SPECIFICITY OF THE DYE IN THE CRYSTAL-VIOLET AGAR REACTION OF STAPHYLOCOCCI

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In a previous paper (Chapman and Berens, 1935), it was shown that staphylococci produce orange, violet or white growths on crystal-violet agar. Because no satisfactory explanation of these color changes has been offered, experiments were undertaken in an effort to learn more about the chemistry of the reaction. Attempts to discover the identity of the active molecule of the dye will be described in the present paper.

The crystal-violet agar had the following composition:

Agar	15 grams
Proteose peptone, Difco	5 grams
Meat extract	3 grams
Crystal-violet, Commission Certified 0.1 per cent (based on the dye content and not on crude weight)	3.3 cc.
Water to make	1,000 cc.
pH	6.8±

The medium is quite inhibitive for staphylococci, making it necessary to inoculate heavily to obtain growth. For this reason, it is suitable only for the study of pure cultures.

Crystal-violet agar prepared from a solution of crystal-violet several weeks old had a rose color, was considerably paler than that prepared from fresh solutions, and gave no sharp color differentiation of staphylococcal growths. Many strains which gave pale violet growths on a medium prepared from this solution gave white growths with violet borders on media prepared from fresh solutions.

A freshly prepared solution of Commission Certified crystal-violet gave best results when added to the agar base to make

TABLE 1

Dyes which, when added to proteose lactose agar, did not give color changes with staphylococci similar to those obtained with crystal-violet

Nitro compounds	Martius yellow, naphthol yellow S
Azo dyes	Acid yellow, chrysoidin Y, chrysoidin R, orange G (LO-3)*, Bordeaux red, Janus green B (NJ-2), methyl orange (NN-4), orange II (NOB-2), amaranth, Sudan III (LY-2), Biebrich scarlet, Bismarck brown (LN-1), Congo red (LQ-4), benzopurpurin 4B, trypan blue, orange I
Oxyquinones	Alizarin, alizarin red S, alizarin blue S, alizarin yellow R
Quinone-imides:	
Thiazins	Thionin (NT-4), azure A, azure B, azure C, azure II, methylene blue (LA-5), methylene violet (2 samples), methylene green, toluidine blue O (NU-1), new methylene blue N
Oxazins	Brilliant cresyl blue (NV-10), gallein, gallocyanin, Nile blue sulfate, cresyl violet (NW-3)
Azins	Neutral red (NX-3), neutral violet, safranin O (LS-4), phenosafranin, nigrosin (2 samples)
Phenyl methanes:	
Diphenyl methanes	Auramin
Triphenyl methanes:	
Diamino	Malachite green (LMg-1), brilliant green (NBg-3), light green SF (NL-3), fast green FCF
Triamino	Basic fuchsin (LF-2), pararosanilin, rosanilin (2 samples), rosanilin HCl, new fuchsin, methyl green (NG-3), acid violet, iodine-green, spirit blue AS, acid blue WS, methyl-blue, methyl violet (LMv-1 and 4 other samples), crystal-violet (NC-9), Hoffman violet (2 samples), ethyl-violet and dahlia
Hydroxy	Aurin (rosolic acid) (2 samples), corallin red (2 samples), corallin yellow (2 samples)
Xanthines:	
Pyronins	Pyronin G
Rhodamins	Rhodamin B
Fluoranes	Fluorescein (acid), fluorescein (sodium salt), eosin Y WS (LE-8), methyl eosin AS, ethyl eosin AS, eosin B (NEB-4), eosin B AS, erythrosin Y, erythrosin B (NEB-4), phloxine B, (NPh-4), rose bengal
Sulphonephthaleins	Thymol blue, cresol red, bromphenol blue, bromthymol blue
Acridines	Acriflavine, proflavine, neutral acriflavine
Natural dyes	Hematoxylin, hematein, orcein, cochineal, indigo carmine, indigo, borax carmine, brazilin, carmine, carminic acid

* The figures in parentheses refer to the certification number of the sample.

dilutions of between 1:300,000 and 1:350,000. Best color differentiation was obtained after 36 hours incubation.

