# PROTOBACTERIAL FORMS OF B. DIPHTHERIAE

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Among the more important of the recent developments within the field of bacteriology must certainly be included the recognition of the fact that under certain circumstances, at least, bacteria pass through, or yield, forms which are filter-passing,—the so-called protobacteria. That such forms exist seems to have been established beyond question, despite the fact that the factors controlling filtrability or non-filtrability are still, in many respects, obscure. It may readily be supposed that further studies of the physical conditions determining filtrability and, perhaps, also of the biological states permitting or facilitating filtration will serve to define more precisely the nature of the beings involved, and to explain the relationships between complexity of organization and antigenic and biochemical attributes.

Just as it has been impossible to consider the bacterium in its visible form without taking cognizance of the environment within which it exists and upon which it subsists and reacts, so also will it be impossible to regard the protobacterial forms as unassociated with their surroundings; for, although they have thus far been regarded as of significance chiefly from the point of view of ontogeny, it is certain that from the standpoint of phenomena of infection and resistance they must play parts of fundamental importance. It is only necessary to recall the work of Friedberger (1927) Bisceglie, (1924–5) and of Fejgin (1927) upon the kryptantigenic viruses to gain some impression of the possible importance of such forms in reaching valid conclusions concerning problems of epidemiology, immunology, and preventive medicine.

In view of the accumulated reports upon the subject, it is so

longer necessary to adduce proofs that bacteria may exist in, or yield, the protobacterial stage. Furthermore, the diversity of the bacterial species for which such forms have been described clearly shows that the monomorphic concept is not one to which there are certain isolated exceptions, but rather that, if exceptions must be taken, the instances where protobacteria have not been demonstrated must be so regarded, and that polymorphism must be accepted as the general rule. It surely would be difficult to find organisms more divergent, from all recognized methods of differentiation, than *B. coli* and *B. pestis*, *B. typhosus* and *B. fusiformis*, the paratyphoid bacilli and the staphylococcus, *B. dysenteriae* and *B. diphtheriae*, *B. proteus* and *B. tuberculosis*, or *V. cholerae* and the streptococcus, and yet for all of these, filtrable forms have been disclosed. It does not seem unreasonable to infer that with the application of proper methods this list can be extended to embrace all species; or, indeed, it may very well be that such study will but serve to show that many of the forms now recognized as species are derivatives, through the filter-passing form, of other species.

Such questions remain to be answered, as does that bearing on the nature of the incitants which may lead to the appearance of protobacteria. Whether they originate solely through the operation of some external force or whether they represent some obligatory or optional stage in cyclogeny is a matter of the greatest significance. Manifestly, with the fragmentary data now available it is premature to attempt broad generalizations or to formulate a theory sufficiently comprehensive to explain the phenomena observed or to suggest the significance of the facts now known.

The period of observation and of the mere recording of facts can well be extended, leaving speculation, attractive as it may be, until further disclosures have measurably filled the obvious gaps in our knowledge. Thus it is that the data presented here comprise the results of studies made upon the presence of protobacterial forms of *B. diphtheriae*, no attempt being made to explain their significance.

