carbon compounds and the proper amount of phenol-red indicator. The plates were inspected daily for a period of ten days and at frequent intervals throughout the incubation period. The fermentation reactions which were to be expected occurred in all cases except sucrose. There was no formation of acid in the case of lactose or mannitol while all strains fermented glucose, maltose and glycerol promptly.

Differences in capacity to ferment sucrose were evident within
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twelve hours. Six strains produced sufficient acid to cause complete reversal to the full acid color of the indicator during this period, while all others became more strongly alkaline, and remained so indefinitely. The acid strains eventually reverted to the alkaline reaction but only after a period of about two weeks.

Since simultaneous formation of acid and alkali or failure to produce acid under aerobic conditions might cause the results obtained for the non-acid strains, these were studied further in test tube cultures. The medium consisted of a 1.0 per cent peptone solution containing 2.0 per cent sucrose plus phenol-red indicator. Cultures were prepared in both liquid medium and in deep tubes of agar.

The liquid cultures of all strains showed some slight change toward the acid side of neutrality within twenty-four hours, but in no case was the full acid color reached except on prolonged incubation. Four strains reverted to the alkaline reaction within forty-eight hours, with no subsequent reversal of reaction to acid. The remaining ten strains reached the full acid color in periods varying from five to fifteen days, after which no reversal occurred.

The cultures in agar tubes became alkaline at the surface in a zone extending downward to a depth of about 1 or 2 cm., but in all cases the deeper layers became acid within twenty-four to forty-eight hours. This shows that these strains do not attack sucrose, or at least not vigorously, in the presence of oxygen but are capable of sucrose fermentation from the beginning under conditions of partial anaerobiosis. If any acid is formed at the surface it is masked by the more vigorous formation of alkali.

The slow fermenting strains of B. mycoides are thus seen to vary toward sucrose in much the same manner as do those of B. typhi to dulcitol, as shown by Penfold (1911), but in a somewhat different manner from the reaction of B. coli-mutabile to lactose. It remains to be shown now that this similarity in fermentation extends also to the matter of secondary colony formation.

Results on agar plate cultures are shown for secondary colony formation in table 1. All of the slow fermenting strains ex-
cept A₂, C and its two variants, 22 and 94, formed secondary colonies on sucrose, but not on any of the other carbon compounds tested. None of the rapid fermenters (A6, 17, 23, 402, 405, 407) produced secondary colonies on any of the media. It

### TABLE 1

<table>
<thead>
<tr>
<th>NUMBER OF STRAIN</th>
<th>COMPOSITION OF MEDIUM</th>
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<tr>
<td></td>
<td>Beef extract, 0.3 per cent</td>
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<tr>
<td>A.T.C. A</td>
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<tr>
<td>B</td>
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<td>C</td>
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<td>424</td>
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+ = Secondary colonies.  - = No secondary colonies.


Strains C, 22, 94 and A2 gave final alkaline reaction in sucrose peptone water.

Strains A.T.C., B, 410, 416, 417, 419, 420, 421, 422 and 424 caused delayed fermentation of sucrose with final acid reaction.
is a point of considerable interest that the slow fermenters which failed to form colonies of secondary origin on sucrose agar had been previously shown to be the only positive strains on plain agar. An explanation for this will be offered in connection with additional experiments. In regard to secondary colony formation, the three groups of strains of B. mycoides react to sucrose in a manner almost identical with that of colon bacteria to lactose. The acid strains are comparable to B. coli which ferments the specific compound promptly but does not form secondary colonies. The strains which cause delayed fermentation are similar to B. coli-mutabile, while the four strains which fail to cause delayed fermentation or produce secondary colonies are more like B. paracoli.

A point of considerable interest was observed in the correlation between right and left hand spiral symmetry and sucrose fermentation. Prompt fermentation correlates, in general, with left hand spiral growth (strains A6, 17, 23, 402, 405, 407), while delayed fermentation correlates with right hand turning of the threads (strains A.T.C., A2, 22, 24, 62, 94, 410, 416, 419, 420, 424). The correlation was not quite perfect, since three strains (417, 421, 422) with left hand spirals were classed as slow fermenters and produced secondary colonies. Without exception the right hand strains proved to be unable to cause prompt fermentation of sucrose. It is no doubt true that the collection contained many duplicates which could not be distinguished from each other, but there were some strains in each group which could be readily distinguished by appearances of the colony and growth rate. Strains A2 and C differed from all other members of the right hand group by forming much longer and less robust threads. They differed also in not being capable of adaptation to sucrose, which was also true of the two variants from C, numbers 22 and 94. Strain 402 resembled other members of its group, but the growth on plain agar was much less vigorous and the colonies soon became scarcely visible as a film of glassy threads. When grown on sucrose agar, it could not be distinguished from other strains. It appeared to suffer from carbon deficiency in the absence of an added source. The sec-
ondary colonies appear rather promptly in all strains capable of forming them. The A.T.C. strain begins to show very minute papillae by the sixth day, while other strains require a somewhat longer period. In all cases where papillation had not occurred by the fifteenth day the result was negative at the end of two months.

