CRYSTAL VIOLET AGAR AS A DIFFERENTIAL MEDIUM FOR STAPHYLOCOCCI[1]

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In a previous paper Chapman, Berens, Peters and Curcio (1934) showed that staphylococci could be differentiated on the basis of their hemolysis and coagulase reactions. It was further shown that a critical interpretation of the hemolysis test depended upon an accurate knowledge of the pigment production of the strain. The existence of intermediate-colored strains or of dissociants showing wide variations in color from the parent strain makes it difficult to interpret hemolysin tests of certain strains. If some correlative reaction were available, it should be useful for comparative purposes. The search for such a reaction is described in the present paper.

There was also demonstrated a correlation between the response of a strain to the reactions described and its pathogenicity for rabbits. The animal experiments were not entirely satisfactory because, in those rabbits which died, the interval between the time of inoculation and death varied considerably, suggesting that factors other than toxicity might have been responsible for some of the lethal effects. In the present study attempts were made to eliminate some of these other factors. It was hoped that this would simplify the interpretation of the animal experiments and result in an improved correlation.

Stimulated by the observation of Burbank (1929) on the effect of sodium carbonate on *Escherichia coli* and streptococci in feces, the effect of sodium carbonate on staphylococci was studied. Strains of staphylococci were treated with varying concentrations

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of this salt and transplants were made at intervals. Most strains grew from transplants taken after one hour's exposure but they failed to grow from transplants taken after three hours' exposure. Moreover, it was found that, depending upon the concentration of sodium carbonate, there was an interval when the growths from the transplants varied considerably for different strains but were characteristic for each strain. The time/concentration of carbonate necessary for the demonstration of this variable growth was somewhat critical. Essentially similar results were obtained by comparison of transplants from centrifuged carbonate supensions, indicating that carbonate carried over had little effect.

The following technic was adopted: To a series of sterile plugged test tubes were added 0.5 cc. quantities of sterile 1.0 per cent sodium carbonate (Merck reagent). A loopful of the growth from proteose lactose agar was added to each tube and thoroughly shaken. Transplants were made immediately to a solid medium to serve as controls, both of the number of bacteria present and of the effect of carbonate carried over. The tubes were placed in the incubator at 37°C. At the end of one, one and a half, two, two and a half and three hours, transplants were again taken from the carbonate suspensions and plated on the solid medium in such a way that the initial or control growth from each transplant could be compared with those obtained after incubation. After overnight incubation the growths on solid medium were critically compared and the results reported as follows: If there was a good growth from the initial transplant and from that obtained after incubation, the reaction was recorded as $++++$; if the transplant obtained after incubation showed a slightly diminished growth as compared with that from the initial growth, it was interpreted as $+++$; if there was a marked diminution of growth after incubation, it was considered a $++$ reaction; when there was no growth after incubation, as compared with an excellent initial growth, it was considered negative.

In tabulating the results to determine the correlation between the carbonate reaction and hemolysis and coagulase tests, hemolytic aureus strains (which may or may not coagulate plasma) and albus or aureus strains which coagulated citrated or oxalated

plasma were considered strains with "positive hemolysis and coagulase reactions." The best correlation was obtained with exposure to the carbonate solution for two and a half hours. However, when 151 additional strains were tested by exposure for two and a half hours, the correlation with hemolysis and coagulase reactions was only 76.8 per cent. This was considered unsatisfactory and further studies were made in an effort to find a test giving better correlation with hemolysis and coagulase reactions.

In considering the mechanism of the carbonate reaction and in an effort to obtain better correlation, it was postulated that, since the ability to grow from transplants was constant for each strain, it might have been due to factors associated with the viability of a strain or its susceptibility to the action of certain chemical agents. If this were true, it should be possible to obtain similar results by using other chemical substances. The strains were tested by the following technic:

Copper sulfate (Baker anal.), mercuric chloride (Merck reagent), crystal violet (National Aniline, comm. cert.) Merthiolate (Lilly), Metaphen (D.R.L.), ferric chloride (Eimer & Amend, T.P.), basic fuchsin (National Aniline, comm. cert.), Lysol, phenol (Merck reagent) and hexylresorcinol (S.T. 37, Sharpe & Dohme) were selected for comparative tests. These reagents were prepared in dilutions of 1:100, 1:500, 1:2,500, 1:12,500, 1:62,500, 1:312,500 and 1:1,562,000. Each dilution of the chemical substance was measured into sterile plugged tubes in 0.5 cc. quantities. Two strains of staphylococci were selected for the tests. One strain was pathogenic for rabbits, reacted positively to both hemolysis and coagulase tests and grew from the carbonate transplants (++++), while the other was nonpathogenic and reacted negatively to the *in vitro* tests. These two strains were mixed with each dilution of the chemical as was done in the carbonate reaction and transplants were made both before incubation and again at the end of one hour. Growths from the transplants were compared after overnight incubation. They were interpreted as in the carbonate reaction.

