

PLEOMORPHISM OF *PROTEUS VULGARIS* DETERMINED BY SODIUM CHLORIDE CONTENT OF CULTURE MEDIUM, AND SIMULATING STAGES IN A LIFE CYCLE.

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(With Plate I.)

THERE is probably no more striking example of alteration in bacterial morphology in response to change in the composition of the culture medium than that of the plague bacillus (*Pasteurella pestis*) when grown on nutrient agar containing from 2.5 to 3.5 per cent. of sodium chloride, and it is of interest that the discovery by Hankin and Leumann (1897) of the effect of salt in hastening the appearance of the involution forms of this organism was the outcome of a chance observation. From a brine pond near an infected village they had isolated a plague-like bacillus which was peculiar in showing involution forms on ordinary agar within 24 hours, and they surmised that its capacity for abnormally rapid involution might be related to the high salt concentration of the pond water in which it had been living for some time previously. Their experiments, however, made it clear that involution of the plague bacillus is induced immediately the salt concentration of the medium on which it is grown is suitably increased, and that it is unnecessary for the organism to have been subjected preliminarily to special environmental conditions in order that this may occur. Matzschita (1900) studied the effect of increased salt concentration on bacteria in general, and he confirmed that the salt involution forms of the plague bacillus are characteristic and unlikely to be confused with those of other organisms grown under similar cultural conditions. He found that different organisms vary greatly in their sensitiveness to sodium chloride, and that the morphology of some species remains uninfluenced even when the salt concentration of the medium is raised to 1 in 10. Other workers described similar morphological changes in the case of certain bacterial groups, e.g. *Vibrio* (Hammerl, 1906), and found that these can be induced not only by sodium chloride but also by salts of other bases, notably lithium, and by carbolic acid. There was no question of ascribing any special biological significance to the abnormal forms which appeared under such conditions, and

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earlier observers accepted them as evidence of "involution" or "degeneration"; but in recent years there has been an increasing tendency to seek a less obvious explanation and to advance pleomorphism as an argument in favour of the occurrence of some form of life cycle similar to what is observed in the group of fungi. Klieneberger (1930) has reviewed the literature bearing on this much-disputed subject and has critically examined the evidence which is adduced in support of the various hypotheses. Like Henrici (1928) he concludes that there is at present no justification for accepting a life-cycle theory as proved, and he confirms the observations of earlier workers as to the action of sodium chloride, lithium chloride, and carbolic acid in inducing morphological changes in bacteria, especially in bacilli of the coli-typhoid group.

In view of the inadequacy of existing evidence to permit a settlement of the controversy it seems desirable to put on record any observations relating to the occurrence of marked pleomorphism in an organism, and to define as clearly as possible the cultural conditions with which its appearance is associated. One cannot but be impressed by the comparative absence of any detailed reference to variations in morphology in current text-books of bacteriology, in spite of the fact that it is common knowledge that many bacteria of everyday occurrence furnish outstanding examples of the very close relationship which exists between the form of an organism and its environment, *e.g.* that of coliform bacilli as seen in a smear of the sediment from urine in a case of cystitis and in a film from the corresponding culture. This relationship was not overlooked by earlier writers, and Hankin and Leumann were careful to indicate that they were unable to give exact figures as to the amount of salt necessary for inducing rapid involution of the plague bacillus; "it probably varies with the nutritive value of the agar-agar and is likely to be found different in different laboratories." It is rare nowadays for this important factor to be mentioned, and it is often by no means evident that it receives sufficient consideration in connection with problems of bacteriological research. There is no such thing as a standard culture medium in general use in different laboratories, nor can any two batches of the same type of medium made in the same laboratory be regarded as identical. Reasonable similarity in composition is the most that can be hoped for, and in the study of bacterial variation, whether of the temporary or hereditary type, it should be regarded as essential to use the same *batch* of medium for the whole of the experiments in any one series of observations; for it cannot be doubted that differences in the nature of the media used may be associated with much greater modification of the characters of an organism than mere alteration in its morphology or in its staining affinities. A strain of *B. typhosus*, for example, investigated in connection with work on a natural antibody (Dunlop, 1928), has been found to show gross difference in the thermostability of its antigenic property, *in vitro*, depending on the type of agar medium on which it is grown (cf. Mackie and Finkelstein, 1930); and loss of virulence of *B. anthracis*

cultures as a result of their tendency to dissociate under laboratory conditions has recently been correlated by Bordet and Renaux (1930) with the calcium content of the medium used.