The first papillae are invariably located on the oldest portion of the colony. For the purpose of examining the progressive development of the parent colony and the secondaries, the A.T.C. strain was found to be most satisfactory. It spreads much more slowly, requiring a period of about two weeks to cover the surface of the plate. The papillae likewise develop slowly and do not reach mature size until about the fifth day after they become visible. At maturity they are remarkably uniform in size, shape, and consistency. The maximum size is about 2 mm. in diameter. They are much whiter than the original threads of the parent colony and contrast sharply with the bluish background. Figures 4, 5, 6 show various aspects of this strain.

At the end of about three weeks the whole surface of the plate is thickly studded with secondary colonies. The parent colony as a whole grows but little, if any, better than on plain agar, and there is never any change in the size or vigor of filaments as they spread out from the line of inoculation. This indicates that only certain cells are capable of variation toward the sugar and that there is no mass transformation, as has been claimed by some previous workers.

Subcultures are easily obtained, since the colonies are relatively large and stand out well above the background of threads. Dilution plates prepared from a suspension of a bit of the colony invariably produce nothing but typical thread-like growths indistinguishable from the parent type. In one experiment forty subcultures were picked to sucrose agar slants from a plate prepared from a single secondary colony. These were subsequently transplanted to sucrose agar plates and tubes of peptone water containing sucrose. The results of this experiment showed that the secondary colony produces a race more active in fermentation of sucrose than the original, and not capable of again producing
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secondary colonies. Thirty-five of the substrains belonged to this type, while five were like the original parent both as to slow acid formation and ability to form the secondary growth.

In this connection it should be pointed out that, with one possible exception, none of the original strains had been in previous contact with sucrose between the time of isolation and the performance of these experiments. The possibility that the active fermenters had acquired this habit through previous cultivation after the manner just described is thus excluded. A complete record of the A.T.C. strain was not obtained, but its behavior would indicate a non-sucrose history. This strain forms an abundance of secondary colonies when cultivated on sloped sucrose agar in test tubes, and it would appear that, after a few transplants made at long intervals, the original slow fermenting type might easily be completely lost.

Subraces were established from secondary colonies of each of the ten strains which produced them. These races not only fermented sucrose more rapidly than the originals, but they grew with greatly enhanced vigor on media which contained sucrose. The relative vigor of strain A.T.C. (original and one of its sucrose variants) is shown in figure 4. The plate was inoculated with a mixture of the two strains. It may be seen that there is not only a striking difference in the vigor of growth, but that secondary colonies which are developed in abundance on the original type are wholly lacking in the variant.

The permanence of the variants has not been determined fully, since the oldest strains have not been cultivated on sucrose-free media for a longer period than approximately three months. During this time ten transfers have been made, and at the same time tests for reversion have been made on sucrose media. Reversion has not occurred during this period in any of the several subraces tested. There has been no loss in capacity to attack sucrose, and secondary colonies are not produced. The variant races, when cultivated on plain agar, are not more vigorous than the original and do not appear to differ from it in any character except ability to ferment sucrose and lack of capacity to form secondary colonies.
The experiments to be reported next were directed at the cause of secondary colony formation in culture media which do not contain carbon compounds in the form of sugars or alcohols. Many such cases have been reported, but it is here that we approach the phase of our problem where little or no experimental evidence is available.

The cases reported by Preisz (1904) have already been mentioned. His explanation that the phenomenon is due to the germination of spores or other more resistant cells within the primary colony appears to have met with wide acceptance and has been frequently cited as the explanation for the resumed or secondary phase of growth.

Eisenberg (1906) described a type of secondary colony formation which he regarded as different in principle from that previously reported by Preisz. He believed that certain cells might become detached from the parent colony and make their way into the depths of the substratum, either by their own power, as in the case of motile forms, or by diffusion in other cases. Such cells may develop secondary foci of growth which first become visible as granulations and in certain cases develop into deeply-lying secondary colonies. These could not be seen in some species until after the surface growth had been scraped away. Such colonies always developed promptly (within the first or second day) and never at the surface of the primary growth. Whether this phenomenon is of common occurrence or not is impossible to say. Eisenberg believed that the nature of the culture medium was of some importance and that such colonies were more abundant when the medium contained either blood serum or egg albumin. Although he was careful to point out that the phenomenon differed from that described by Preisz, some recent writers have made no such distinction. Thus, Hadley includes the entire list given by Eisenberg in his compilation of secondary, colony-forming species. It is obvious that if such colonies originate in the manner described by Eisenberg they have nothing in common with secondary colonies which form only after the primary growth has reached the period of senescence, and that they are of no particular significance.
In order to determine the extent of secondary colony formation in the absence of specific sugars and alcohols, the several strains of \textit{B. mycoides} were cultivated on a variety of culture media. These included agars prepared from casein digest according to the directions given by Kristensen (1925), tryptophane broth (desiccated bacto product) 1.0 per cent, peptone alone 1.0 per cent, bacto beef extract alone 1.0 per cent, and aminoids of beef 1.0 per cent. The results are shown in Table 1 and Figure 2.

