OBSERVATIONS ON THE GROWTH OF BACTERIA ON MEDIA CONTAINING VARIOUS ANILIN DYES.*

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In recent communications Churchman (1) has described a selective action on bacteria of gentian violet. Stated in general terms, he found that bacteria were divided into two groups, of which one fails to develop in media containing gentian violet, while the other grows abundantly in the presence of this dye. The former group corresponds roughly with the Gram-positive group, the latter with the Gram-negative group of bacteria. His conclusions are based mainly on results obtained with dilutions of 1 to 100,000 of the dye.

A striking variation was noted in some enteriditis strains. These strains were identical by all ordinary cultural tests; one strain, however, persistently refused to grow on gentian violet agar. A subsequent examination showed that this strain could be differentiated from the others by agglutination.

Our interest in the use of media containing dye stuffs was further aroused by an article by Signorelli (2). He stated that on agar containing dahlia, cholera vibrios develop colored colonies by absorption of the dye with decolorization of the medium, whereas non-cholera vibrios give colorless colonies. We (3) were unable to verify these findings. In the course of the work we found two non-cholera vibrios which uniformly refused to grow on agar containing dilutions of dahlia which had no influence whatever on the growth of similar vibrios. Because of the above results, a few preliminary observations with various anilin dyes were made, which showed differences among related bacteria, and also differences in the reaction of the same bacteria to different dilutions of the same dye. The results of the comparison of the action of various dyes are given in this paper.

For the tests, the common pathogenic bacteria which grow freely

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on ordinary agar were selected. *Bacillus subtilis* was included as it is very sensitive to the presence of gentian violet. Other bacteria requiring serum or other similar additions were tried in a limited way, but this was discontinued as the albumins interfered with the activity of the dye.

As a routine a batch of agar was selected which was found especially suitable for the more feebly growing types. To the hot agar an appropriate volume of a watery solution of dye was added to give the final dilution desired. The same agar was used in each experiment.

The most convenient and economical method was found to be as follows. One unopened Petri dish was used to tilt up one side of a second dish. In the opposite side was poured just sufficient agar to give a satisfactory slant. This dish was covered and used to tilt up a third dish, and so on in a row. After the agar had set, slants were poured in the other side of the Petri dishes which were tilted in the reverse direction. In this way two mixtures of agar could be used in the same dish, very little agar being required for each slant.

In the general observations that follow two dishes were used containing the control agar, and agar with 1 to 500,000, 1 to 100,000, and 1 to 50,000 dilutions of the dye. The weakest dilution was used in the plate with the control.

For inoculation a fresh broth culture was usually employed. A small loop was used and a streak made on the agar slant, a fresh loopful being taken for each slant. Very heavy seeding as from agar slants tends to give irregular results, probably because of lack of close contact between the bacteria and dyed agar.

Table I gives the results of a series of tests after eighteen to twenty-four hours' incubation.

Some allied bacteria showed variations as noted in the table; namely, the streptococcus-pneumococcus group, the dysentery group, the capsulatus group, and diphtheria and the morphologically allied types. More complete tests of these groups were made.

Naphthylamin blue R gave an apparent difference between diphtheria and diphtheroid bacilli. Thirty-four strains of these bacilli were, therefore, planted on agar containing this dye. There was

TABLE I.