In the present instance the organism which has been the object of study was obtained in pure culture from a catheter specimen of urine. The patient, a lad of eighteen, was suffering from cystitis, and he had a diverticulum of the bladder. The first examination was made in November, 1927, and films of the urinary sediment showed scanty polymorphonuclear leucocytes and large numbers of Gram-negative bacilli which varied considerably in length, some being almost coccal while many were filamentous. A few streptococci were also present. The primary culture on casein-digest agar was examined after 48 hours' incubation at 37° C. and copious growth had occurred, but the organism had no resemblance to the bacillus seen in the direct smears of the urinary sediment and no streptococci were found. The growth consisted wholly of irregularly spherical bodies of varying size ( $3\mu$  to  $20\mu$ ), and most of them were ghost-like in appearance, resembling involution forms of the *Pasteurella* group, although a few stained intensely and uniformly with basic dyes. Sub-cultures were made on agar (with casein digest as a basis) and on MacConkey's agar, and in each instance the resulting growths were pure. In films from the MacConkey culture after 24 hours' incubation the picture was that of a short Gram-negative bacillus, comparatively regular in length and thickness and uniform in its staining (Fig. 1). In corresponding films from the agar culture the bacilli showed extreme variation in all their morphological characters: long filaments stretching across several microscopic fields (Fig. 2); thick faintly staining ghost-like forms; irregularly pyriform masses often of enormous size, some staining faintly, others staining deeply, and many staining unevenly, but all obviously bloated bacilli (Fig. 3); intensely staining club-shaped forms, and filamentous individuals with one or more large bulbous swellings in their continuity (Fig. 4); Y-shaped or branching forms (Fig. 5); and many bacilli with rounded bud-like projections which apparently became detached (Fig. 6), or with deeply staining granules which gave them a beaded appearance—often such deeply staining granules were seen as projections from a faintly stained individual, or lying free. The same pictures were repeated in subsequent sub-cultures (agar  $\rightarrow$  agar; agar  $\rightarrow$  MacConkey; MacConkey  $\rightarrow$  agar; MacConkey  $\rightarrow$  MacConkey), MacConkey's medium invariably yielding short bacilli while agar gave bacilli showing all degrees of variation in size, shape, and staining affinity. The naked-eye characters of the two cultures differed also: on MacConkey's medium the growth in 24–48 hours was comparatively scanty, showing individual colonies with a tendency to spread at the edge in the form of a delicate film which encircled the colony as with a halo: on agar the growth was luxuriant and the spreading character was absent. The purity of the growths was confirmed by serial platings from single colonies, and two cultures were finally maintained, one on casein-digest agar and the other on MacConkey's agar. These will be referred to as "agar strain" and "MacConkey

strain" respectively, and they were sub-cultured at weekly intervals during the following 6 months.

#### PLEOMORPHISM.

*Agar strain.* After two or three sub-cultures the general pleomorphism of the organism was much less marked, and poorly staining giant forms and bacilli showing irregular swelling, which were abundant in the earlier cultures, became more and more difficult to find and ultimately ceased to appear. It is noteworthy that while they persisted they were much more numerous in young cultures (18–24 hours at 37° C.) than in the same cultures after longer incubation, or after standing at room temperature for a day or two. Y-shaped individuals and longer forms showing lateral buds continued to be present, and they still occur, although scantily, 4 years after the isolation of the strain.