It may be seen from the table that no secondary growth occurred on either beef extract alone or aminoids of beef, while on the digested casein media and peptone alone some strains proved consistently positive and others negative. It is a point of interest that six strains (A6, 17, 23, 402, 415, 407) which had previously been found negative on sucrose proved to be positive on the peptone and digested casein media, and that all strains (A2, C, 22, 94) which had previously been found positive on plain agar but negative on sucrose were positive on peptone and casein digest. With three exceptions (417, 421, 422) all strains which formed secondary colonies on sucrose agar were found negative on these media. It should be noted that these are the strains in which the direction of spiral growth and sucrose fermentation failed to correlate. Their behavior on peptone and casein digest shows that they respond to nutrients in a different manner from all other strains, since they alone were positive on sucrose and peptone. As in the case of sucrose agar, secondary colonies did not appear in any case until a period of from six to eight days had elapsed.

Various brands of peptone were tested, including bacto, Witte, Jensen, Fairchild, Parke Davis and proteose. No difference whatever could be observed in the case of the bacto, Fairchild and Parke Davis products, which proved to be the best suited for this purpose. Proteose peptone supported more vigorous growth, but the secondary colonies appeared less promptly and never became as large or well developed. Primary growth on Witte's peptone was less vigorous than with other products, and the phenomena of secondary colony formation were somewhat different. The primary growth failed to spread in the characteristic
fashion and had little resemblance to a colony of *B. mycoides*. Threads grew out eventually for a distance of about two centimeters as a very thin almost imperceptible halo. This was followed by development of secondary colonies which remained small, discrete and very numerous on the thin portion, but larger at the extreme margin where they became confluent and thus formed a wall or border. A second fringe developed which in time also produced secondary colonies. This same phenomenon was observed on some, but not all, of the strains on both casein digest and tryptophane agar. Jensen’s peptone supported a more vigorous growth of all strains, but no secondary growth occurred. It thus resembled the beef extract and aminoids of beef rather than the other brands of peptone.

Subcultures were prepared from all positive strains by plating methods. In all cases the thread-like typical form was obtained, but the subraces grew with enhanced vigor and failed to produce secondary colonies either on the same or on a different brand of peptone.

When sucrose was added to 1.0 per cent peptone the strains which had produced secondary colonies on beef extract peptone agar containing sucrose were all found to be positive, while all other strains positive on peptone alone or peptone plus beef extract became negative. No secondary colonies were formed on 1.0 per cent peptone plus 2.0 per cent glucose, maltose, or glycerol, nor by any but the four already mentioned (A2, 62, 22, 94) when beef extract was added. In this case less than 0.2 per cent was not sufficient to suppress their formation in the remaining peptone-positive strains (A6, 17, 23, 402, 405, 407). The addition of 0.2 per cent sodium citrate, tartrate or acetate to 1.0 per cent peptone agar did not cause suppression of secondary colony formation in any of the peptone-positive strains.

These experiments show that secondary colony formation may be caused in some but not all strains by nutritive substances other than sugars and alcohols. This fact does not appear to have been fully appreciated in the past. Both peptone and casein digest are complex substances which contain some fractional parts which are more readily available as nutrients than
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others. Until experiments are conducted with synthetic media and purified derivatives it will not be possible to designate the precise substances concerned. The results indicate that the more highly digested products, such as aminoids of beef, are more completely utilized by all of the cells and that variation is therefore less likely to occur.

The results presented thus far show that differences exist in the nutritive requirements necessary for the formation of secondary colonies by the several strains of *B. mycoides*. Such colonies may be produced under the following conditions: (1) when sucrose and protein derivatives are both in the medium but not on a medium otherwise identical which lacks sucrose (7 strains); (2) when the agar contains protein derivatives alone but not when sucrose or certain other readily available sources of carbon are also present (10 strains); (3) when protein derivatives alone are present, and in a medium of the same composition but containing sucrose also (3 strains). The presence of a non-utilizable carbon compound has no effect. The distribution of the strains has been previously discussed and is shown in the accompanying table.