The initial purpose of the study was to investigate the possible

TABLE 1

| STRAIN DESIGNATION | SOURCE | MORPHOLOGICAL TYPES (INITIAL CULTURES) | MORPHOLOGICAL TYPES (CULTURES FROM RESIDUES) |
|---|---|---|---|
| Co... | New Haven Hospital | a, a′, $a^2$ | $c^2$, coccoid forms |
| Ha... | New Haven Hospital | a, $a^2$, c, d | $c^2$, $d^2$ |
| Tr... | New Haven Hospital | a, a′, $a^2$, $c^2$, $d^2$ | $d^2$ |
| En... | New Haven Hospital | c, $c^2$ | $c^2$, $d^2$ |
| Pa... | New Haven Hospital | c, $c^2$ | No growth |
| Hay... | New Haven Hospital | a, a′, $a^2$, c, c′, d, d′, $d^2$ | $a^2$, $c^2$, $d^2$ |
| Hay-1...* | | a, a′ | d, $d^2$ |
| Hay-2...* | | c, c′, d, d′ | $a^2$, $c^2$ |
| Park-Williams No. 8 | | | $d^2$ |
| Park-Williams No. 8 + resistant staphylococci | | | $d^2$ |
| Park-Williams No. 8 + susceptible staphylococci | | | $d^2$ |
| No. 721 | Connecticut State Board of Health | c, c′, d, d′, $d^2$ | c′ (few), $d^2$ |
| No. 955 | Connecticut State Board of Health | d, $d^2$ | $d^2$ (atypical) |
| No. 5 | Connecticut State Board of Health | c, c′, d, d′ | d′, $d^2$ |
| No. 9 | Connecticut State Board of Health | d, $d^2$ | $d^2$ |
| No. 959 | Connecticut State Board of Health | $c^2$, $d^2$ | No growth |
| No. 720 | Connecticut State Board of Health | $a^2$, $c^2$, d, $d^2$ | $c^2$, $d^2$ |
| No. 723 | Connecticut State Board of Health | c, $c^2$, d | d′, $d^2$ |
| No. 721–10 | Connecticut State Board of Health | c, d | c, c′, $c^2$ |

* Cultures Hay-1 and Hay-2 were derived from the original Hay culture as obtained from a clinical case of diphtheria. As is indicated above, this culture showed a wide variety of morphological types. Platings from a single colony yielded colonies of two types: 1, a large colony comprised of large, heavily staining club-shaped bacilli exhibiting marked pleomorphism; 2, a minute colony in which small beaded bacilli predominated with but an occasional swollen cell. The original culture, which was made from a single colony, and the two derivative cultures were treated as three strains.

relation between diphtheria toxin, and toxin production, and the capacity of diphtheria strains to yield filtrable forms, a problem obviously suggested by the work of Hauduroy (1927) who succeeded in demonstrating filter-passing forms in a number of samples of diphtheria toxin. These organisms, derived from his Chamberland filtrates, possessed the morphological and biochemical characteristics of *B. diphtheriae*, but were lacking in pathogenic properties for the guinea pig, an alteration in character which seems to be shared by the majority of protobacterial forms.

In order to initiate the study, pure cultures of 17 strains of *B. diphtheriae* were obtained; including 8 isolated from clinical material in the New Haven Hospital, 8 strains obtained from the Connecticut State Board of Health Laboratories, and the standard Park-Williams No. 8 which is used in toxin production. That the capacity to yield filtrable forms might be in some way related to type was, of course, obvious; hence the types of organism present in the original cultures were made a matter of record, as appears in table 1.

For obtaining toxin production the several strains were grown, with repeated daily transfers, in a nutrient broth in order to habituate the organisms to the medium somewhat prior to the inoculation of the larger quantities requisite to obtaining the desired amount of toxin. For the latter, large erlenmeyer flasks were employed, thus presenting a large surface for aerobic growth. With the cultures which readily developed a pellicle a portion of this was floated upon the surface; with the strains where pellicle formation was restricted, sterile, thin cork discs were coated with agar, and these, after inoculation with the cultures in question, were floated upon the surface of the broth. In all instances growth, with pellicle formation, took place.

In all save two instances the flasks were inoculated with known pure cultures of *B. diphtheriae;* the two exceptions being flasks planted with known mixed cultures, one containing the diphtheria bacillus (P-W No. 8) in association with a bacteriophage-susceptible strain of the staphylococcus, the other with the same strain of diphtheria organism in combination with a bacteriophage-resistant derivative of the same strain of staphylococcus. The

particular purpose in this procedure was, of course, quite outside of the primary purpose of the study, namely, to investigate the question of bacterial associations as related to the development of toxins and their behavior in the skin test. These problems have no bearing upon the matter under discussion, but are mentioned simply by way of explaining the reason for employing such mixed cultures and, as will be seen in the following discussion, it is possible that such associations may be of some significance in the origin of protobacterial forms. Indeed, it may very well be that under normal circumstances, biologic antagonisms or symbioses may be incitants of cyclic changes in the beings involved.

It may be well, before presenting the results of the studies with these diphtheria cultures, to state that the primary object—a study of the toxins produced—has not been reached, consequently no data will here be presented bearing upon the possible relation between toxin and protobacterial forms.