COMPARATIVE TESTS WITH OTHER DYES

Expecting that the active molecule of crystal-violet was a simple one and that it would be found in dyes containing similar chemical groups, a number of such compounds were tested. The results were negative. Therefore, an attempt was made to determine the active chemical group by testing 108 dyes, representing each of the different groups of biological dyes (table 1). Because the optimum dilution of crystal-violet was critical, it was expected that the optimum dilution of other dyes having similar differential properties would also be critical. Therefore, enough of each dye was added to the agar base to make dilutions of 1:10,000, 1:100,000, 1:250,000 and 1:500,000. Each dye agar was inoculated heavily with 10 strains of staphylococci which were selected because of their different hemolytic, coagulating and crystal-violet agar reactions. After 36 hours incubation, the colors of the growths were compared with those which had been produced on crystal-violet agar.

Inconclusive results were obtained with most of the dyes, any color changes being associated with *albus-aureus* differences, i.e., related to pigment. However, significant color changes were noted with the following dyes: 5 samples of methyl-violet, 1 of crystal-violet and 1 of Hoffman violet which gave results similar to those obtained with the original sample of crystal-violet; and bromthymol blue which gave color changes parallel to, but chemically different from, those produced on crystal-violet agar. The results with bromthymol blue agars depended upon the indicator property of the dye.

STUDIES OF METHYL-VIOLET AND RELATED VIOLET DYES

These results suggested that the active molecule of the crystal-violet agar reaction is contained in crystal- and methyl-violet and some samples of Hoffman violet. Therefore, tests were made with a larger series of violets. Because of the close relationship between Hoffman and ethyl-violets and, because one dye was

labeled "methyl-violet (methylene violet)," samples from these groups were also included. A total of 60 samples was studied among which were "Dahlia," "Dahlia B," crystal-, methyl-, gentian-, ethyl-, Hoffman and methylene violets. The results are summarized in table 2.

With the exception of a few samples of methyl-violet which, since they were labeled "R," "2R," etc., appeared to be mostly tetramethyl pararosanilin, dyes labeled "methyl," "gentian" and

TABLE 2

Chromatic properties of 60 samples of violet dyes when tested for their value in the crystal-violet agar reaction of staphylococci

MANUFACTURER'S DESIGNATION OF DYES TESTED	ABILITY TO DIFFERENTIATE STAPHYLOCOCCAL GROWTHS				
	Standard		Poor	Slight or none	No parallel
	a*	b†			
Crystal—violet	6		4		
Methyl—violet	24		7		
Gentian—violet	3		1		
Dahlia B	1				
Hoffman violet	1	1		3	
Ethyl—violet		2		3	
Dahlia		1			
Methylene violet					3

* Colored growths on these dye agars were similar to those on standard violet agar. The intensity of the color of an 0.1 per cent solution was approximately equal to that of the standard sample of crystal-violet.

† Colored growths on these dye agars were similar to those on standard violet agar. However, the intensity of the color of an 0.1 per cent solution was equal to only 54 to 75 per cent of the standard sample of crystal-violet. The solutions had rose tints.

"crystal" violet gave color changes typical of crystal-violet. Dyes considered as tetramethyl pararosanilin gave poor color differentiation.

Methylene violets, which included one sample labeled "methyl-violet (methylene violet)," gave atypical color differentiation.

