A METHOD OF STAINING LYTIC AREAS PRODUCED BY THE BACTERIOPHAGE

PHILIP HADLEY

Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

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In carrying out the so-called "enumeration test" of d'Herelle with the bacteriophage working in its appropriate culture film on an agar substratum, it frequently happens that the lytic colonies of the "small" type are difficult to observe, especially if one or more secondary or resistant colonies arise at the site of lysis and partially fill in the area, as sometimes happens. This is more likely to occur if the lytic agent is weak in energy or if the culture mass is to some degree resistant. The staining method to be described has the advantage (1) of staining the areas of lysis so that they can be easily seen, and (2) of revealing colonies of secondary or resistant culture arising on the lysed areas.

In brief, the method involves pipetting about one cc. of a well polychromed, Loeffler's methylene blue over the agar slant culture and tilting the tube so that the stain comes in contact with the bacterial film for about one minute. The stain is then washed out by adding distilled water with a pipette, blowing the stream with some energy over the slanted surface. The tube may then be rinsed with more water, or immersed in a jar of mercuric chloride (1:1000) and dried with a cloth. This procedure serves to stain the lysed areas red or reddish violet while the unlysed areas are either colorless (the color of the agar substratum) or a faint greenish blue. When stained in this manner the areas of lysis, viewed by transmitted light, stand out much more clearly than in unstained preparations. Moreover, some

1 Hadley: This Journal, p. 397.
lytic areas that may easily be overlooked in unstained tubes are now detected. These effects are seen to best advantage in the case of the d’Herelle bacteriophage acting upon the Shiga dysentery bacillus.

Although d’Herelle\(^{2}\) has stated that the areas of lysis are always sterile, and that at no time do there exist within them microscopically visible cells, in the experience of the present writer, a secondary culture of the organism frequently arises in the form of minute colonies on the lysed surface. These usually appear only after some days. Although the primary, unlysed culture does not stain on the agar slant, the secondary colonies, at the end of the staining process, appear as bluish or bluish purple points against the red lytic areas. These, in turn, contrast with the greenish blue background of the agar. The nature of these blue colonies will be referred to again subsequently.

One may gain some knowledge of the manner in which this staining process works by staining in a similar manner (1) a slant agar tube carrying a growth of normal Shiga culture, and (2) a sterile tube of slant agar of the same composition. In the first case, after washing the slant with distilled water or mercuric chloride, the entire surface of the agar formerly covered with culture shows the greenish blue color. In the second case the agar surface stains a bluish purple, not greatly different from the color of stained lytic areas. It is thus clear that the mere fact of staining of the lytic areas is not due to any modification of the agar surface resulting from lysis, since normal agar surface stains in a somewhat similar manner. Moreover, it is clear that the reason why the agar surface underneath the unlysed culture appears greenish blue after staining is because it remains relatively free from stain; in fact, it has been more or less protected against the stain by the covering of normal culture. When the tube is rinsed in water or mercuric chloride the normal culture is washed off, so that the only staining which the underlying agar receives is due to the brief contact with the stain during the process of washing. The color is greenish

blue rather than bluish because of the yellowish tone of the agar. This may, however, vary with reaction of the medium and with the length of the staining time.

Although the color of the stained lytic areas may resemble that of a stained normal agar surface, when not previously covered with culture, a difference can be detected. While the normal agar surface stains bluish purple, the lytic areas stain reddish-purple to red. There is a difference due to lysis. This difference suggests a difference in reaction at the lytic sites, as if these areas were more alkaline. In addition, the deeper tone of the stain in the lytic areas is probably due to the presence of alkaline debris remaining from the lysed bacterial cells.

The staining features of the secondary colonies of the dysentery bacillus call for further mention. It is recalled that the normal, unlysed culture washes off the agar surface when the tube is rinsed in water or mercuric chloride. The secondary growth, however, is not so affected; it remains adherent and stains blue to bluish-purple. One can see the colonies as bluish points against the red areas of lysis, which in turn contrast with the greenish blue of the agar background. In explanation of this difference, all that can be said at present is that the resistant colonies are strongly adhesive to the agar substratum. They cannot be washed off and are removed only with difficulty by the aid of a platinum needle. Indeed they differ in many respects from the normal Shiga culture, but the actual cause of these changes is at present only a matter of conjecture.

In conclusion a few guard points may be mentioned: 1. Ordinary methylene blue and a non-polychromed Loeffler's methylene blue do not give the typical differential coloring; particularly they fail to differentiate the lytic areas. A polychrome methylene blue, originally divided by Novy and used for many years at the University of Michigan as one of the three solutions in staining by a modified Romanowsky method, has given results surpassing in clearness those obtained by the ordinary polychromed methylene blue.
2. For good differentiation the first wash water must be poured off quickly before the denuded agar surface can take up the diluted stain.

3. Staining is more intense if the stain is heated in a tube to about 60°C. before added to the agar.

4. The stain must not run down the sides of the tube between the agar and the wall. This may be prevented by heating the line of junction of agar and glass with a fine blast lamp flame, and then cooling.

5. The results are clearer when staining is carried out twelve to twenty four hours after the lytic areas have attained their maximum size.

SUMMARY

1. A method is described for staining areas of lysis produced by the bacteriophage acting upon a bacterial film on agar slants.

2. The method serves to turn negative pictures of lytic colonies into positive ones, and to reveal for enumeration some of the smaller colonies which might otherwise escape notice.

3. The method also reveals the presence of colonies of the secondary or resistant strain of the lysed culture arising on the lytic sites.