VARIATIONS IN THE SCOURS TYPE OF BACILLUS COLI FROM THE STANDPOINT OF BACTERIOPHAGIC ACTION.

By JOHN B. NELSON, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Certain strains of the scours type of Bacillus coli, described by T. Smith and Bryant, are subject to variation, on solid media, characterized by the appearance of indented areas at the periphery of the colonies. These areas may be v-shaped or hemispherical and are underlaid by a secondary growth which differs from the primary, mucoid, opaque form in that it is non-mucoid and translucent. The bacteria comprising it are actively motile whereas those of the parent colony are non-motile or sluggishly so. Both forms produce a diffuse turbidity in bouillon. Subcultures made from the secondary growth are pure with respect to the characters enumerated and continue to breed true in subsequent generations. Subcultures from the parent colony are unstable and subject to variation under certain conditions.

Hadley describes a somewhat similar type of variation occurring in colonies of Friedländer's pneumobacillus and of various intestinal bacteria marked by the appearance of peripheral, bluish, translucent invaginations. These may extend radially to produce a fringe or backward into the colony which may eventually be wholly consumed. The secondary growth is comprised largely of the R type of culture and upon subculture yields thin, irregular, translucent colonies. This form of variation, termed by Hadley marginal dissociation, may occur suddenly and spontaneously in cultures which have previously given a normal growth on solid media. Its analogy with the present form of variation appears close but not complete. With the colonies of the

1 Smith, T., and Bryant, G., J. Exp. Med., 1927, xlvi. 133.
scours type of *B. coli* the peripheral indentations are always present in the initial culture, made directly from the calf. They are to be observed in the majority of the colonies. The exceptions, which appear normal in that generation, invariably undergo variation upon subculture. The secondary growth, unlike that described by Hadley, has not shown characters which would suggest the R form of culture, at least when first isolated. The colonies do not present an irregular border and do not flocculate in liquid media. There is an indication, however, that the secondary growth may readily undergo partial transformation to the rough type which flocculates spontaneously and yields an irregular colony.

d'Hérelle\(^a\) has intimated that all fixed bacterial mutations are produced through the action of bacteriophage. The changes which occur in the colonies of the scours organism do bear a superficial resemblance to changes found in the colonies of susceptible bacteria exposed to bacteriophagic action. The fact that they regularly recur in generation after generation is also suggestive of the influence of some transmissible agent, carried on in subcultures or generated anew with each transfer. Unlike the eroded areas of lytic colonies, however, the indentations of the scours growth tend to increase in size as the colony ages. Moreover they are sometimes delayed until the colony is relatively old. With such a relationship in view a typical strain of the scours organism has been examined for evidence which might indicate the presence of bacteriophage.

Attempts to demonstrate a transmissible principle in cultures of the primary, mucoid type, using lysis as an indicator, have regularly met with failure. Filtrates made from 6, 24, and 48 hour bouillon cultures of a typical mucoid strain, *B. coli* 223 A, possessed no demonstrable lytic action.

In practice, 1:10 and 1:100 dilutions in 5 cc. of bouillon were made from the filtrates and inoculated with 0.05 cc. amounts of 18 hour bouillon cultures picked from individual colonies of the homologous type. At 37°C. growth was normal macroscopically and microscopically through 48 hours. Plates streaked from 1:10 dilutions after 6 hours of incubation showed normal colonies comparable to those of a control plate streaked from a plain bouillon culture. Successive passage of

\(^a\) d'Hérelle, F., The bacteriophage and its behavior, translated by Smith, G. H., Baltimore, 1926.
the mucoid type through a filtrate likewise failed to elicit any demonstrable lytic action. The filtrate of a 48 hour bouillon culture of B. coli 223 A, diluted 1:10 with bouillon, was inoculated with 0.05 cc. of an 18 hour culture of the same type. After 48 hours at 37°C. the culture was filtered, the filtrate again diluted 1:10, and reinoculated. This procedure was continued for six passages. The final filtrate was tested, as before, by low serial dilution in bouillon seeded with equal amounts of a young culture of 223A. Growth was normal in the dilution tubes. There was no inhibition, no macroscopic alteration in the nature of the growth, and no microscopic change in the morphology of individual organisms. Plates streaked from the 1:10 dilution likewise showed normal growth. Both the original and passage filtrates were also inactive for the non-mucoid variant.