Type.	Bacteria tested.	Hoffman violet. 1:50,000	Hoffman violet. 1:100,000	Hoffman violet. 1:500,000	Crystal violet. 1:50,000	Crystal violet. 1:100,000	Crystal violet. 1:500,000	Dahlia. 1:50,000	Dahlia. 1:100,000	Dahlia. 1:500,000	Fuchsin. 1:50,000	Fuchsin. 1:100,000	Fuchsin. 1:500,000	Rosanalin violet. 1:50,000	Rosanalin violet. 1:100,000	Rosanalin violet. 1:500,000	Cresylecbtviolett. 1:50,000	Cresylecbtviolett. 1:100,000	Cresylecbtviolett. 1:500,000	Anilin violet. Gentian B. 1:50,000	Anilin violet. Gentian B. 1:100,000	Anilin violet. Gentian B. 1:500,000	Naphthylamin blue R. 1:50,000	Naphthylamin blue R. 1:100,000	Naphthylamin blue R. 1:500,000	Bleu de Lyon. 1:50,000	Bleu de Lyon. 1:100,000	Bleu de Lyon. 1:500,000	Säureviolett (Kühne). 1:50,000	Säureviolett (Kühne). 1:100,000	Säureviolett (Kühne). 1:500,000	Methylviolet 6 B. 1:50,000	Methylviolet 6 B. 1:100,000	Methylviolet 6 B. 1:500,000
Gram-positive	Staphylococcus aureus	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−*	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
	Streptococcus (green)	−	−	+	−	×	+	−*	×	±	×	±	+	−	±	+	+	+	+	−	×	+	+	+	+	+	+	+	+	−	±	+
	Streptococcus (hemolytic)	−	−	±	−	−	±	−	−	+	−	−*	+	−	−	±	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+
	Streptococcus mucosus capsulatus	−	−	+	−	−	±	. .	. .	. .	−	×	+	−	−	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+
	Pneumococcus	−	−	±	−	−	+	−	−*	+	−	±	+	−	−	+	−	−	+	−	×	+	+	+	+	+	+	+	+	−*	+
	B. diphtheriæ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	×	−	−	−	±	±	+	+	+	+	−	−	−
	Diphtheroid bacilli (Cameron)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−
	Diphtheroid bacilli (Cunningham)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	. .	. .	. .	−	−	−	−	−	+	+	+	+	−	−	−
	B. hoffmanni	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	±	+	+	+	+	−	−	−
	B. subtilis	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−
	B. lactimorbi	−	−	−	−	−	−	−	−	−	−	−	+	−	−	±	×	±	+	−	−	−	+	+	+	+	+	+	−	−	−*
	B. anthracis	−	−	−	−	−	−	−	−	−	−	−*	+	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−
Gram-negative	Cholera	−	−	+	−	−	+	−	×	+	+	+	+	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	−	×	+
	Vibrio (indol-positive)	−	−*	+	−	−	+	×	±	+	+	+	+	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	−	×	+
	Vibrio (indol-negative)	−	−	+	−	−*	+	×	±	+	+	+	+	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	−*	+*	+
	B. proteus	±	+	+	−*	×	+	±	+	+	+	+	+	+	+	+	±	+	+	±	+	+	+	−	±	+	+	+	+	−*	+*	×
	B. pyocyaneus	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Shiga)	−*	×	+	−*	±	+	×	±	+	−	−*	+	−*	±	+	+	+	+	+	+	+	+	+	±	+	+	+	±	+	+	+
	B. dysenteriæ (Flexner)	×	±	+	−	×	+	−*	±	+	±	±	+	−*	±	+	+	+	+	±	+	+	+	+	+	+	+	+	×	±	+	+
	B. dysenteriæ (Mt. Desert)	±	+	+	−	±	+	+	+	+	×	±	+	±	+	+	+	+	+	±	+	+	+	+	+	+	+	+	±	+	+	+
	B. typhosus	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	. .	. .	. .	+	+	+	+	+	+	±	+	+	+
	B. paratyphosus, A	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, B	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. enteriditis (Gaertner)	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communis	±	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communior	±	+	+	±	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Friedländer's bacillus	±	+	+	×	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. lactis aerogenes	+	+	+	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
	B. ozænæ	×	±	+	−	−	+	. .	. .	. .	+	+	+	×	±	+	+	+	+	−*	+	+	+	+	+	+	+	+	×	±	+
	B. pullorum	+	+	+	×	±	+	. .	. .	. .	±	+	+	. .	. .	. .	+	+	+	+	+	+	. .	. .	. .	+	+	+	+	+	+	+	+

+ = growth like control; ± = restrained growth; × = markedly restrained growth; − = no growth; −* = some growth developed later.

irregular restraint at 1 to 100,000, more marked or complete in some instances at 1 to 50,000. There was, however, no evidence of a grouping according to the different types of bacilli.