To obtain comparative results with ethylated compounds, such as ethyl or Hoffman violet or Dahlia, it was necessary to use higher concentrations of the dyes. Such media were similar in

intensity of color to those prepared from methylated pararosanilins. With these concentrations, 2 of 5 samples of ethyl-violet, 2 of 5 samples of Hoffman violet and 1 sample labeled "Dahlia" gave color reactions typical of crystal-violet. If many of these dyes are improperly labeled, as suggested by Conn (1929) then the typical color reactions given by some samples may be explained on the basis of their containing crystal-violet or a higher homolog of methyl-violet. The fact that the solutions were rose-colored and that considerably more dye was required to bring the color to match standard crystal-violet agar in intensity indicates that they also contained either ethyl or Hoffman violet (Dahlia) as claimed on the labels. On this assumption, ethylated dyes giving color differentiation typical of crystal-violet are believed to be mixtures containing methylated pararosanilins. If this is true, then the only dyes which give the color reaction are those which contain moderate or large amounts of methylated pararosanilins.

The active chemical group can be still further defined from a consideration of the relationship between methyl-, crystal- and gentian-violets. Methyl-violet is a mixture of tetra-, penta- and hexa-methyl pararosanilin, the exact shade depending upon the proportion of different homologs. The redder shades, designated "R," "2R," etc., which are composed mostly of tetramethyl pararosanilin did not give characteristic color changes. Therefore, the active groups should be sought in the higher homologs. The two higher homologs (pentamethyl and hexamethyl pararosanilin are not clearly differentiated in biological dyes. This is due to the lack of precise control of the degree of methylation, so that samples offered on the open market may contain these two compounds in different proportions and may also contain the tetramethyl compound. On the other hand, crystal-violet which is usually made by a different synthesis is composed almost entirely of the hexamethyl compound. Gentian-violet, a term which is becoming obsolete in this country, is used rather loosely to designate various types of methyl- or crystal-violet.

These observations lend support to the belief that the active group in the crystal-violet agar reaction of staphylococci is either

pentamethyl or hexamethyl pararosanilin, or both. This is further supported by the following observations.

Dyes such as malachite green or auramin, which have only two amino groups, do not react. This shows the importance of the three amino groups. These groups must be substituted, because dyes such as the fuchsins in which they are not, fail to give the color reaction. Ethylated rosanilins and pararosanilins, such as brilliant-green and ethyl-violet, do not give characteristic color changes. However, samples believed to contain either pentamethyl or hexamethyl pararosanilin, or both, give typical color differentiation due to the presence of such compounds. This shows the value of methyl substitution.

Dyes such as Janus green B, methylene-blue, methylene-green, neutral-violet, thymol-blue and bromthymol blue, which contain 4 or more methyl groups, do not react. This proves that it is not methyl substitution alone, but the attachment of methyl groups to another specific group which furnishes the basis for the reaction.

Dyes such as methyl-violet 2R, considered as lower homologs, either do not react or give poor differentiation when they do react. This eliminates tetramethyl pararosanilin. The introduction of further methyl or other groups into crystal-violet as in methyl-green, iodine-green and methyl-blue, prevents the color reaction. This demonstrates the unfavorable effect of further changes in the crystal-violet molecule.

Because it is more likely to be uniform, it is suggested that Commission Certified crystal-violet be used for the preparation of crystal-violet agar.

SUMMARY

Heavy inoculation is necessary to secure growth of staphylococci on crystal-violet agar.

Solutions of crystal-violet used for the preparation of crystal-violet agar should be freshly prepared. The optimum dilution is between 1:300,000 and 1:350,000.

Although representatives of the different groups of biological dyes were tested with staphylococci, color changes similar to those

produced on crystal-violet media were obtained only with media prepared from dyes containing methyl-, gentian- or crystal-violet.

The active group of the dye used in the crystal-violet agar reaction of staphylococci was found to be either pentamethyl or hexamethyl pararosanilin, or both.

CONCLUSIONS

The violet agar reaction of staphylococci is highly specific as far as the dye is concerned and depends upon a reaction of growth products of staphylococci with either pentamethyl pararosanilin or hexamethyl pararosanilin. To insure best results, only fresh solutions of crystal-violet, preferably Commission Certified, should be used for the preparation of crystal-violet agar.

REFERENCES

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