The broth inoculations with the diphtheria strains were made in the month of April, 1929. Because of circumstances it was impossible to prepare the necessary filtrates for testing, both for toxin and for protobacterial forms, as had been planned. Indeed, it was not until October, six months after inoculation, that they received attention. During the intervening period the flasks had remained at room temperature, carefully closed, but not sealed in a way to prevent evaporation, and when subjected to further examination the contents had in most instances been reduced through loss of water to a thick syrup or gum-like residue.

To permit further study and filtration this concentrated material was redissolved in sterile distilled water. Of the resuspended material smears and cultures, as well as filtrates, were made. Neither the smears nor the cultures showed evidences of contamination and, aside from the two instances which yielded no growth, the cultures derived corresponded in type fairly well, after several transplants, with the types present in the initial cultures inoculated. The types observed are recorded in table 1 in parallel with the initial types; and if such observations have significance it would seem that the tendency

for organisms of the $d^2$ type to persist or to appear in the cultures is the only salient feature, aside from the fact that there is an almost complete disappearance of the beaded and barred forms.

In view of the known transformations from one type of morphology into another, this change from the beaded and barred types into the solid-staining forms is not surprising, particularly since organisms of this type are considered as the more saprophytic forms and thus are presumably more competent to withstand adverse environmental conditions. However this may be, it is significant that throughout a period of six months the organisms remained viable in a medium constantly becoming more and more concentrated, and consequently exerting a greater and greater physical force upon the organisms present within it. It would seem that a degree of adaptation must have been brought about; and it is, of course, possible that such transformations in physical state must needs have their counterpart in changes in morphological structure. In contrast to this rather surprising viability of these cultures under such adverse conditions it is of interest to note that pure cultures of the initial strains which had been in sealed tubes of Loeffler's medium in the ice-box throughout this same period failed to yield growth when subcultured.

That such changes in morphology as are evidenced by the data presented in table 2 are not necessarily permanent is shown by the fact that with certain of the cultures derived from the medium residues a regression to the original type took place when these were subjected to further cultivation under the ordinary conditions of bacterial growth. Thus, with certain of the strains which have been repeatedly transplanted the solid-staining character has been lost, and after a period of three months they present the Wesbrook morphological types indicated below. In the table are listed the initial types present, the types developing from the concentrated medium upon their first cultivation, and the types ultimately to appear.

With a few of the recovered cultures virulence determinations were made, showing that this attribute seems to bear little if any relation to any other discovered factor; thus, strains Co...,

TABLE 2

| STRAIN DESIGNATION | INITIAL TYPES | TRANSITIONAL TYPES | ULTIMATE TYPES |
|---|---|---|---|
| Co... | a, a′, $a^2$ | $c^2$, coccoid forms | a′, $a^2$ |
| Ha... | a, $a^2$, c, d | $c^2$, $d^2$ | d |
| P-W. No. 8 | | $d^2$ | $d^2$ |
| No. 721 | c, c′, d, d′, $d^2$ | c′ (few), $d^2$ | a′, c′ |
| No. 955 | d, $d^2$ | $d^2$ (atypical) | d′ |
| No. 5 | c, c′, d, d′ | d′, $d^2$ | c′, d′ |
| No. 9 | d, $d^2$ | $d^2$ | $a^2$ |
| No. 720 | $a^2$, $c^2$, d, $d^2$ | $c^2$, $d^2$ | a′, c′, $c^2$, d′ |
| No. 723 | c, $c^2$, d | d′, $d^2$ | a′, c′ |
| No. 721–10 | c, d | c, c′, $c^2$ | a |