On *cresylechtviolett* the strain of pneumococcus failed to grow whereas other allied cocci grew. Forty strains of this group were tested. A variable restraint or inhibition of individual cultures was noted, but this had no relation to the subgroups.

In the dysentery group there is a general tendency for the paradysentery types to show less restraint than the Shiga type. The result on fuchsin seemed to show a definite difference between the types. The inhibition caused by fuchsin was also of interest because of its presence in Endo agar. This is the most commonly used medium for the isolation of dysentery and must, therefore, have been found satisfactory for this group; at least we know of no unfavorable criticism of the growth on this medium. The question arises as to how far the influence of the dye itself might be lost after its reduction by sodium sulphite to the leuko-base.

Table II gives the results on fuchsin agar with the available strains of dysentery and paradysentery. The table gives the range of growth observed in several repetitions.

It is probable that some of the cultures under similar names, but obtained from different institutions,[1] are duplicates. As we were unable to determine this positively they are given separately in the table. They act as probable controls on the degree of variation of growth in the presence of the dye.

It is evident from table II that the reaction to fuchsin is not a completely specific difference between the dysentery and paradysentery groups. There is a shading of one group into the other. There is, however, a marked tendency to a general group reaction.

Two strains, one of the Shiga type and the other of the paradysentery type, which reacted almost alike were plated and ten fishings of each made in broth. These were planted on fuchsin agar to determine the probable range of variation under identical conditions. A moderate variation was noticed in some instances slightly greater than those already noted in the table.

[1] We are indebted to the Museum of Natural History, to The Rockefeller Institute for Medical Research, and to Professor Hiss and Dr. Torrey for these cultures.

TABLE II.

Growth of Dysentery Bacilli on Fuchsin Agar.

Culture.	Mannite fermentation.	Time of incubation and dilution of dye.										
		18 to 24 hours.				48 hours.				72 hours.		
		25,000	50,000	100,000	150,000	25,000	50,000	100,000	150,000	25,000	50,000	100,000
Shiga, Gay-Shiga, Shiga	−	−	−	−	− or ×	−	−	s.c. or ×	±	−	s.c.	±
Kruse, Kruse	−	−	−	− or s.c.	− or ×	−	−	s.c. or ×	±	−	s.c. or ×	±
Kruse, Shiga	−	−	−	− or ×	×	−	−	× or ±	±	−	×	+
Shiga	−	−	−	− or ×	±	−	− or s.c.	× or ±	±	−	±	+
Torrey 21	−	−	−	× or ±	±	−	× or ±	+	+	− or 1 c.	× or ±	+
Cruz 1	−	−	− or ×	± or +	+	− or ×	±	+	+	− or ×	±	+
Sydenham	+	−	− or ×	± or +	+	−	× or ±	+	+	−	±	+
Torrey 4, Torrey 5–6, Hiss Y	+	−	×	± or +	+	s.c. or ×	± or +	+	+	× or ±	±	+
Hiss Y, Flexner-Harris, Medburg, Ferra-Hiss, Torrey 16–3, Torrey 32, Y, Baltimore, Strong, Flexner, Mt. Desert	+	×	± or +	± or +	+	× or ±	± or +	+	+	±	± or +	+
Strong, Gay-Flexner, Silk, Baltimore, Flexner, Gray, Torrey 3, Torrey 41, Flexner-Harris	+	±	± or +	+	+	± or +	+	+	+	± or +	+	+
Gray B, Hiss 48	+	+	+	+	+	+	+	+	+	+	+	+

− = no growth; 1 c. = colony; s.c. = several colonies; × = very slight growth; ± = evident restraint; + = practically like control.

Influence of Decolorization on Fuchsin.—An agar was made containing decolorized fuchsin diluted 1 to 25,000. The strains showing no growth in this concentration of the unaltered dye after twenty-four hours' incubation were planted. Three strains grew as well as on the control, four strains showed slight to moderate restraint, and one strain showed marked restraint. With a similar medium containing the decolorized dye in a dilution of 1 to 50,000, one strain showed some restraint, but at 1 to 100,000 no restraint was evident with any of the strains.

