

THE SELECTIVE ACTION OF GENTIAN VIOLET IN BACTERIOLOGICAL ANALYSIS.

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In 1912 Churchman¹ reported a new differential test for the *Schizomycetes*, depending upon the selective bactericidal action of gentian violet. The action of this stain in high dilution upon various organisms planted in media containing the dye was found to correspond closely to the Gram staining reaction, the forms inhibited—"violet positive"—being in the majority of cases forms that retain the stain; those growing—"violet negative"—usually being strains that decolorize when treated by the Gram method. His actual results on 318 different strains of bacteria are shown in the following table:

318 Strains.	Gram positive.	Violet positive.	Violet negative.
	182	165 (90%)	17 (10%)
	Gram negative.		
	136	15 (11%)	121 (89%)

The characteristic behavior of bacteria cultivated in the presence of the dye in high dilution (1:100,000) is "so constant and clear cut that it must be regarded as a fundamental biological characteristic." The Gram stain has ever been an unsatisfactory test with certain groups of organisms, especially the Coccaceæ. Differences in the age of the cultures, time of application of the various reagents, and the temperature may influence the result. It is sometimes extremely difficult to interpret the result of the stain as, some individual cells will retain the stain and others in the same field or even in the same chain as contiguous cells will have decolorized. As an instance of discrepancy in interpretation of results we may cite Kligler's work² who by the same method (Churchman's) recorded 13 of 17 strains of saprophytic cocci as certainly Gram negative, while four stained uncertainly, as opposed to Churchman's recording of all 17 strains as Gram positive. In my own work on 240 strains of streptococci I found 21 to stain irregularly and occasionally successive stains of the same culture at different times would give totally

¹ Jour. of Exp. Med., Vol. XVI, No. 2, p. 221.

² Jour. of Exp. Med., Vol. XVII, No. 6, p. 653.

opposite results. The violet reaction is in striking contrast to this "notoriously uncertain" staining test and though not assuming to be a parallel or substitute test, it is a valuable differential reaction.

Work on various other staining agents has shown many to exhibit a definite selective inhibitive action. The Conradi-Drigalski medium for isolation of *B. typhosus* from water, stools, etc., has as its basis the restraining action of the crystal violet towards various cocci and bacilli, without influencing at all the growth of the typhoid-colon group. Krumwiede³ and Pratt⁴ and Churchman⁵ have made observations on the growth of bacteria on media containing various closely related aniline dyes and have found their action to correspond closely to that of the gentian violet. Smith⁶ has shown the violet test to be specific for certain of the phytopathological bacteria.

Aside from the significance of this test as a classificatory feature of great value it might be expected to have some practical application in sanitary bacteriological analysis, as most of the intestinal bacteria that we presume indicate pollution by sewage are Gram negative and, therefore, with few exceptions, are violet negative. Many of the common saprophytic bacteria found normally in water and in milk are Gram positive and so would in the majority of cases fail to grow in the presence of the stain. Churchman in his work on the collection at the American Museum of Natural History found the following organisms to be Gram negative and with two exceptions also violet negative:

- 3 strains of *B. coli communis*.
- 5 strains of *B. coli communior*.
- 5 strains of *B. paracoli*.
- 2 strains of *B. coli varictus*.
- 14 strains of *B. typhosus*.
- 18 strains of *B. paratyphosus*.
- 5 strains of *Vibrio cholera*.
- 3 strains of *B. dysenteriae*.
- 5 strains of *B. enteritidis*.
- 3 strains of *B. cloaca*.

Curiously enough *B. welchii* and *B. sporogenes*, both Gram positive, proved to be violet negative. *Subtilis*, *mycoides*, *megatherium*, *liodermos*, *mesentericus* and many of the saprophytic cocci are violet positive.

³ Ztschr. of Hyg., Vol. XXXIX, p. 283.

⁴ Proc. N. Y. Path. Soc., Vol. XIII, p. 43.

⁵ Jour. Exp. Med., Vol. XVII, No. 4, p. 373.

⁶ Phytopathology, Vol. II, No. 5, p. 213.

A priori, then, we might expect that the addition of gentian violet to our culture media in proper dilution would result in eliminating many saprophytic bacteria, still permitting those forms of sewage origin to flourish. If we used a sugar medium and added litmus we could still further emphasize the colon group, as these are acid-forming organisms. The violet stain partly masks the coloration of the litmus indicator, but not sufficiently to make the picture of acid fermentation uncertain.