Growth curves were plotted for each chemical and they indicated that the strain which was pathogenic for rabbits and gave

positive hemolysis, coagulase and carbonate reactions could be recovered from more concentrated solutions than the strain which reacted negatively to the *in vitro* and *in vivo* tests (fig. 1).

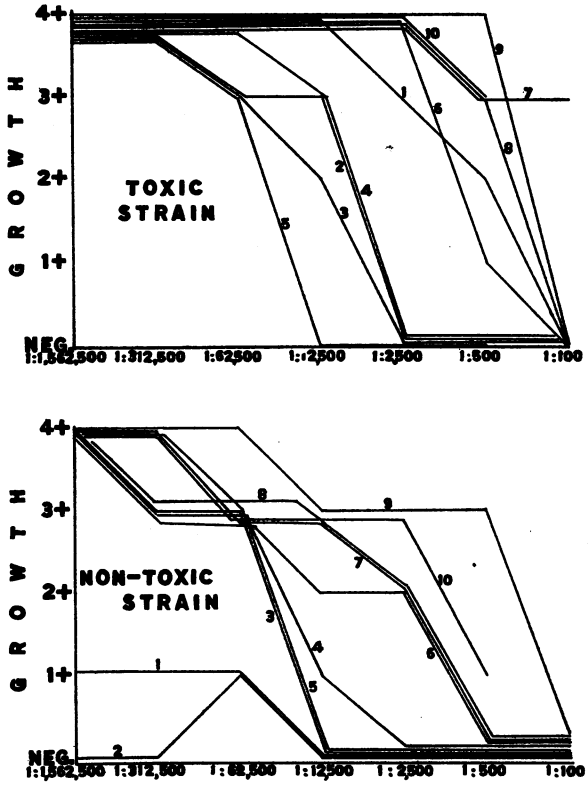

FIG. 1. EFFECT OF VARIOUS DILUTIONS OF DIFFERENT CHEMICALS ON A TOXIC AND A NON-TOXIC STRAIN OF STAPHYLOCOCCUS

One hour's exposure used throughout.

Chemicals used: (1) Copper sulphate, Baker anal.; (2) crystal violet, National —comm. cert.; (3) mercuric chloride, Merck reagent; (4) Merthiolate; (5) Metaphen; (6) ferric chloride, Eimer & Amend—tested purity; (7) basic fuchsin, National—comm. cert.; (8) Lysol; (9) phenol, Merck reagent; (10) S.T. 37.

The best differentiation between the two strains was obtained with crystal violet, but when 49 additional strains were tested with a dilution of 1:50,000 for one hour, the correlation with hemolysis and coagulase tests was only 59.6 per cent.

In the meantime, we had reasoned that, if a solid medium containing crystal violet would inhibit the growth of strains giving negative hemolysis and coagulase reactions but would permit the growth of positively reacting strains, it should be of value for the selective isolation of such positive strains. Crystal violet (National Aniline, comm. cert.) was added to proteose lactose agar in a concentration of 1 part dye substance in 100,000 parts of medium. Several strains were plated on this medium but the growths were poor. The concentration of dye was reduced to 1:1,000,000 (0.1 cc. of 1.0 per cent dye substance to 1 liter of medium). All strains grew fairly well when a large inoculum was used. It was noted, however, that growths from strains giving positive hemolysis and coagulase reactions had acquired a violet color. A few of the growths were golden colored, sometimes with violet fringes. These strains had produced an intense golden color on proteose lactose agar and had reacted positively to hemolysis and coagulase reactions. Strains of this type were usually highly pathogenic for rabbits.

A series of 594 strains were plated on the crystal violet agar to determine the degree of correlation between hemolysis and coagulase tests and the ability to acquire the violet color of the dye. The results are indicated in table 1. The best correlation (95.4 per cent) was obtained with strains producing white growths on violet agar. Orange colored growths gave almost as good correlation (92.2 per cent) but here the discrepancy may possibly be explained by the fact that the basis of comparison was the intense golden color (which obscured the violet reaction). Consequently it is possible that the 4 strains which gave negative hemolysis and coagulase reactions might have been "white" strains. The intermediate group of violet colored strains gave a correlation of 87.5 per cent. This type of organism is often of the albus variety and appears to be intermediate in other characters. This group requires further study. When the entire series of 594 strains was considered as a whole, there was an agreement with the hemolysis and coagulase reactions in 92.6 per cent.