Evidently decolorization robs the dye of some of its action. A series of plates was made containing the decolorized dye in the 1 to 50,000 dilution. These were kept at room temperature and in the light to bring back the color gradually. Several of these plates, with controls, were planted each day. When planted the day after making, some restraint was evident. Those planted thereafter gave no growth except with a strain which grew in the presence of undecolorized fuchsin. The reaction of three members of the dysentery group to the violet dyes has been given. Because of the results on fuchsin the reaction of all the strains was tried, gentian violet and dahlia being used. With both dyes, individual strains showed more variation than with the fuchsin. There was no apparent difference between the dysentery and paradysentery group, the individual strains of both groups showing, however, markedly different degrees of restraint in the presence of the dye.

Reaction of the Streptococcus-Pneumococcus Group to Dyes.—In the results already given there is a marked quantitative difference in the reaction of the streptococcus-pneumococcus group as compared with the other Gram-positive bacteria. Because of this about a hundred strains of the group were tried;[2] these included typical pneumococci, streptococci, *Streptococcus mucosus,* types from chronic endocarditis, streptococci from various diseased conditions in man, a series isolated from the conjunctiva, a series isolated from domestic animals, and a few from the external world. Two dyes were used, gentian violet and Hoffman violet. The results were practically the same with both dyes.

[2] We are indebted to Miss Jean Broadhurst for most of the streptococcus cultures.

In dilutions of 1 to 500,000 of the dye the members of the group grew as freely as on the control, with a few exceptions where slight restraint or delayed growth was evident. By increasing the concentration of the dye, the amount of restraint becomes greater, and some of the strains fail to grow, the number increasing with the concentration. When a dilution of 1 to 100,000 is reached a few strains still grow but the growth is restrained or delayed. One exception was a strain of *Streptococcus lacticus,* which even at 1 to 600,000 showed inhibited or delayed and feeble growth.

The Gram-positive organisms used for comparison, including sixteen strains of staphylococci, failed to grow at 1 to 500,000 dilution of the dye. In a few instances a slight growth developed along the edge of the slant, probably due to the dilution of the dye by the water of condensation.

The streptococcus-pneumococcus group, even the feebly growing strains, is, therefore, more resistant to the action of the violet dyes than other Gram-positive bacteria, growing at dilutions which still inhibit the other Gram-positive bacteria. This may be found of value in isolation from mixed material.

The above observations explain the irregular results in the streptococcus-pneumococcus group given in Churchman's protocols. Stowell, Hilliard, and Schlesinger (4) found regular inhibition of nearly all their strains.

The influence of decolorization by sodium sulphite on the restraining action of gentian violet was very slight. In a concentration of 1 to 50,000 or 1 to 100,000 of the dye, there was no growth of either the staphylococcus or hay bacillus; when a dilution of 1 to 400,000 was reached, there was very feeble growth of the staphylococcus, but not of the hay bacillus.

While the above work was in progress, a further article by Churchman appeared (5). He found similar results, using allied dyes. As he used only four strains for testing, and, as far as we can determine, only one dilution of the dye, he naturally found none of the variations in individual dyes, in which we were especially interested.

SUMMARY.

Gentian violet and allied anilin dyes have a similar influence on bacterial growth, dividing bacteria into two groups corresponding in general to their reaction to the Gram stain.

Among Gram-negative bacteria a strain is occasionally encountered which will not grow on violet agar, differentiating it from other members of the same species or variety.

The reaction is quantitative, although the quantitative character is more marked with some species than with others.

The streptococcus-pneumococcus group differ from other Gram-positive bacteria in their ability to grow in the presence of amounts of dye sufficient to inhibit the other species.

The dysentery bacillus group shows marked variation in the presence of dyes. In the case of fuchsin the variation approaches closely a specific difference between the dysentery and paradysentery groups. The variations of the latter groups with other dyes show no correlation with the common differential characteristics. A closer study might reveal variations in other characteristics which would parallel the different reactions to dyes. Decolorization with sodium sulphite robs the dyes of some of their inhibitive powers.

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