*MacConkey strain.* This was maintained for over 2 years, during which its characters remained unchanged. Morphological variation was limited to difference in the lengths of individual bacilli, similar in degree to what occurs commonly in the coli-typhoid group, and no swollen, budding, or beaded individuals were ever observed. Sub-cultures of this strain on *ordinary* agar invariably showed greater pleomorphism than corresponding sub-cultures of the "agar strain"; Y-shaped and budding forms, streptobacilli, and long bacilli of thick, deeply staining, irregularly swollen type occurred within the first 24 hours. All of these were difficult to find, or else could not be found, in the same cultures after longer incubation.

The resemblance of films from the primary agar culture to the picture of salt involution forms of plague together with the absence of pleomorphism on MacConkey's agar, which contains no added sodium chloride, were so striking that the effect on the morphology of the organism of growing it on media with different NaCl concentrations was studied. A simple peptone-water agar [Witte's peptone, 2 per cent. *plus* agar fibre, 2.5 per cent.; reaction adjusted to + 12.5 (Eyre)] was prepared, filled into tubes in amounts of 5 c.c., and sterilised. Sodium chloride was added to the tubes later so as to yield the following range of NaCl concentrations: nil, 0.5, 1, 2, 3, 4 and 5 per cent. These media, varying in their NaCl content but all prepared at the same time from the same batch of peptone-water agar, were sterilised and the tubes sloped. Subsequently they were inoculated with the "agar strain" or with the "MacConkey strain," and films were made from the resulting cultures at intervals during a period of a fortnight (24–48 hours at 37° C.; thereafter at room temperature). From a 24 hours' culture of either of the strains on the medium containing no added NaCl the organism appeared long and filamentous, and no mis-shapen or swollen forms were found. In the presence of 0.5 per cent. NaCl a totally different picture was obtained, and the bacilli showed variation in all their morphological characters, such as has already been described. With 1 per cent. and with 2 per cent. NaCl the picture was similar, but the pleomorphism was even more marked, while with higher concentrations of salt

the growth was comparatively scanty and consisted of short bacilli only. Re-examination of the cultures after a further period of incubation, or after standing at room temperature for 24–48 hours, revealed no new features, but most of the aberrant forms in the presence of 0.5–2 per cent. NaCl were ghost-like in appearance, and the films therefore seemed to show less pleomorphism than previously. On still later examination, films from the different cultures were indistinguishable one from another, and the morphology of the organism showed little variation except for the presence of an occasional Y-shaped individual and a few poorly staining forms. The growths in the presence of 4 and 5 per cent. NaCl developed an orange-yellow pigmentation in the course of 10–14 days at room temperature, but this disappeared on sub-culture to medium with lower salt concentration although it reappeared in fresh sub-cultures on 5 per cent. NaCl media. It was apparent from these observations that variation in the sodium-chloride content of the medium was an important factor in determining the pleomorphism of the organism, and this was further confirmed by comparing its morphology when grown on MacConkey's agar and on the same medium *plus* 1 per cent. NaCl. Cultures on the former were coliform in type; but on the latter, swollen, beaded, and branching individuals were abundant during the first 24–48 hours.

It must be noted, however, that although the appearance of highly aberrant forms, as detailed above, was definitely related to the NaCl content of the medium, other variations in morphology were dependent on other factors. Thus on MacConkey's agar, in the preparation of which NaCl is not used, the organism was consistently of short bacillary type, while it grew in the form of very long filaments on ordinary agar from which the customary proportion of NaCl had been purposely omitted. Similar filamentous growth, resembling a leptothrix in its characters, occurred on potato (ordinary or alkaline), but as the culture aged this gave place to short bacilli such as were usual when the organism was grown on other routine bacteriological media. Changes in the H-ion concentration of the medium over a wide range (from acid to litmus to alkaline to phenolphthalein) were without effect on the general morphology of the organism, but a tendency to polar staining was noted in cultures on peptone-water agar whose reaction had been adjusted to - 100 (Eyre).