It has also been shown that subraces from the secondary colonies vary from the parent race in capacity to attack the specific compounds which must be present to cause their origin and development. This variation does not appear to extend to other characteristics such as the form of the colony or size, shape, and structure of the cells. When cells were examined from the secondary and primary colonies, some differences could be noted, but when subraces which varied with sucrose were cultivated on sucrose-free medium and examined, they could not be distinguished from the original, when cultivated in the same medium. It is believed, therefore, that these differences in the morphology of cells taken directly from secondary colonies are no greater than can frequently be observed when the same strain of an organism is cultivated on different media. Secondary growth occurs on a medium which is quite different from that which supports the primary growth. This is due in part to changes which have occurred in the medium itself such as ac-
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cumulation of metabolites, depletion of the nitrogen-bearing compound and desiccation. But the enhanced ability of the organism to utilize sucrose is probably of more importance than any changes which have taken place in the medium. It is not to be expected that an organism would show precisely the same details of morphology when cultivated on sucrose agar and on agar not containing sucrose. This is very well illustrated by a decrease in spore formation and by more pronounced tendency to pleomorphism shown by strains of *B. mycoides* when cultivated on media containing utilizable carbohydrates or alcohols. These same tendencies are shown by the secondary colonies borne on sucrose agar cultures. This point does not appear to have been fully appreciated and has doubtless led to some confusion in the past.

Since variation has been shown to occur in all of the cases of secondary colony formation considered here, the experiments which follow have been directed at its nature and its causes. Although variation has been studied since the earliest days of bacteriology, there is probably no phase of the subject concerning which there are so many conflicting opinions. There is no intention at present to treat of this subject in a general way, but it is necessary to consider the factors involved in the special type of variation which leads to the formation of secondary colonies.

There is little or no difference of opinion that enhancement of fermentative power occurs only when the organism is cultivated in contact with the specific compound with which the variation occurs. It is likewise recognized that the variation depends also on inherent potentialities of the organism, since no amount of contact can cause variation in all cases.

Henderson Smith (1913) expressed some doubt as to the validity of the former statement. He regarded it as more probable that *B. typhi*, which varies in dulcitol tends to produce variants even in plain broth, but since dulcitol is absent and the characteristic is not well pronounced at the beginning, it is lost through lack of a selective agent. There seems to be no experimental evidence which supports this theory.

A somewhat similar explanation but differing from it in some
important details is the well-known theory of "pure lines" or biotypes. According to this view, a mass culture of bacteria may consist from the beginning of some cells which have the fermentative capacity well developed and others which have no such capacity. The sugar or other compound serves merely as a selective agent which picks out the pure line which has been present from the beginning. Results obtained by single cell isolations from such forms as B. coli-mutabile seem to exclude the possibility of pure line selection in many cases.

A still different explanation for this type of variation is based on the theory that all cells within a mass culture are alike at the beginning, wholly unable to attack the compound, or to do so but feebly. Any enhancement of this power must, therefore, be due to a specific variation by some or all of the cells. According to this view, the sugar serves as a specific inciting substance which stimulates a feeble or latent metabolic capacity to activity. Since the variation is at the same time beneficial, it is perpetuated in successive generations. This view appears to be in harmony with many observed facts.

The earlier workers, Neisser (1906), Massini (1907), Burri (1910), Reiner Müller (1909), arrived at different conclusions as to whether the full fermenting capacity is gained abruptly, as in the mutations of higher plants and animals, or whether it occurs by a slow gradual process of enhancement. More recently Stewart (1927) concluded that variation is caused either by mutation, which occurs but rarely, or by Mendelian segregation in such cases as the so-called mutabile strains of bacteria. Mellon (1925) explained it as due to chromatic rearrangements caused by isogamous conjugation.

Some light is thrown on these questions by the behavior of B. mycoides strains when cultivated in media which contain sucrose. Mere contact with the sucrose for given periods of time, which might apparently be prolonged to any degree, was not found to cause variation. This was determined by the method of daily serial transplants on media containing sucrose. Strain A.T.C. was carried through a series of twenty-five daily transplants on tubes of sucrose agar without acquiring any enhance-
ment of power to ferment sucrose or any lessening of the time period required for secondary colony formation.

Similar behavior was noted on the giant colonies which grew on sucrose agar in petri dishes. After a period of five to six days, the colonies began to appear as minute papillae on the oldest portion of the colony. As these papillae enlarged to form the secondary colonies new papillae appeared daily on the zone immediately beyond, until the whole surface became covered with the mature secondary colonies. There was never any formation of papillae until the primary threads had reached an age of five or six days. When the behavior was first noted and before much attention had been given to the matter, it was thought that this denoted origin of the colonies from spores, but in view of some experiments yet to be reported this is no longer believed. Stewart (1927) reported that a strain of *B. coli-mutabile* could not be caused to form secondary colonies sooner by preliminary cultivation in lactose broth for several hours before plating. The colonies were obliged to reach a certain stage of maturity without change of environment before the papillae began to develop. Papillation could be hastened or delayed depending on the number of colonies present. This was thought to be due to a shortening of the growth phase due to environmental conditions. Large colonies required a longer period for papillation and it could be delayed indefinitely by serial transplanting at short intervals. Mellon (1925) reported results the reverse of this. He found that crowded colonies frequently failed to form secondary colonies and explained this as due to the reaction itself or some metabolic product associated with it.