The association of a bacteriophage with the non-mucoid variant seemed less probable. However, 24 and 48 hour filtrates of the variant and later a single filtrate subjected to serial passage were tested in low dilution against the homologous type and also against the mucoid type. Growth was followed at intervals through 48 hours and plates were streaked after 6 hours. No inhibition of growth and no change in the character of growth were observed with either type. There was no indication that the variant, non-mucoid form of B. coli 223 was lysogenic.

The exposure of a susceptible organism to bacteriophagic action is frequently followed by the appearance of a secondary growth which is relatively resistant to lysis. The variant form of the organism under discussion might represent such a secondary growth arising in response to the action of a lytic agent. If such were the case it should possess some degree of resistance to bacteriophagic action while the parent, mucoid form should be susceptible. An effort was made to recover an active lytic agent, from the animal host, as an indicator of the susceptibility of the two culture types.

Fecal samples were obtained by swab from a series of ten calves. Two individuals, Nos. 3 and 4, were scouring when the samples were taken. The remainder were recovered cases from which feces were secured within a week following the disappearance of active symptoms. Heavy suspensions were made from the swabs in approximately 15 cc. of bouillon and incubated at 37°C. After 3 to 6 hours an agar plate was streaked from each culture. After 48 hours they were filtered through paper and a Berkefeld N candle. The filtrates were tested for lytic activity against the A and E types of B. coli 223 and against at least one strain, presumptively identified as B. coli, isolated from the corresponding plate. Growth was observed in 1:10 and 1:100 bouillon dilutions of each filtrate and on
agar plates streaked from them. The activity of the individual filtrates against
the three strains is given in Table I.

Only one sample from each calf was examined and a second passage
of the cultures was not attempted with individual filtrates which
showed no initial activity. Hence the results indicate only roughly
the distribution of bacteriophage within the series. The filtrate from
Calf 1 was selected for further study. Preliminary examination had
failed to indicate any difference in the strength of the two filtrates
which were active for the scours type.

TABLE I.
Action of Calf Filtrates on B. coli Strains.

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<th>Calf No.</th>
<th>Type of B. coli</th>
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The above filtrate was retested against the non-mucoid E type of B. coli 223.
Dilutions ranging from $10^{-1}$ through $10^{-12}$ were made in 5 cc. amounts of bouillon
and inoculated with 0.05 cc. of an 18 hour bouillon culture. A culture control
was included. Separate pipettes were employed throughout for each mixing.
After 1 hour at 37°C. there was a faint, diffuse turbidity in all tubes. Micro-
scopically, by hanging drop, the first two dilutions showed numerous small clumps
together with motile free forms. After 2 hours there was a fine, macroscopic
flocculation in the first two tubes. Microscopically there was an increase in the
size and number of clumps. In addition, many individual bacteria, both clumped
and free, were definitely increased in size. After 6 hours the supernatants of the
first two dilutions were nearly clear, a few floccules remaining suspended. There
was an abundant granular sediment at the base. The third dilution showed no
visible flocculation but a scant granular sediment. The turbidity of the remain-
ing dilutions and control was diffuse, with no floccules and no sediment. Micro-
scopically the first two dilutions, after shaking, showed clumps of varying size with
comparatively few free forms. The majority of the bacteria displayed a swollen appearance with an increase in size of from 2 to 5 or more diameters. There was a marked variation in the shape of individual cells. Spherical, elliptical, and drumstick shapes were most common. The larger forms frequently showed one or more central vacuole-like areas of different refraction from the remainder of the cell. In addition, indistinct granules were sometimes visible. The larger of the swollen forms bore a resemblance to yeast cells. Decreasing numbers of these forms and clumps were seen through dilution $10^{-4}$. The remaining dilutions and the control showed no swollen forms and only an occasional small clump.

After 24 hours there was a noticeable decrease in the bulk of the sediment at the base of the first two dilution tubes, which could not be attributed simply to gravity. The supernatants showed a faint turbidity with floccules in suspension. Dilution $10^{-3}$ displayed a heavier sediment with a turbidity distinctly less than that of the control. With dilutions $10^{-4}, 10^{-5}$, and $10^{-6}$ the turbidity was equal to that of the control, but there was a scant granular sediment with floccules suspended in the lower third. The remaining dilutions and the control showed a heavy, diffuse turbidity and a very scant, compact, button-like deposit quite different from that of the preceding dilutions. Microscopically the first two dilutions showed a marked decrease in the number of swollen forms. Clumps were still visible. Some showed sharply outlined bacteria but in many cases the individual units appeared indistinct, often granular in shape. Dilutions $10^{-3}, 10^{-4}, 10^{-5}$, and $10^{-6}$ showed many clumps together with free normal bacteria and an occasional swollen form. The bacteria in the remaining dilutions and the control were evenly distributed in the fields with a few small clumps and no swollen forms.

