

A NEW STAIN FOR THE DIPHTHERIA BACILLUS
AND A CONSIDERATION OF THE PRINCIPLES
INVOLVED IN THE STAINING OF
THIS ORGANISM *

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From the time of Loeffler's discovery of the diphtheria bacillus in 1884, its staining has been a matter of great interest. Many special staining methods have been devised all of which have aimed at making the diagnosis of diphtheria easier and more definite.

For some time, Loeffler's methylene blue was the only stain used for the clinical differentiation of the diphtheria organism. In 1893, Neisser developed a stain the purpose of which was to bring out the polar granules more definitely. He believed that his stain would differentiate the true diphtheria bacillus from pseudoforms. It was subsequently shown that these polar granule forms of the diphtheria bacillus were not always found in cases of diphtheria and that they were sometimes found in cultures of Hoffman's bacillus. However, in Hoffman's bacillus the granules are smaller, not so numerous and appear later during the incubator growth than do those of the diphtheria bacillus. Neisser's method was a distinct advance and a great help to the inexperienced observer. It has remained a standard method up to the present time.

It has always been the working policy of the contagious hospital to call bacilli from the nose and throat, which show the typical polar granules, diphtheria bacilli, and to regard individuals showing these organisms as contagious until it could be proved definitely by animal inoculation whether these bacilli were of the virulent type. There will be only a small percentage of error with this plan of procedure and one will err on the safe side. In some cases of diphtheria, no polar granule organisms can be found, but the percentage of such cases is very small. In practically all patients with diphtheria, seen at the contagious hospital, it has been possible to find some bacilli with polar granules either from smear or culture. The polar granules appear almost invariably if the culture is incubated several hours longer. This is so constant that for several years it has been the custom in the contagious hospital to study all cultures in this way.

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When a culture from the nose or throat in a case of diphtheria is examined hourly after incubation, it is usually found that up to the tenth or eleventh hour no polar granules can be seen. The bacilli are all of the solid forms. About ten hours after incubation, small polar granules can be seen in a few of the bacilli. Then the number of organisms with polar granules increases rapidly, and the individual granules, also, become larger. After twenty-four hours, involution forms appear, and other organisms override the diphtheria bacilli. Occasionally, polar granules are not observed until twenty-four or even thirty hours after incubation, but this is very uncommon.

Unquestionably, the bacteriologic diagnosis of diphtheria is simplified by a stain which reveals the polar granules the clearest, and differentiates the morphologic and tinctorial characteristics of these organisms.

The Polar Granules.—The diphtheria bacillus is one of a group of bacilli which, when treated with certain stains, shows various granules within the cell. These granules are designated as polar or meta-chromatic granules; the latter term being used because of the fact that they can be made to stain a different color from the substance of the cell body.

The nature and function of these granules is not definitely known. This is, probably, because the granules are not of the same character in all bacilli containing them. Meyer¹ has shown by microchemical reactions that some of these granules are fat or lipid, some carbohydrate and others protein in nature. This may account for the varying views of their physiologic function (reserve food supply, centrosomes, indicators of virulence, etc.).

The nature of the polar granules is of interest, particularly, as little is known concerning them. A number of specimens of the diphtheria bacillus, in pure culture, obtained from active clinical cases, were stained with sudan III, Lugol's solution and with an acid solution of methyl green which is a specific dye for chromatin.² From this investigation, one would determine whether the granules were composed of fat, carbohydrate or chromatin. The sudan III series were entirely negative.³ Staining with Lugol's solution was negative.

1. Meyer: (Flora **86**:428, 1889.) Abstr. in Centralbl. f. Bakteriol. **6**:339, 1900. Zweite Abteilung.

2. Lee's Vade Mecum, Ed. 6, 1905, p. 196.

3. It is a question whether this stain can be accepted as of equal value in the recognition of fat in micro-organisms and in tissue sections. It was introduced by Dorset (New York M. J. **69**:148 (Feb. 4) 1889) as a specific stain for the tubercle bacillus which is known to contain fat. But Cowie (New York M. J. **71**:16, 1900) has shown that because of the many varieties of sudan III, all of which do not possess the power of staining the tubercle bacillus, it cannot be relied on for this purpose.

Staining the fresh, unfixed diphtheria bacilli with an acid solution of methyl green was positive, showing that the granules are chromatin in nature, and that they are richer in chromatin than is the body of the bacillus.

That these granules are composed of chromatin is further shown by their reaction to acid and alkaline solutions. When the unfixed bacilli are treated with a weak solution of a mineral acid (HCl 0.1 per cent.) their staining reactions are unaffected. On the other hand, when they are allowed to remain unfixed in a weak solution of an alkali (KOH 1 per cent.) for some time, the polar granules stain poorly. Chromatin is soluble in weak alkalis, but not in weak mineral acids.⁴ The results of these few experiments show that the polar granules of the diphtheria bacillus contain chromatin, but do not aid us in determining their physiologic significance.

Metachromasia.—The diphtheria bacillus affords a valuable and instructive medium for studying this phenomenon. Very early in the use of Loeffler's methylene blue, it was noted that there were granules in some of the diphtheria bacilli which stained purplish red against a blue background. This phenomenon did not occur when a watery or alcoholic solution of pure methylene blue was used.