Results reported by Drudgeon and Pulvertaft (1918), who studied the delayed fermentation caused by several strains of *B. coli*, show that the reaction was not hastened when the bacilli were transplanted twice daily in lactose broth for a period of ten days.

Twort (1907) was able to “train” various species so that after a time they became able to ferment compounds which at the beginning they could not attack. His method consisted of subculturing at rather long intervals. He reasoned that a sufficient
period must elapse during which the protein constituents of the medium would become depleted and that the organisms with any such capacity would then attack the sugar. That this procedure was based on a sound physiological principle so far as depletion of the peptone is concerned has subsequently been shown by Berman and Rettger (1918) who studied, by precise methods, the influence of carbohydrates on nitrogen metabolism of various bacterial species. As would be expected, a non-utilizable carbohydrate, lactose, had no effect on the protein metabolism of *Proteus vulgaris*, since the protein was completely broken down in its presence.

It does not of necessity follow that Twort's expectation of a subsequent attack on the sugar would always apply. Numerous attempts by others to duplicate his results on different strains of *B. typhi* have failed. This was explained in part by Penfold (1911) who found that the original strain employed by Twort did not ferment lactose but showed greater powers of fermentation for certain other compounds than was to be expected from a typical strain of *B. typhi*.

There is some evidence (Bronfenbrenner and Davis, 1918, and Kennedy, Cummings and Morrow, 1932), that daily transplants may in some cases cause enhancement of fermentation, but this is probably due to differences in capacity of the strains at the beginning. There is no doubt that this method is wholly without effect in the strains of *B. mycoides* which are considered here.

This could be explained in the case of *B. mycoides* on the assumption that secondary colonies arise from spores and that variation is in some way caused in the process of sporulation. Such an explanation has been suggested by Stewart (1927), who regarded variation as due to an autogamic method of reproduction which, in the spore-forming species, takes place in sporulation. But this is based on an assumption which has every appearance of being false.

The fact that *B. coli-mutabile* could not be caused to form secondary colonies until the primary colony reached a certain stage of development was explained by Stewart (1927) as follows:
Now it is just before or shortly after that the papillae appear and papillae are daughter colonies which continue the growth of the parent without any change of surroundings. Are we not justified in suspecting that they originate from individuals which have conjugated and that the intrinsic factor which arrests the growth of the colony is the completion of a cycle by an outburst of conjugation . . . . We can, therefore, admit that the growth and arrest of growth of the bacterial colony is determined by an intrinsic rhythm.

The principal evidence presented in support of this hypothesis was that the causes generally accepted to account for arrest of growth are not sufficient and that the papillation of colonies of B. paracoli on media containing lactose or of B. coli-mutabile on sugar-free media are not explained by the principle of beneficial variation. This evidence does not stand critical analysis. The factors involved in arrest of growth are well known and universally accepted and the papillation in question was doubtless due to some peptone fraction in the medium.

A better explanation can be offered which is in harmony with all of the observed phenomena and with fundamental physiological principles. Arrest of growth is due not to intrinsic but to extrinsic factors chief of which are depletion of nutrients and the accumulation of harmful metabolites. It is probable that no general law is applicable to all cases but that different extrinsic factors may cause arrest of growth in different organisms. The behavior of the so-called mutabile strains indicates that sufficient nitrogen remains to support growth in the presence of an available source of carbon. It appears that in a complex culture medium containing more than one source of carbon (either peptone fractions alone or peptone plus a carbohydrate or alcohol) the more readily available source of carbon is attacked first while the less available or non-utilizable remains. It appears to be probable that no variation occurs until a sub-critical stage is reached when some or all of the cells begin to utilize the carbon compound. This sequence of events accounts for the fact that secondary colonies are not formed until the primary colony reaches a definite stage of development and explains the failure to obtain enhancement of fermentation by rapid serial transplants in a medium containing peptone and a carbohydrate.
Before Twort's theory of the depletion of preferred nutrients can be accepted as the final explanation for the secondary phase of growth it must be determined whether or not such a phase occurs in synthetic culture media containing single sources of carbon and nitrogen. Other species of bacteria are better suited for this purpose than \textit{B. mycoides}. The behavior of \textit{B. coli-mutabile} in a synthetic culture medium containing ammonia and lactose, or \textit{B. paratyphi} B in a medium containing ammonia and raffinose, as the sole sources of nitrogen and carbon should afford interesting results. Since it appears to be true that variation in a complex medium results from utilization of residual nitrogen and the second carbon compound, it is quite probable that variation may be found to occur also in a synthetic medium containing the same source of carbon and a non-carbon bearing source of nitrogen. It is to be expected, therefore, that secondary colonies may be found to develop on simple synthetic agar.

A final factor of importance must be noted. In order for the secondary phase of growth to occur, the conditions, aside from available food, must have remained suitable for growth. Such conditions are probably never as satisfactory as in fresh media and it is doubtless true that in many cases growth ceases before a condition of critical nutritive stress is reached. The most important factor involved here is probably the change in hydrogen ion concentration, but other products of metabolism are doubtless of some importance.