In April, 1928, an opportunity occurred of obtaining a further catheter specimen of urine from the patient and bacteriological examination again yielded a pure growth of the same organism which, in its sensitiveness to the concentration of NaCl in culture media, behaved similarly to the strain isolated 6 months previously. This new strain was maintained as "agar strain, 2." During 1929 the three strains of the organism which had been propagated under laboratory conditions for from 12 to 20 months were re-examined with reference to their morphology on different media, and with special reference to their sensitiveness to NaCl concentration. The findings already recorded were confirmed, and the only changes worthy of note were that pleomorphism

of the "agar strain" and "agar strain, 2" on ordinary agar (0.5 per cent. NaCl) had practically disappeared, and that 2 per cent. NaCl was now necessary to determine the occurrence of the strikingly aberrant giant and ghost-like forms which had previously been obtained with from 0.5 to 1 per cent. NaCl. These changes, may have been partly dependent on the use of a different nutrient agar, but there would appear to be considerable justification for associating them with a certain tolerance for NaCl, acquired by the organism as a result of prolonged growth on salt-containing media (0.5 per cent.). Thus the "agar strain" now showed pleomorphism when sub-cultured on peptone-water agar *without* added NaCl; no pleomorphism on the same medium *plus* 0.5–1.5 per cent. NaCl; and intense pleomorphism in the presence of 2 per cent. NaCl. The "MacConkey strain," on the other hand, grew as a filamentous coliform bacillus on simple peptone-water agar (no added NaCl) and showed no aberrant forms; with the addition of 0.5–1.5 per cent. NaCl to the medium, its morphology changed completely and it appeared as a thick, deeply staining, spirillar organism, often irregularly swollen; while in the presence of 2 per cent. NaCl its pleomorphism was extreme and even more marked than that of the "agar strain" on the same medium.

Results similar to those of 1929 were obtained in 1930 and the "agar strain" has recently been examined again. Its pleomorphism is now less extreme than it was originally, but it is still a very striking feature and it is still wholly different in its characters from the morphological variations ordinarily encountered in bacteriological work. There is, however, an important modification in the behaviour of the organism, for, although variation in the NaCl content of the medium remains the factor which induces pleomorphism, aberrant forms of the irregularly swollen type only occur in large numbers when a stock culture several weeks old provides the inoculum; they tend to be scanty if a recent growth is used. The organism thus appears to be able to adapt itself more readily to change in the NaCl content of its environment if it has been growing actively beforehand than it does if it has been resting. Furthermore, pleomorphism is now most marked on media containing no added NaCl, and it persists in such cultures for several days. It is minimal or absent, however, in subsequent sub-cultures on the same salt-free medium. Increase in the NaCl concentration of the medium (up to 5 per cent.) is associated with a change in the general morphology of the organism towards a spirillar form, such as was noted previously with the "MacConkey strain," and the growth is scanty, coherent in consistence, and does not appear until the second day of incubation. Budding and branching individuals can usually be found easily in such cultures, but swollen forms are less common. In the course of 4 to 5 days at 37° C. films show many poorly staining individuals tending to occur in clumps and, later still, appearing as masses of granular or structureless material containing ghost-like bacillary remains (cf. "sympiasm" of life-cycle theories). Sub-cultures on ordinary medium (0.5 per cent. NaCl) yield abundant growths which may or may not show marked pleo-

morphism but in which a few Y-shaped individuals and thick, deeply staining, filamentous forms usually occur. Lithium chloride, instead of sodium chloride, has been used with success in several experiments, but it is no more effective than the latter in inducing pleomorphism.

#### BIOLOGICAL CHARACTERS.

The organism is a feebly motile Gram-negative bacillus which grows well on all ordinary media, either at 37° C. or at room temperature. It survives in culture for at least 6 months at room temperature, but it is readily killed by exposure to 60° C. (10–15 min.). It does not cause haemolysis on blood agar (human or rabbit). It liquefies gelatine and Löffler's serum, and it digests the casein of milk without the occurrence of preliminary acidity or clotting. Indole is formed in peptone water. Growth in sugar-free media is not associated with change in their reactions, as determined with indicator dyes. On MacConkey's agar growth is less rapid than on ordinary agar and the reaction of the medium becomes slightly alkaline. As has been noted previously, the organism has a marked tendency to develop growth of spreading type and, although this occurred only on MacConkey's agar originally, it is now observed on ordinary agar also. It is a specially striking feature on media containing blood and the appearances coincide with those described by Hadley (1927) as "regeneration fringes," although no differences have been observed in the characters of the bacilli from the different zones. In view of Hadley's theory of "Microbic Dissociation" it is to be noted that, with the exception of ability to produce gas in certain sugar media, the cultural characters of the organism have remained unchanged for nearly 4 years; and that, so far as its pleomorphism is concerned, similar variations in morphology are described by Arkwright (1921) in the coli-typhoid group as occurring independently of "rough" or "smooth" characters.