TABLE 3

| STRAIN DESIGNATION | CULTURAL TYPES | VIRULENCE (CULTURE) | TOXICITY (FILTRATE) | EVIDENCE OF SECONDARY GROWTH |
|---|---|---|---|---|
| Co... | $c^2$, coccoid forms | 0 | 0 | + |
| Ha... | $c^2$, $d^2$ | 0 | 0 | ++ |
| Tr... | $d^2$ | | 0 | ± |
| En... | $c^2$, $d^2$ | | 0 | 0 |
| Pa... | No growth | | 0 | + |
| Hay... | $a^2$, $c^2$, $d^2$ | | 0 | 0 |
| Hay-1... | d, $d^2$ | | + | ++ |
| Hay-2... | $a^2$, $c^2$ | | 0 | ++ |
| P-W No. 8 | $d^2$ | 0 | 0 | ++++ |
| P-W No. 8 + R St. | $d^2$ | | 0 | + |
| P-W No. 8 + S St. | $d^2$ | | 0 | 0 |
| No. 721 | c′ (few), $d^2$ | + | + | 0 |
| No. 955 | $d^2$ | 0 | 0 | + |
| No. 5 | d′, $d^2$ | | + | + |
| No. 9 | $d^2$ | 0 | 0 | ± |
| No. 959 | No growth | | 0 | 0 |
| No. 720 | $c^2$, $d^2$ | + | + | ++++ |
| No. 723 | d′, $d^2$ | + | 0 | ± |
| No. 721–10 | c, c′, $c^2$ | 0 | + | 0 |

Ha..., P-W No. 8, No. 955, No. 9, and No. 721–10 proved to be avirulent, while strains No. 721, No. 720, and No. 723 were virulent.

As stated above, after redissolving the medium residues and

making cultures from them, these residues were subjected to filtration. In all instances filtration was carried out with the Martin arrangement of apparatus, Chamberland $L_5$ candles being employed. Inasmuch as these candles had been used before, as they have since, in other filtration work without giving evidence of being in any way defective, there is no reason to suppose that the results obtained in this series are due to faulty filters or technic.

The filtrates were all prepared between November 4 and November 8, and cultures made in broth and in deep agar immediately after the filtration failed in every instance to yield growth. Tests for the toxicity of the filtrates, made by the intracutaneous method in guinea pigs, clearly showed that certain of them were definitely toxic, others were not (table 3).

Following the making of bacteriological controls, the filtrates were allowed to stand for periods of between two and three weeks, with frequent observation in order to detect the appearance of the faint clouding or of the fine sedimented precipitate characteristic of the development of secondary cultures in filtrates prepared in bacteriophagy experiments. In the majority of filtrates this evidence of growth, although stained smears showed nothing, appeared between November 19 and 30. In table 3 the presence of such a sediment, as well as the relative volume, is indicated, and for correlative purposes are given the morphological types present in the residue cultures, the virulence of these cultures, and the results of the intracutaneous toxicity tests.

It is thus apparent that the development of turbidity in filtrates bears no direct relation to the Wesbrook type present in the cultures yielding such filtrates, or to cultural virulence or filtrate toxicity. Apparently these several factors vary independently.

Following the technic of Haudùroy (1927) these filtrates were spread upon plates containing beef infusion agar of the following composition: 1 per cent peptone, 0.08 per cent $Na_2HPO_4$, and 1 per cent lactose, with phenol red as indicator. The medium contained 1.5 per cent agar. In the inoculation about 0.2 cc. of the filtrate was used, it being spread evenly over a small area of the medium. The plates were incubated at 38°C., and follow-

ing this the area so inoculated was carefully washed off, whether visible change had taken place or not, with a small quantity of pneumococcus broth, and this material was again spread upon a fresh plate. This procedure was repeated at twenty-four to forty-eight-hour intervals, as indicated in table 4. In such serial inoculations the first change to take place in the medium, indicative of growth of any type, was the occurrence of a slight dulling of the otherwise shiny surface. With transmitted light this change was not apparent, but could be detected only by so holding the plate that the light struck it obliquely. In the earlier transfers showing such dulled areas stained smears prepared from the washings failed to show any organized material suggestive of bacterial growth, but with later transfers, as colonies developed, even though they were microscopic in size, smears revealed forms which were, unquestionably, bacteria. These morphological changes will be discussed later.

The number of transfers necessary to yield the dulled surface was variable and seemed to bear no definite relation to the amount of sedimented precipitate present in the original filtrate used as inoculum. Table 4 states the dates of the several spreadings of each of the filtrates, together with appearance of the areas inoculated; + indicating but a very slight dulling of the surface, ++++ meaning that the dulling was pronounced, and "colonies" meaning the appearance of microscopic colonies which, when stained, showed definitely formed microörganisms.