According to this view, secondary growth within the primary colony is due to a beneficial, variational response to hunger. It is obvious that the variant cells, in some cases, obtain additional advantages such as reversal of an unfavorable alkaline reaction due to subsequent formation of acid by the variant cells. The secondary colony could be expected to follow the same general method of development as a primary colony and in turn reach its own senescent phases. Whether a second secondary phase occurs in all cases must be regarded as doubtful. Such a condition, designated as tertiary colonies, was reported by Preisz (1904) for \textit{B. anthracis}, by Enderlein (1925), and Hadley (1927) for other species. Such tertiary colonies have not been seen in
any of the strains of \textit{B. mycoides}. The formation of tertiary colonies would depend on whether cells of the secondary colony are capable of still further enhancement through variation and whether conditions within the colony have remained suitable for growth.

An experiment of some interest in this connection was performed to determine the effect of reaction and other metabolites on growth of the primary colony and the formation of secondary colonies. The original strains were cultivated in standard nutrient gelatin for a period of sixty days. The medium was then restored to full nutrient value by the addition of peptone and beef extract, but the reaction was left unchanged. This liquid, containing the original growth and metabolites, received 2.0 per cent of agar and was then sterilized at 15 pounds pressure. Each medium was inoculated with its homologous culture and poured into Petri plates or poured and then inoculated on the surface. The resulting colonies bore little or no resemblance to typical colonies of any of the strains. Streak cultures on the surface invariably formed a dense restricted mat with no tendency to spread over the surface. After about two weeks, secondary colonies developed on some of the strains but not on others (fig. 7). The colonies on seeded plates remained small and compact and could not be easily distinguished from true secondary colonies (fig. 3). It is thus shown that the changes which occur in the medium, aside from depletion of nutrients, influence the form of the secondary colony. It is also shown that digested gelatin contains substances which, like other digested protein, suffice for secondary growth. On a medium prepared in a similar manner but converted into agar at the end of two weeks, the form of growth was influenced somewhat but was more nearly typical. Again secondary colonies appeared (fig. 1). The strain illustrated (strain B) did not form secondary colonies on peptone alone or on peptone plus beef extract.

There has been a tendency to regard the secondary colony as arising in some way through a response to these subsidiary unfavorable conditions, but to disregard the nutrient relationships, the importance of which had been clearly indicated by the work
of Neisser (1906), Massini (1907) and others of the earlier workers. It seems very doubtful whether secondary growth could ever be due to the development of cells better able to withstand unfavorable conditions or which possessed greater vitality, as assumed by Preiss for Vib. cholerae and B. diphtheriae, or by cells which are able to overcome an inhibition, Arkright (1930), unless the unfavorable condition or the inhibition is conceived of as due to deficiency in the food supply. Quite similar to this is the notion that salt in sufficiently high concentration may have the same effect. This has not been tested experimentally for B. mycoides, but Enderlein (1916) reported that secondary colonies were formed by Serratia marcescens on agar containing an excess of sodium chloride. The experiment could not, however, be confirmed by Ramchandani (1929). The principle of so-called drug fastness or enhancement of capacity to resist harmful chemicals would appear to offer a means of testing this matter. In any case the variation should correspond with the substance and in all cases which have been carefully studied the variation has been to a specific nutrient substance.

It may now be pointed out more definitely how the principles just enumerated correlate with and enable us to explain the behavior of the three groups into which the strains of B. mycoides may be separated. These groups have previously been shown to correlate with the composition of the culture medium. The strains of group one obtain their carbon principally from the nitrogen bearing compounds during the early phases of growth but are capable of variation in response to sucrose. Accordingly, when the period of nutritive stress is reached, variation occurs, and this results in the development of secondary colonies. The strains of group two behave in a similar manner when cultivated on agar containing protein derivatives alone, but in this case variation appears to be related to some unused fraction of the digested protein. When sucrose is added, no secondary growth occurs. This is due to the well known protein-sparing effect of a utilizable carbohydrate. The rapid fermenting strains of this group are not capable of further enhancement in fermentative power, and colonies are not formed due to response to sucrose.
Since the course of nitrogen metabolism is hindered by formation of acid and other metabolites, growth is completely arrested before the critical stage is reached and the protein fraction which, in the absence of sucrose causes secondary growth, is not attacked. The slow fermenting strains of this group utilize the carbohydrate, but have no power to become enhanced in power through variation. The strains of group three which are capable of secondary growth with or without sucrose, vary to the sucrose in preference to the protein. The addition of a non-utilizable carbon compound is without effect in all of the groups, since nitrogen metabolism goes on unhindered and the organism lacks the capacity to vary in response to the added carbon compound. This was found to be true not only for carbohydrates and alcohols but for organic acids as well.