After 48 hours there was no appreciable change in the macroscopic or microscopic appearance of the first six dilutions. Dilutions $10^{-7}$ and $10^{-8}$ were slightly less turbid than the control with scant granular sediment and suspended floccules in the lower portions. The remaining dilutions and the control showed comparable turbidity with no flocculation and no change in the sediment. Dilutions $10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}$, and $10^{-5}$ were filtered through Berkefeld candles and retested in one dilution only, $10^{-4}$. The four highest dilutions showed normal growth macroscopically and microscopically through 48 hours at $37^\circ$C. Dilution $10^{-8}$ showed a few clumps and small swollen forms after 6 hours. After 24 hours there was a decreased turbidity with granular sediment and suspended floccules. The $10^{-4}$ dilution faithfully reproduced the original reaction.

A portion of the same filtrate was likewise tested against the mucoid A type, of *B. coli* 223. The same method was employed but only five dilutions from $10^{-1}$ to $10^{-4}$ were included. The tubes were examined at the same intervals as before through 48 hours at $37^\circ$C. Growth was normal in all dilutions from the start and at no time showed any variation from that of the control. Swollen forms were not seen in hanging drop preparations. Plates streaked from the $10^{-1}$ dilution and from the control showed no significant difference in the number of colonies and no deviation from normal in their appearance. Since the original filtrate displayed no initial lytic activity for the mucoid type an attempt was made...
to adapt it. Six passages of the mucoid type were carried out with 48 hour intervals between filtrations. The final filtrate showed no demonstrable lytic action against the passage type of the organism when tested as before.

Serial passage was also resorted to in attempts to increase the activity of the original filtrate for the non-mucoid E type. Six passages were made with 48 hour intervals between inoculations. The final filtrate was tested in a dilution series with a range from $10^{-1}$ to $10^{-15}$. The lytic strength of the filtrate was not appreciably affected. It displayed no greater tendency to cause complete lysis of the homologous bacteria, with permanent inhibition of growth, than did the original filtrate. There was, however, an increase in titer. A marked reaction was shown in dilution $10^{-5}$ after 6 hours and a scant reaction in dilutions $10^{-3}$ and $10^{-16}$ after 48 hours. The limiting dilution was determined, as before, by filtering and retesting in low dilution after 48 hours. Fourteen additional passages were carried out with a 24 hour interval between inoculations. The final filtrate showed no significant difference in action or in titer from the previous one. Like the original filtrate it was inactive for the parent, mucoid type of culture.

**DISCUSSION.**

The activity of the filtrate employed in the preceding work was weak but nevertheless definite. A growing culture of the non-mucoid variant upon exposure to a low dilution of the filtrate was first agglutinated and then altered morphologically. Individual cells became markedly exaggerated in shape, size, and internal structure. By prolonged examination it was at times possible to observe the rupture of the swollen cells. There was immediate shrinkage, with a minute granule-like residue. Complete dissolution apparently did not occur in most instances. Hanging drop preparations made from 24 hour filtrate cultures showed clumps composed in part of minute, poorly staining granules, together with an occasional swollen form and sometimes clearly outlined, deeply staining rods. The factor accountable, in part or in whole, for the cellular agglutination was slightly active for bacteria killed by heat. With dead bacteria, however, the agglutination was slow, occurred only in low dilutions, and was not accompanied by any morphological alteration of the cells. The active principle of the filtrate was relatively sensitive to heat. Exposure of the undiluted filtrate to a temperature of 55°C. for 30 minutes resulted in partial inactivation. At 65°C. for 30 minutes there was complete inactivation.

The weak lytic activity of the filtrate was demonstrable only in the
presence of the non-mucoid variant type of culture. In the presence of the parent, mucoid type it was inactive. The anomalous susceptibility of the two forms of growth together with the inactivity of their filtrates is opposed to the natural association of bacteriophage with the described scours organism as the agent responsible for variation.

CONCLUSIONS.

1. A lytic agent was not demonstrable in culture filtrates of either the parent or variant type of the scours organism.
2. The parent type was resistant to the action of a "weak" bacteriophage, obtained from the animal host, while the variant type was susceptible.
3. Exposure of the variant type to the scours bacteriophage was attended by agglutination of the cells, marked swelling, and an alteration of the contents prior to lysis.
4. The manifestations of variation which regularly occur on agar plate cultures of the scours organism do not appear to be the result of bacteriophagic stimulation.