Examination of the table shows that of the nineteen cultures carried through the serial spreadings, colony development took place more or less promptly with eleven. In two instances they appeared on the fifth transfer, in two on the sixth, in two on the seventh, in one on the ninth, in one on the tenth, in one on the eleventh, in one on the twelfth, and in one on the thirteenth. It is probable that colony formation would have been secured in other instances, perhaps, indeed, in all, had it been possible to continue the subculturing. This was, however, impossible, for the study and further cultivation of the positive cultures obtained involved so much technical work that it seemed wiser to make sure of those already recovered, rather than risk losing them through trying to gain growth from all filtrates.

These results do not indicate in any way whether the readiness with which colony formation is obtained is referable to strains which can be cultivated from the protobacterial form with particular ease or whether quantitative relations are significant here, as they are in the growth of the visible forms of bacteria where variations are made in the volume of the inoculum. It is also of significance that in some cases where the plates showing no dulling of the surface were allowed to stand, dulling and colony formation later appeared. This suggests that, had the intervals between transfers been greater, that is, had the plates

| STRAIN DESIGNATION | FIRST SPREADING | | SECOND SPREADING | | THIRD SPREADING | | FOURTH SPREADING | | FIFTH SPREADING | | SIXTH SPREADING | | SEVE SPREA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Co... | 11.19 | 0 | 11.21 | 0 | 11.25 | + | 11.26 | 0 | 11.27 | 0 | 11.29 | 0 | 12.2 |
| Ha... | 11.19 | + | 11.20 | 0 | 11.25 | 0 | 11.26 | 0 | 11.27 | 0 | 11.29 | 0 | 12.2 |
| Tr... | 11.30 | 0 | 12.2 | 0 | 12.3 | ± | 12.4 | 0 | 12.7 | 0 | 12.9 | 0 | 12.10 |
| En... | 11.25 | + | 11.27 | ± | 11.29 | 0 | 12.4 | 0 | 12.7 | ± | 12.11 | ++++ | 12.14 |
| Pa... | 11.21 | 0 | 11.25 | ++ | 11.26 | ++ | 11.29 | ++++ | 12.2 | colonies | | | |
| Hay... | 11.19 | 0 | 11.21 | 0 | 11.25 | 0 | 11.26 | 0 | 11.27 | ± | 11.29 | ± | 12.2 |
| Hay-1... | 11.19 | + | 11.20 | 0 | 11.25 | 0 | 11.26 | 0 | 11.27 | 0 | 11.29 | + | 12.2 |
| Hay-2... | 11.19 | + | 11.21 | 0 | 11.25 | ++++ | 11.26 | ++++ | 11.27 | ++++ | 11.29 | colonies | |
| P-W No. 8 | 11.19 | 0 | 11.21 | 0 | 11.25 | 0 | 11.26 | ++ | 11.27 | +++ | 11.29 | colonies | |
| P-W No. 8 + R St. | 11.21 | 0 | 11.25 | ++ | 11.26 | ++ | 11.27 | ++ | 11.29 | ++ | 12.2 | 0 | 12.3 |
| P-W No. 8 + S St. | 11.19 | ± | 11.21 | 0 | 11.25 | 0 | 11.26 | + | 11.27 | colonies | | | |
| No. 721 | 11.19 | 0 | 11.25 | ± | 11.26 | ++ | 11.27 | ± | 11.27 | 0 | 11.29 | 0 | 12.2 |
| No. 955 | 11.19 | 0 | 11.21 | 0 | 11.25 | ++ | 11.26 | +++ | 11.27 | + | 11.29 | ++ | 12.2 |
| No. 5 | 11.19 | 0 | 11.21 | 0 | 11.25 | ++ | 11.26 | ++ | 11.27 | + | 11.29 | 0 | 12.2 |
| No. 9 | 11.19 | 0 | 11.21 | 0 | 11.25 | ++ | 11.26 | ++ | 11.27 | ± | 11.29 | 0 | 12.2 |
| No. 959 | 11.19 | 0 | 11.21 | 0 | 11.25 | 0 | 11.26 | 0 | 11.29 | 0 | 12.2 | 0 | 12.4 |
| No. 720 | 11.19 | ± | 11.21 | ± | 11.25 | 0 | 11.26 | 0 | 11.27 | 0 | 11.29 | 0 | 12.2 |
| No. 723 | 11.21 | 0 | 11.25 | ++ | 11.26 | ++ | 11.27 | 0 | 11.29 | 0 | 12.2 | 0 | 12.3 |
| No. 721-10 | 11.19 | 0 | 11.21 | 0 | 11.25 | 0 | 11.26 | 0 | 11.27 | 0 | 11.29 | 0 | 12.2 |

been held longer before washing, colony appearance might possibly have been encountered in some of the earlier serial transfers.