The organism ferments glucose, saccharose, and maltose; but it has no action on lactose, mannite, or dulcitol. Fermentation tests were carried out immediately after its isolation and on at least three occasions during the subsequent 8 months (peptone water or casein-digest broth being used as basic media) when acidity alone was produced in the presence of the sugars fermented. During 1929 it was found that the same sugars were attacked, but that their fermentation was now associated with the production of both acid and gas, and that this change in the character of the organism had affected all three strains which had been maintained ("agar strain"; "agar strain, 2"; "MacConkey strain"). Such an observation is apparently very unusual and the somewhat similar instance recorded by Sørensen (1912) is the only other example of spontaneous acquisition of gas-producing powers which has been traced.

The cultural characters of the organism thus correspond with those of *Proteus vulgaris*, but it differs from most strains of this species in being only feebly motile. Until quite recently it was regarded as definitely non-motile,

having been observed during several series of rapid sub-cultures in fluid media without showing motility at any stage of growth; but by the use of soft agar medium (0.5 per cent. agar) motility has now been noted and peritrichous flagella stained<sup>1</sup>.

## COMMENT.

The *Proteus* group, as its name implies, is well known to be associated with variability in the morphology of its members, but in neither of the recent studies devoted to the group (Bengtson, 1919; Wenner and Rettger, 1919) is there any reference to pleomorphism of the type described above. Moltke (1928) mentions the occurrence of "sick, big, bloated, partly vacuolised cells, often definitely branching" in cultures of *B. proteus vulgaris* (Hauser) grown on carbolic acid agar, and these were probably similar to some of the larger forms observed in the course of the present work. They resulted from a deliberate attempt to damage the organism seriously, short of killing it, and thus they lend support to the view that such extremely aberrant individuals are probably moribund. The biological significance of other types of pleomorphism such as have been observed in the organism under consideration is the problem which the various life-cycle theories, which are fully discussed by Klieneberger, have tried to explain. The occurrence of such types constitutes an integral part of the argument of workers who favour a life-cycle theory, and aberrant forms apparently identical with many of those seen in the present instance figure in illustrations as examples of methods of reproduction other than simple fission (cf. Hort, 1919-20; Mellon, 1925; Broadhurst, Moriyama, and Pease, 1931). Since evidence in support of this contention is as yet unconvincing it seems important to emphasise that this strain of *Proteus vulgaris* underwent similar morphological variation in response to a simple physical or physico-chemical alteration of its environment, and that its capacity for reacting in this way has persisted for more than 3 years. It is sensitive to alteration in the NaCl concentration of the medium on which it is grown, and *any* change of this nature tends to be associated with some variation in its form. Within limits, the greater the difference between the NaCl content of the medium to which the organism has been accustomed and that of the medium to which it is sub-cultured the more marked is the resulting pleomorphism; and diminution of NaCl is as effective in this respect as increase. Furthermore, no alteration of the ordinary environmental conditions other than that of the salt concentration has succeeded in inducing these extreme morphological abnormalities; and they are usually scanty or absent in cultures which grow poorly on account of the use of a medium whose NaCl content (5 per cent.) approaches the limit of tolerance. The writers find it difficult to correlate such observations with the view that an altered method of reproduction, a life cycle or a process of dissociation on the part of this organism provides a satisfactory explanation

<sup>1</sup> We are indebted to Mr J. Kirkpatrick for the methods for demonstrating motility and flagella.



of its continued pleomorphism; if such should be the case, one can induce the change at will and by a very simple procedure.