The phenomenon of metachromasia is an interesting one. There are three main theories to explain it: (1) That it is a specific chemical reaction; (2) that it is an optical phenomenon; (3) that it is due to impurities in the dye. Proof for the last theory was accidentally encountered in working with the various dyes. The sample of methyl green used gave a metachromatic reaction with the diphtheria bacillus, the polar granules staining red and the cell body blue. Methyl green is a metachromatic stain only when it is impure. It usually contains a small amount of methyl violet, as can be demonstrated easily by the purity test described by Mayer.⁵ When the sample used, at first, was tested, it was found to be impure. Another lot of the dye, obtained from a different firm, gave absolutely no metachromatic reaction with the diphtheria bacillus, and this brand was found to be pure. Michaelis⁶ has shown that the metachromatic properties of methylene blue are due to methylene azur, which is an oxidation product of methylene blue and a red dye. Pure methylene blue is not metachromatic. Oxidation is favored by adding alkalis, as in Loeffler's alkaline methylene blue and Unna's polychrome methylene blue and

4. Lee's Vademecum, Ed. 6, 1905, p. 348.

5. Mayer (Mitth. Zool. Stat. Neapel **12**:313, 1896): Quoted by Lee, p. 195.

6. Michaelis: Centralbl. f. Bakteriol. **29**:763, 1901.

also by boiling methylene blue with an alkali. Michaelis makes a polychrome or metachromatic methylene blue by boiling the dye with weak caustic soda and then acidifying with sulphuric acid.

Other metachromatic dyes have not been investigated as to their purity, but it probably could be shown that they all owe their valuable properties to slight amounts of impurities. In fact, this is a very well known belief. It explains why some lots of dyes give beautiful staining reactions while others are very unsatisfactory. A metachromatic dye can be obtained by mixing two pure dyes together. When methylene blue and basic fuchsin are combined in an acetic acid solution, a very sharp metachromatic reaction can be obtained. The polar granules stain red and the cell body blue. This suggests that, although there is chromatin in both the cell and the polar granules, there is some chemical or physical difference between the two.

Acid and Alkaline Dyes.—Acid and alkaline solutions of the various coal tar dyes have been used to stain the diphtheria bacillus. Good results have been obtained by both methods. The acid solutions, as a rule, have been the most popular for the reason that a sharper differentiation between polar granule and cell body is thus obtained. Solutions of basic fuchsin, methylene blue, methyl violet, gentian violet, crystal violet, methyl green and pyronin were made up with water in the proportion of 1 to 1,000. When these dyes were made very strongly acid with acetic acid, the polar granules stood out distinctly, and the cell body was practically unstained. By diminishing the acidity a point was reached where the polar granules stained intensely, and the cell body stained well, although of a lighter shade than the polar granules. Even with a neutral solution of these dyes, the polar granules stained a deeper shade than the cell body, but the differentiation was not so good. A fairly good differential stain for diphtheria can be obtained with any of these dyes in the proportion of dye 0.1 gram, glacial acetic acid 3 c.c., distilled water 100 c.c. However, with a pure dye there is no metachromatic reaction.

A further differentiation is seen in the chemical nature of the polar granule and the cell body by the difference in the intensity with which they take the stain, when treated with an acid solution of a basic aniline dye. This sharp differentiation of the polar granule with acetic acid suggests that the chromatin in the polar granules must be relatively more abundant than in the cell body.

Stain for Diphtheria Bacillus.—These observations would seem to show that the best stain for the recognition of the diphtheria bacillus would be a weak solution of a metachromatic dye acidified with acetic

acid. In trying the various metachromatic dyes,⁷ I found that kresylecht-violet with methylene blue was the most satisfactory in the following proportions:

Kresylecht-violet	0.07 gr.
Methylene blue	0.1 gr.
Glacial acetic acid.....	3.00 gr.
Water	100.00 gr.

Dissolve the dye in the acetic acid and water solution, and filter. The stain keeps well. In making the preparation, a modification of Ponder's⁸ technic was used: To a drop of water on a cover glass, make a smear of the growth with a platinum wire. Dry gently over flame. Place one drop of the stain on the cover glass. Allow it to remain for from one to two minutes, and then shake off the excess of stain. Place a drop of water on the cover and make a hanging mount with a well slide. The preparation can be sealed with the excess of water. The polar granules are purplish red and the cell body is greenish blue. The Hoffman bacillus stains a light greenish blue. Most of the other organisms stain very lightly, excepting a few strains of cocci. The hanging drop method makes the organisms appear much larger than they do in the ordinary preparation; a single bacillus is easily seen and differentiated from the surrounding organisms.

The stain can be used with the usual slide method. The polar granules by this method are purplish red and the cell body is light blue, and the contrast is a little better. This stain is the most successful I have used in searching for diphtheria bacilli in a throat smear preparation. Smears can be made directly on a slide or cover glass, or, better, an emulsion of the membrane with water can be made on a slide and a portion of this transferred to a cover glass and stained in the usual manner. The hanging drop method can be used for other organisms.

In presenting this new stain for the diphtheria bacillus, emphasis is not placed so much on the stain itself as on the principles involved, the principle of metachromasia, the nature and function of the polar granules and the value of the acid solutions of dyes. With a knowledge of these principles, anyone can make for himself a satisfactory differentiating stain for the diphtheria bacillus with whatever dyes he has at hand.

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7. The stains used were purchased from Eberbach & Son, Ann Arbor, Mich.

8 Ponder: *Lancet* **2**:22, 1912.