A correlation of the positive cultures with the other characteristics of the strains shows that the tendency to yield protobacterial forms such as can be cultivated agrees most closely with the tendency of the filtrates to develop a sedimented precipitate upon standing. Even here the correlation is not complete, but agreement between these two characters is far more consistent than between colony formation and filtrate toxicity, derived

morphologic type or original morphologic form. But three of the five filtrates which showed toxicity yielded colonies, and in these growth was not particularly prompt.

The behavior of the Hay... cultures and of the Park-Williams No. 8 mixtures is of interest, but possibly of no great significance. The original Hay... culture showed no filtrate precipitate and failed to dull the surface of the medium or yield colonies, while of the two derivative strains, Hay-1 and Hay-2, both gave a moderate amount of filtrate precipitate and both yielded colonies, Hay-1 (the large colony, club-shaped bacillus strain) on the

| EIGHTH SPREADING | NINTH SPREADING | | TENTH SPREADING | | ELEVENTH SPREADING | | TWELFTH SPREADING | | THIRTEENTH SPREADING | | FOURTEENTH SPREADING | | FIFTEENTH SPREADING | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 12.7 | 0 | 12.9 | 0 | 12.11 | 0 | 12.14 | 0 | 12.17 | 0 | 12.19 | 0 | | |
| 0 | 12.4 | ++ | 12.7 | 0 | 12.9 | ++ | 12.10 | 0 | 12.14 | 0 | 12.17 | 0 | | |
| 0 | 12.17 | colonies | | | | | | | | | | | | |
| 0 | 12.26 | 0 | 1.6 | colonies | | | | | | | | | | |
| ± | 12.4 | 0 | 12.7 | 0 | 12.9 | 0 | 12.10 | 0 | 12.14 | 0 | 12.17 | 0 | | |
| 0 | 12.7 | 0 | 12.9 | 0 | 12.11 | 0 | 12.14 | 0 | | | | | | |
| ++ | 12.9 | 0 | 12.9 | 0 | 12.11 | 0 | | | | | | | | |
| 0 | 12.4 | ++ | 12.7 | +++ | 12.9 | +++ | 12.11 | colonies | | | | | | |
| 0 | 12.4 | 0 | 12.7 | 0 | 12.9 | 0 | | | | | | | | |
| 0 | 12.9 | 0 | 12.11 | 0 | 12.13 | 0 | 12.14 | 0 | 12.17 | 0 | 12.19 | 0 | 12.26 | 0 |
| 0 | 12.4 | ± | 12.7 | 0 | 12.9 | 0 | 12.11 | ± | 12.17 | colonies | | | | |
| 0 | 12.7 | 0 | 12.9 | 0 | 12.14 | colonies | (colonies later developed on the 12.9 plate) | | | | | | | |
| ± | 12.4 | ++ | 12.7 | 0 | 12.9 | ++ | 12.17 | 0 | 12.26 | 0 | | | | |

seventh passage, and Hay-2 (the minute colony composed of beaded bacilli) on the sixth spreading. The colonies made their appearance suddenly in the case of Hay-1, whereas with Hay-2 a marked clouding of the surface had been evident from the third passage.

In the case of the Park-Williams strain, a race of diphtheria bacilli well known to be lacking in virulence, but of high toxigenic power, colonies developed on the sixth transfer. The results with this strain grown in association with staphylococci of the bacteriophage-susceptible and bacteriophage-resistant varieties were

clearly different. Such variations in behavior as those associated with these cultures may very well have been quite accidental. The only statement that can be made with any assurance is that with eleven out of nineteen filtrates cultures have been recovered which may very well represent development from protobacterial forms of *B. diphtheriae*. The causes and mechanisms leading to this result are obscure.