Whether the variation occurs abruptly or by slow gradual process of change does not appear to be of any special significance, although it has been a matter of interest. If variation is due to isogamous conjugation, as assumed by Mellon (1925), or to autogamy, as claimed by Stewart (1927), then we would expect it to occur suddenly, as in the case of true mutation or Mendelian segregation; but if this does not occur, which is altogether more probable, and the enhancement is due simply to an awakening of a latent function, then either might occur. Although slight differences have been observed in pure subraces isolated from secondary colonies of different age, the results were somewhat conflicting and no definite conclusions are possible.

In accordance with the work of Preisz on sporogenic and non-sporogenic races of *B. anthracis*, it has generally been believed that secondary colonies arise from spores in the sporogenic species, but the evidence has been meager. Since all of the strains of *B. mycoides* included in this study were found to be about equal in capacity to form spores, none of the differences already reported could be due to this cause.

The sucrose variant type was never obtained by plating methods from five day sucrose agar cultures when suspended in water and heated to 85°C. for a period of ten minutes, but was invariably present when the suspension was plated before heating.
Similarly, five day cultures on sucrose agar plates held at 65°C. for a period of two hours failed to form secondary colonies, although the spores were capable of growth when transplanted to fresh media. We are obliged to conclude from these experiments that the secondary growth of B. mycoides is due not to spores but to vegetative cells.

This is not wholly surprising, and in the light of all preceding experiments it would, indeed, seem to be more probable on theoretical grounds alone. Among the considerations which favor the origin from vegetative cells are the following: (1) there is no real reason to suppose that spores would be more likely to germinate in the media which support development of secondary colonies than in others which do not; (2) it is unlikely that variation is in any way connected with the process of sporulation or that it could occur after the spore is formed; (3) the method of secondary colony formation appears to be identical with the same process in non-spore forming species such as B. coli-mutabile, and others; (4) the conditions which cause sporulation are not perfectly known, but there is no reason to suppose that such conditions would be favorable to germination; (5) if variation occurs before sporulation, the added advantage should result in continued vegetative activity.

GENERAL DISCUSSION

Although the method of secondary colony formation which has been reported for B. mycoides has long been known, there has been a general impression that its application extends only to a few special cases of enteric organisms when cultivated in the presence of certain carbohydrates or alcohols. The experiments reported here have shown a much wider application of the principle than has been fully recognized in the past, and cast some doubt on various explanations that are based on principles which are not so well established.

The conclusion has been reached by various workers that some special biological significance must be assigned to this phenomenon and that this is to be found in a method of reproduction other than ordinary fission. Although such explanations are
based more especially on assumptions rather than experimental investigation, a certain type of experimental evidence has been presented in some cases. It is desirable to subject some of the various explanations to critical analysis in the light of all the known facts.

Mellon (1922) writes as follows:

As regards the biological significance of these secondary colonies, I might state tentatively as the result of observations covering several years that they represent a special stage in the development of the culture and are to be regarded as the macroscopic counterpart of what we speak of microscopically as pleomorphism.

In a later communication (1925) he speaks of *B. coli-mutabile* as a transitional or developmental stage between "the wild non-lactose fermenting *B. coli*" and the normal fermenting strain from which the latter arose. Pleomorphism, according to Mellon's well known view (1925d), is due to isogamous conjugation. In harmony with this view he considered *B. coli-mutabile* as a form derived from *B. coli* by a process of sexual reorganization resulting in the loss of capacity to ferment lactose. "The loss of *B. coli*’s ability to ferment lactose means that the developmental phase bearing this character (the secondary colonies) is absent or undeveloped."

It is not made clear whether the presence of lactose was regarded as exerting any influence on the sexual process or not, but the impression is gained that the sexual method of reproduction leads to variation which becomes manifest by "phases" which may or may not ferment the sugars. There is no ambiguity, however, in the statement that "bacteria are fungi whose life cycle is not completely known or at least not always completely manifested under the conditions of observation." This is directly in harmony with the notion of a complex life history and cyclogenic phases analogous to the fruiting or so-called perfect stages of certain fungi.

A more definite explanation of the relation of sexual reproduction to secondary colony formation is found in the work of Stewart (1926, 1927). Starting with the general hypothesis by which he
explained growth and arrest of growth as a process of "intrinsic rhythm" involving a vegetative and reproductive phase, he arrived at the conclusion that bacteria follow a Mendelian manner of inheritance. According to this view, B. coli-mutabile or any other species of similar behavior is to be regarded as the heterozygous form which gives rise to homozygous dominant and recessive strains through segregation of characters. The subrace which ferments the sugar or alcohol but does not again vary in response to it is regarded as the homozygous recessive, while the race which neither ferments the compound nor gives rise to a fermenting race is the homozygous dominant.