In an effort to improve the accuracy of the violet reaction, other concentrations of crystal violet were tested. With concentrations

of 1:100,000 or more, the color of the medium was so intense that it was difficult to determine the color of the growths. On the other hand, with dilutions of 1:1,000,000 or more, the color of the growth was too pale to be distinguishable. The optimum concentration was 1:300,000. Proteose lactose agar containing this concentration of crystal violet will be referred to as "violet agar" and the phenomenon, called the "violet reaction." With this amount of dye, the violet colored growths were readily recognized after overnight incubation. The color was intensified after 48

TABLE 1

Correlation of growth of staphylococci on crystal violet agar 1 : 300,000 with hemolysis and coagulase reactions

COLOR OF GROWTH ON VIOLET AGAR	NUMBER OF STRAINS TESTED	ON BASIS OF HEMOLYSIS AND COAGULASE TESTS	NUMBER	AGREEMENT
				per cent
Orange (usually with violet fringe)	51	+	47	92.2
		0	4	
Violet	193	+	169	87.5
		0	24	
White	350	+	16	95.4
		0	334	
Total	594			92.6

hours but, with longer incubation periods or after storage in the refrigerator, even the pale growths became colored.

The effect of solutions of crystal violet on the color of suspensions of staphylococci was studied by plating strains on proteose lactose agar, washing off the growths the following day with 10.0 cc. of a 1:300,000 aqueous solution of crystal violet and pouring the suspensions into test tubes for comparison. Certain suspensions showed an immediate change of color and the sediments in these tubes were of a distinctly reddish hue. When the tubes were arranged according to the violet agar reactions of the organisms, the following correlation was noted. Those strains which produced white colonies on violet agar tended to bleach the aqueous

solutions and the suspensions, which settled rapidly, were blue. Suspensions from strains giving violet growths were violet, with a reddish tinge. The phenomenon is probably similar to that responsible for differential staining as described by Tolstoouhov (1929).

It was possible that some related dye might have given a sharper differentiation. When malachite green and brilliant green solutions were tested color changes occurred, but there appeared to be no correlation between the type of color change and the results of hemolysis and coagulase tests. Eosin, methylene blue, acid fuchsin, basic fuchsin, methyl green and neutral acriflavine gave results of doubtful value.

For purposes of classification, those strains which produced violet colored growths on violet agar were called "violet" strains; those which produced white growths were called "white" strains; while those strains which produced pale blue or pale violet growths were called "doubtful."

In comparing the violet reactions of staphylococcal strains with their pathogenicity for rabbits, experiments were undertaken using filtered killed suspensions in place of live unfiltered cultures. It was hoped by this method to secure more clear cut results than had been obtained previously (Chapman *et al.*, 1934).

Preliminary experiments showed that the lethal effect of whole killed cultures for rabbits varied considerably. However, most of the strains which killed rabbits when whole live cultures were injected intravenously, also killed rabbits when suitable concentrations of killed cultures were injected intravenously. Only animals weighing 1500 $\pm$ 75 grams were used in the animal experiments.

The suspensions for rabbit inoculation were prepared in as concentrated a form as possible in sterile distilled water. Serial dilutions were plated to determine the number of bacteria present. The suspensions were then treated with 1.0 per cent phenol and allowed to stand for several days in the refrigerator. Since viable organisms were still recovered, a stronger antiseptic, such as Merthiolate, was substituted. It was necessary to use a concentration of 1.0 per cent, buffered with 1.4 per cent sodium borate

to obtain sterile suspensions. After the suspensions were sterile, they were filtered through sterile cotton and injected intravenously into rabbits using 1.0 cc. quantities. Lethal effects were obtained in only a few rabbits and the inoculum was increased to 5.0 cc. Apparently the Merthiolate itself had no

TABLE 2

Correlation of hemolysis and coagulase reactions and color of growth on crystal violet agar 1:300,000 with lethal effect of 5.0 cc. of Merthiolated cultures of staphylococci for rabbits when injected intravenously

Results obtained with strains producing white or pale growths on crystal violet agar.