Study of the characteristics of the organisms ultimately developing from the filtrates involves a consideration of their morphology, their colony type and pigmentation, and their biochemical activities. Serological studies upon these strains have not yet been completed.

As stated above, the development of colony formation upon the plates is usually preceded by a progressive dulling of the surface of the medium throughout the successive transfers. Smear preparations from such dulled areas fail uniformly to show bodies having a recognizable morphology. The stainable amorphous material may present an occasional Gram-positive granule more deeply stained than the surrounding mass which is Gram-negative, but these granules possess no distinctive characteristics and they may or may not represent the precursors of the organisms later to develop. With approaching colony formation on the plates, the smears first show large, diffuse, fairly round bodies, usually about $4\mu$ in diameter; and somewhat later these tend to give place to an abundance of granules, definitely coccoid in form, and often appearing in pairs, or they may be supplanted by clearly defined, large, clumsy rod-shaped structures. The latter exhibit a marked degree of pleomorphism and extreme variation in size. Swollen club-shaped forms predominate, and, as colony development on the plates progresses, these rods may in turn give way to the development of bacillary forms which morphologically can not be differentiated from certain types of *B. diphtheriae* or from the diphtheroids. They are Gram-positive, and not acid-fast, and when suitably stained they may or may not show metachromatic granules.

In the development of the colonies upon the plates a progressive increase in the size of the colony and luxuriance of the growth becomes apparent with the first few transfers. The initial colony

to appear is microscopic in size, smooth in character, flat, even in contour, transparent, and dull. With subculturing, growth becomes more and more luxuriant and pigmentation of the colony may become an outstanding characteristic, together with the assuming of a markedly mucoid character. These characters resemble closely those so commonly attributed to certain varieties of the diphtheroids, and are not unlike the pigmented strains of diphtheria described by Barratt (1924–5).

The organisms composing the final, derived cultures exhibit a wide degree of morphology, with, in almost all instances, an admixture of coccus-like forms and rod-shaped bacilli. A tabulation of the records made with reference to each of the derived strains shows the following:

Strain Tr... Colonies white and transparent. Organisms granular and coccoid, with solid staining rods. Colonies appeared on ninth passage.

Strain En... Colonies minute, white. Organisms small cocci, irregular in size, diplococci, rods. Colonies appeared on ninth passage.

Strain Pa... Colonies yellow-white, growth scanty. Organisms irregularly-shaped coarse rods, solid-staining, and occasional diplococci. Colonies appeared on fifth passage.

Strain Hay-1... Colonies yellow-orange. Organisms granules, cocci, and cocco-bacilli. Colonies appeared on seventh passage.

Strain Hay-2... Colonies white, with small daughter colonies orange in color superimposed upon the white growth in older cultures. Organisms, granules and cocci, large rods, with swollen ends, containing granules. Colonies appeared on sixth passage.

Park-Williams No. 8... Colonies yellow and mucoid (apparently identical with those recovered from the broth culture after standing). Organisms very small and faintly staining rods in the first passages, later of the typical $d^2$ type. Colonies appeared on sixth passage.

Park-Williams No. 8 in combination with bacteriophage-resistant staphylococci. Colonies yellow and mucoid. Organisms small rods at first, later larger and of the $d^2$ type. Colonies appeared on fifth passage.

Strain No. 955... Colonies white or gray; almost transparent. Organisms coccoid, large, irregularly-shaped rods. Colonies appeared on seventh passage.

Strain No. 5... Colonies yellow and mucoid. Organisms cocci and rods with granules. Colonies appeared on twelfth passage.

Strain No. 720... Colonies small, transparent. Organisms heavy club-shaped rods. Colonies appeared on thirteenth passage.

Strain No. 723... Colonies very minute, non-pigmented. This growth appeared on the plate of the eleventh passage after it had been inoculated for two weeks. Subcutures failed to grow. Organisms small wedge-shaped rods and diplococci.

It is difficult to detect any direct connection between the final type of colony and the morphology of the organisms composing it, and the nature of the initial cultures or of the cultures recovered from the residues; nor does there seem to be any relation between the readiness with which growth was obtained and the type of organism present.