It might be admitted that the three types of colon bacteria, B. coli, B. paracoli and B. coli-mutabile, or the three groups of B. mycoides strains, behave in a manner suggesting segregation, but that this is merely a superficial resemblance is easily shown. All workers have reached the conclusion that variation in these cases can occur only when the specific compound is present in the culture medium. It is impossible to reconcile this fact with Mendelian inheritance as we know it in higher plants and animals. This is not, however, the only objection or even the most damaging which may be raised. There is no evidence that the "homozygous dominant" form ever arises in cultures from these mutable strains. This was explained as due to overgrowth by other forms in the mixed population arising in the culture by segregation, but the explanation does not appear to be well founded.

In order for Mendelian segregation to occur there must be a mechanism for segregation and recombination similar in principle to that already well known for the genes of higher organisms. This mechanism is given in a second hypothesis of segregation and autogamic conjugation. In this process it is necessary to make the assumption that under the stimulus of the sugar "the dominant allelomorphs are dissipated (as primitive polar bodies) and the bacterium varies." Both this and the preceding hypothesis are based on assumptions which are obviously false.

Other workers who have attempted an explanation of secondary colony formation based on a different method of reproduction include Hadley, Delves and Klimek (1932) and Enderlein (1925).
According to the former, secondary colonies may be due to asexual reproductive bodies, gonidia, which are produced in certain cells at a period of "reproductive maturity." The gonidia may, by continued vegetative division, produce a secondary colony which is made up of cells unlike the parent type both morphologically and physiologically. Such colonies yield strains having cells of filterable dimensions, feeble growth, and low fermentative powers. Aside from the uncertainties which exist as to the method of reproduction by means of gonidia, it is difficult to see how such cells, which can be cultivated only with great difficulty, could find suitable conditions for growth as a secondary colony. There is no reason to believe that such a method of secondary colony formation is common.

Enderlein (1925) has written at considerable length on the secondary colony as a stage in a complete cyclogenic life history, which includes both a haploid and diploid phase, comparable to that which occurs in higher plants and animals. The diploid vegetative cells produce gonidia which, by reduction to the haploid condition, become gametes ("spermits and oits"). These are incapable of further multiplication as such, but union of the gametes in fertilization restores capacity for growth which now proceeds with renewed vigor, producing a secondary colony. Nothing is said concerning variation or the relation to nutrients. Enderlein's theory is not convincing, since little or nothing is known concerning the principles on which it is based and it fails to account for all of the known facts.

It is impossible to conclude that any other explanation of the secondary phase of growth has so much in its favor or is backed by such a large amount of experimental evidence as Neisser's theory of variation and adaptation to nutrients. It does not appear necessary to resort to uncertain hypotheses in order to account for a phenomenon which has every appearance of being but the manifestation of well-known principles of physiology and variation.

The biological significance of the secondary phase of growth can be interpreted on this theory as beneficial variation to a specific nutrient compound.
SECONDARY COLONIES OF BACTERIA

SUMMARY

Twenty strains of B. mycoides have been studied with reference to the factors involved in the phase of growth which results in the formation of secondary colonies.

The secondary phase of growth is due to depletion of preferred nutrients and subsequent utilization of unused substances by certain cells. Ability to attack unused nutrients is acquired through variation due to the specific stimulus exerted by the substance concerned. The substances found suitable for promoting secondary growth were sucrose and protein fractions of unknown identity contained in digested meat, casein, or gelatin.

The strains may be divided into three groups on the basis of variation. Group one varies in response to sucrose, but not protein; group two, to protein, but not sucrose; group three, to both. The subraces established from secondary colonies show enhanced capacity for utilization of the compound to which variation occurred and do not again produce secondary colonies in its presence. The variants are relatively stable.

Variation does not involve other characteristics, such as form of the colony, shape, size or structure of the cells. There is no evidence that the secondary colonies in this species are due to methods of reproduction other than fission, or that they represent a special phase of a complex pleomorphic life history through which the organism must pass in a cyclogenic method of development.

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PLATE 1

The colonies are shown natural size in figures 4 and 5. All other colonies are magnified 3.5 times.

Fig. 1. Portion of a giant colony of strain B on agar prepared from gelatin culture two weeks old. The colony was photographed on tenth day.

Fig. 2. Colony of strain 421 at end of two weeks on bacto peptone 1.0 per cent.

Fig. 3. Colonies of a poured dilution plate, strain A, on agar prepared from sixty-day gelatin culture by addition of agar 2.0 per cent, peptone 0.5 per cent, and beef extract 0.3 per cent. The plate was photographed at end of tenth day.

Fig. 4. Strain A.T.C. at end of two weeks on standard nutrient agar plus sucrose 2.0 per cent. Mixture of original and sucrose variant types.

Fig. 5. Strain A.T.C. original type on standard nutrient agar plus sucrose 2.0 per cent. Photographed at end of three weeks.

Fig. 6. Portion of the same colony shown in figure 5. The original threads show but faintly at this age.

Fig. 7. Strain A at end of thirty days on agar prepared as described for figure 3.
(I. M. Lewis: Secondary colonies of bacteria.)