#### SUMMARY.

A strain of *Proteus vulgaris*, recovered from a case of cystitis, showed extreme pleomorphism in a primary culture on ordinary agar.

Pleomorphism was not found in sub-cultures on media free from added NaCl, but it appeared in the presence of a limited range of NaCl concentrations; with still higher concentrations it was not observed, although growth occurred. It was rarely found in cultures which grew with difficulty.

Pleomorphism, occurring under the conditions mentioned as to salt concentration, was most marked in young cultures which grew vigorously; it usually disappeared as the cultures aged; and it became less and less evident in serial sub-cultures on medium containing the same amount of salt.

The morphology of the organism differed on different media, e.g. filamentous on salt-free agar, short bacillary on MacConkey's agar, but it was practically constant in repeated sub-cultures on any one type of medium.

Pleomorphism could be re-induced on any of the media used by suitable alteration of its NaCl content (increase or decrease). Pleomorphism was not determined by variation of the H-ion concentration of salt-free media; nor by variation of the temperature of incubation of cultures; nor by growth in fluid media as opposed to solid media.

After nearly 4 years under laboratory conditions the organism is still sensitive to variation in the NaCl concentration of its environment, as evidenced by pleomorphism; but pleomorphism is now less extreme than it was originally.

Pleomorphism is still most marked in young sub-cultures on media with suitably altered salt concentration, but it is sometimes difficult now to induce pleomorphism unless a stock culture several weeks old is used as the inoculum.

The pleomorphism comprises aberrant forms similar to many of those which are regarded by certain observers as evidence of stages in a life cycle; but, in the case of the organism described, these forms can be induced by the simple expedient of altering the salt concentration of the medium on which it is grown. The occurrence of such forms is thus, of itself, no proof of a life cycle.

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### DESCRIPTION OF PLATE I.

The microscopic fields have been specially chosen to exemplify some of the morphological changes induced in *Proteus vulgaris* by varying the NaCl concentration of its environment, but it must be understood that all these varieties of pleomorphism can usually be found in the same film preparation.

Fig. 1 may be used as a standard for comparison with the other figures. It illustrates the appearance of the organism on any medium to which it has become accustomed as a result of repeated sub-culturing.

All film preparations are stained with dilute carbol-fuchsin, and are from cultures incubated for 24 hours at 37° C. Magnification: × 1000.

- Fig. 1. Culture on MacConkey's agar. Typical appearance of the organism on this medium. No pleomorphism.
- Fig. 2. Sub-culture on peptone-water agar (no added NaCl) from culture on ordinary nutrient agar (0.5 per cent. NaCl). Shows marked increase in size of organism (cf. Fig. 1), and presence of ghost-like forms, filamentous forms, and irregularly swollen individuals.
- Fig. 3. Culture on casein-digest agar (second sub-culture after isolation of organism). The enormous body in the centre of the field is a swollen bacillus. (All stages in the development of such giant forms from typical bacilli could be traced.) These very abnormal forms were only found in sub-cultures shortly after the isolation of the organism.
- Fig. 4. Sub-culture on peptone-water agar (0.5 per cent. NaCl) from culture on peptone-water agar (no added NaCl). Bulbous swelling of a large individual. Ghost-like forms, branching, and irregular staining are also seen.
- Fig. 5. Sub-culture on peptone-water agar (1.5 per cent. NaCl) from culture on ordinary nutrient agar (0.5 per cent. NaCl). Y-shaped branching at each end of an individual which shows slight irregular swelling. (Short Y-shaped forms can usually be found in any culture of the organism on ordinary agar medium, irrespective of its NaCl concentration, but they have not been observed on MacConkey's agar.)
- Fig. 6. Sub-culture on peptone-water agar (2 per cent. NaCl) from culture on ordinary nutrient agar (0.5 per cent. NaCl). Shows variation in size of bacilli, and presence of rounded bodies as lateral buds (cf. Fig. 5). (Similar rounded bodies are often found lying free.)

(Photomicrographs by Mr John Kirkpatrick of the Pathological Department, University of Glasgow.)

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