None of the cultures recovered from the filtrates possess virulence. This finding is, of course quite in accord with the results of most observers who have regained cultures from protobacterial forms. The serological and antigenic behavior of these derivative cultures, as well as of the stages intermediary between the protobacterial form and the final form, remain to be determined.

It may be unwise to mention the fermentative reactions exhibited by the cultures recovered from the filtrates, for if bacteriologic experience indicates anything it shows that fermentation characters are most unstable; that in dissociative phenomena the derivative strains may possess attributes quite unlike those exhibited by the parent strain. Thus, without placing emphasis upon this point, the activities of nine of the diverse strains recovered from the protobacterial forms upon a few of the more common carbohydrates are here recorded (table 5).

Whatever significance these results may have, it appears that three types of organism were derived; three strains corresponding to *B. diphtheriae* in so far as glucose and dextrin fermentation is concerned: 5 strains corresponding to *B. hoffmanii*, and one

strain, which, because of its ability to ferment lactose, may perhaps belong to the enzymicus group. More important than allocation to groups, however, is the fact that derivatives from the protobacterial forms show different attributes; indicating that such a transformation in state offers further means for variability rather than a mechanism for regaining or re-establishing a fixed type.

It is impossible to consider the development of the diphtheroid forms from filtrates of cultures, which by all ordinary criteria must be classed as diphtheria (it may be remarked, that every culture used in this series was designated as *B. diphtheriae* by some other laboratory before it reached our hands) without

TABLE 5

| STRAIN DESIGNATION | GLUCOSE | LACTOSE | SUCROSE | MALTOSE | SALICIN | DEXTRIN |
|---|---|---|---|---|---|---|
| Tr... | + | + | + | + | − | − |
| En... | − | − | − | − | − | − |
| Pa... | + | − | − | − | − | + |
| Hay-1... | − | − | − | − | − | − |
| Hay-2... | − | − | − | − | − | − |
| P-W No. 8 | − | − | − | − | − | − |
| No. 955 | − | − | − | − | − | − |
| No. 5 | + | − | − | − | − | + |
| No. 720 | + | − | − | − | − | + |

recalling the work of Mellon (1917) upon this group, and the more recent work of Walker (1929), and of Ramsin (1926), Hauduroy (1927), and others. The frequency with which organisms of this type appear, whatever may have been the nature of the original culture type under study, suggests that the diphtheroids represent a developmental stage in the cyclogeny of many bacterial species rather than a species *sui generis*. This suggestion has already been made by Koch and Mellon (1930) and the results here reported merely add to the evidence already accumulated.

## CONCLUSIONS

From broth cultures of *B. diphtheriae* which have undergone aging, with loss in water through evaporation, filtrates may be

obtained which upon serial transfers yield visible growth. From eleven out of nineteen such cultures positive results have been obtained.

The morphological types of the organisms ultimately to develop include forms which can not be differentiated from bacilli of the diphtheroid group.

In the development of visible forms of bacteria from the protobacterial forms, a more or less constant sequence of morphologic types obtains: a granular stage, followed by the appearance of giant cocci which are replaced by micro- and diplococci, and ultimately by pleomorphic bacillary forms.

The readiness with which such protobacterial forms develop can not, at present, be correlated with any attribute of the organisms subjected to test.

## REFERENCES

BARRATT: Jour. Hyg., 1924–5, **23**, 241.
BISCEGLIE: Ztschr. f. Immunitätsf., 1929, **62**, 437.
FEJGIN: Compt. rend. Soc. de biol., 1927, **96**, 1490.
FRIEDBERGER: Ztschr. f. Immunitätsf., 1927, **53**, 339.
HAUDUROY: Compt. rend. Acad. de Sci., February 14, 1927, **184**, 409.
KOCH AND MELLON: Jour. Bacteriol., 1930, **19**, 25.
MELLON: Jour. Bacteriol., 1917, **2**, 81 (and subsequent papers of that series).
RAMSIN AND GIVKOVITCH: Compt. rend. Soc. de biol., 1926, **95**, 952.
WALKER: Jour. Prev. Med., 1929, **3**, 167.