My work to date has not been extensive enough to warrant any definite conclusions, but it is at least suggestive. I have analysed various samples of water taken chiefly from the Wabash River, which is rather highly polluted at Lafayette. Duplicate plates of proper dilutions have been made of litmus-lactose agar and litmus-lactose-violet agar, the latter being the same as the former with the exception of the addition of a standardized loop full of gentian violet solution to the agar tube just before pouring. The plates have been examined after 24 hours incubation at 37° C. The total number of organisms growing, the total number of red colonies—acidifiers—and the presumptive coli colonies growing on the two media have been recorded. The suspected coli growths have been “fished” and planted in lactose-peptone-bile for confirmation and almost without exception the fermentation of this media has checked the presumptive colony growth.

The colonies on the violet plates appear somewhat smaller and the acid production is less distinct. The stain is picked up by the cells so that the colonies appear, especially the sub-surface colonies, as distinctly purple growths. Viewed under the microscope the cells show a light purple color, indicating vital staining.

So far I have found pretty generally what was expected, viz., that the total count is materially reduced on the violet plates but that the number of red colonies, and especially of coli, are approximately the same on the two media. It has been found possible to plate a larger sample of water and to intensify the picture of presumptive pollution by the use of the violet. A few typical examples of actual tests will illustrate this:

<i>Sample.</i>	<i>Media.</i>	<i>Total counts.</i>		<i>Total red.</i>	<i>Coli.</i>
Wabash (polluted).	L. L. A.	15,000	5,000	3,000	
	L. L. V. A.	8,000	6,000	6,000	
Wabash.....	L. L. A.	10,000	1,800	600	
	L. L. V. A.	3,100	1,100	500	

<i>Sample.</i>	<i>Media.</i>	<i>Total counts.</i>	<i>Total red.</i>	<i>Coli.</i>
Wabash.....	L. L. A.	3,500	2,000	700
	L. L. V. A.	1,500	1,300	600
Wabash.....	L. L. A.	8,700	2,600	1,600
	L. L. V. A.	4,200	2,000	1,550
Tap (driven wells).	L. L. A.	16	0	0
	L. L. V. A.	0	0	0

The last test noted in the above table suggests that the ratio of the count on lactose agar with and without the violet present may be a valuable diagnostic feature. Polluted waters show about 50 per cent. reduction of the total count on violet media, while unpolluted water containing more of the saprophytic violet positive organisms show a much greater reduction; 100 per cent. in the case of the tap water at Lafayette. Gram stains of centrifuged specimens of fresh sewage shows the ratio of Gram positive to Gram negative cells to be anywhere from 1:5 to 1:100. This does not check the 50 per cent. reduction very closely but many factors of a variable nature enter into the two tests. The significant point is that the majority of sewage organisms are Gram negative and therefore may be expected to be violet negative.

Further work is being done to determine the quantitative relations of pure strains of typhoid and coli studied by this method and to test the effect of attenuation of these forms in relation to the violet when held in suspension in water under various conditions of temperature, light, etc. So far the results seem to indicate that sojourn of a week or more has no selective inhibitive effect; in fact, the violet media seems to be favored by the organisms after this treatment.

One interesting point has been brought out by this latter study. In working with several strains of coli suspended in water, variation in counts on the two media—lactose agar plus violet, and without the violet—was so great that I decided to test the individual strains. I found one, No. 41 received from the American Museum of Natural History and thoroughly tested by myself, to be absolutely inhibited by the violet stain. A study of the culture showed it to be a motile, Gram negative bacillus, fermenting bile rather weakly, not liquefying gelatin after ten days, and giving other characteristics typical of coli. Churchman and Michael⁷ have described work on

⁷ Jour. Exp. Med., Vol. XVI, No. 6, p. 822.

B. enteritidis where one form, indistinguishable from the others by any morphological, cultural or agglutination characters was singled out nevertheless by this delicate affinity of the violet dye. The observation, he states, is an isolated one, but my experience with this colon culture seems to confirm the fineness of this selective affinity.

Although my work is too meagre to warrant any definite conclusions, yet it seems to be suggestive, at least, of the value of selective bactericidal or bacteriostatic dyes as valuable adjuncts in sanitary bacteriological analysis.