STRAIN NUMBER	COLOR	HEMOLYSIS	COAGULASE	VIOLET AGAR	NUMBER PER CUBIC CENTIMETER (PLATED)	EFFECT ON RABBIT	POSSIBLE ERROR
20	Albus	0	0	White	7.2×10^{12}	1. Died 12 hours 2. Survived* 3. Survived	Rabbit
24	Albus	0	0	Doubtful		Survived	
6194	Albus	0	0	White		Survived	
8	Albus	0	0	White		Survived	
6209	Albus	++++	0	White		Survived	
6195	Albus	++++	0	Doubtful		Survived	
6183	Albus	+++	0	White		Survived	
18	Albus	+++	0	White		Survived	
11	Albus	0	0	White		Survived	
2	Albus	0	0	White		Survived	
117	Albus	+	+	White	3.5×10^{10}	Survived	Coagulase
121	Albus	0	0	White	1.0×10^{10}	Survived	
126	Albus	+	0	Doubtful	2.2×10^{12}	Survived	
130	Albus	0	0	White	2.8×10^{8}	Survived	
139	Albus	++	0	White	3.0×10^{7}	Survived	
143	Albus	0	0	White	1.0×10^{7}	Survived	

* Survived four days.

lethal action under the conditions of these experiments because, even when a volume as large as 5.0 cc. was injected, no effect was noted in the test animals, provided strains considered nonpathogenic on the basis of the injection of live cultures were used.

Using the technic outlined, 28 strains were injected into 48 rabbits. With a few exceptions, which will be discussed later,

those strains with negative reactions did not produce death, while those with positive reactions produced lethal effects in rabbits.

In considering the correlation of the violet reaction with the lethal effect of the killed suspensions for rabbits it was noted that, with the exception of one rabbit, in which case the effect was probably an error, "white" or "doubtful" strains did not kill

TABLE 3

Correlation of hemolysis and coagulase reactions and color of growth on crystal violet agar 1:300,000 with lethal effect of 5.0 cc. of Merthiolated cultures of staphylococci for rabbits when injected intravenously

Results obtained with strains producing violet or golden growths on crystal violet agar.

STRAIN NUMBER	COLOR	HEMOLYSIS	COAGULASE	VIOLET AGAR	NUMBER PER CUBIC CENTIMETER (PLATED)	EFFECT ON RABBITS	POSSIBLE ERROR
1	Albus	++	++++	Violet		Died overnight	
15	Aureus	++++	+++	Violet		Died overnight	
22	Aureus	++++	+++	Violet		Died 24 hours	
25	Albus	++++	0	Violet		Died 36 hours	Coagulase?
124	Albus	+	+++	Violet	2.3×10^{10}	Died overnight	
133	Aureus	++++	+++	Golden	4.8×10^{10}	Died overnight	
138	Albus	++	+++	Violet	1.8×10^{8}	Died overnight	
145	Aureus	+	+++	Violet	6.5×10^{8}	Died overnight	
5	Aureus	0	++++	Violet	8.9×10^{16}	1. Died 36 hours 2. Died 48 hours 3. Survived	Rabbit
98	Albus	++++	0	Violet	2.4×10^{10}	Survived	Violet?
146	Aureus	++++	+	Violet	6.3×10^{14}	Died 4th day	
150	Albus	++	++++	Violet	3.8×10^{12}	Died 48 hours	

rabbits in four days (table 2). Among these strains was one which gave a positive coagulase reaction. Three of the strains with doubtful violet reactions were included in this group but they had no effect on the rabbits and should, therefore, be considered as white strains. Confirming the work reported in the previous paper (Chapman *et al.* (1934)), most of the killed cultures of hemolytic non-coagulating albus strains had no apparent effect on rabbits.

In contrast with this group of white or doubtful strains, golden or violet strains were with one exception, uniformly toxic for rabbits (table 3). Most of the animals injected died overnight, a few died in one to two days, and one did not die until the 4th day. Strain 98 failed to affect the rabbit and, since the other *in vitro* tests were negative, the violet reaction was probably in error in this instance. One of the strains (no. 25) gave negative hemolysis and coagulase reactions but the violet reaction was

TABLE 4

Summary of correlation between different tests

Methods used: Crystal violet, exposure to 1:50,000 crystal violet for one hour; sodium carbonate, exposure to 1.0 per cent Na_2CO_3 for two and one-half hours; hemolysis, hemolysis of rabbit blood agar; coagulase, coagulation of oxalated human plasma; violet agar, color of growth on 1:300,000 crystal violet agar; rabbit inoculation, intravenous inoculation of 5.0 cc. of a heavy killed suspension.

TESTS COMPARED		STRAINS TESTED	AGREEMENT
(a)	(b)		
			per cent
Crystal violet	Sodium carbonate	47	68.1
Crystal violet	Hemolysis and coagulase	47	59.6
Hemolysis and coagulase	Sodium carbonate	151	76.8
Violet agar	Sodium carbonate	.101	82.2
Violet agar	Hemolysis and coagulase	1034	93.0
Violet agar	Rabbit inoculation	28	96.4
Hemolysis and coagulase	Rabbit inoculation	28	92.8
Rabbit inoculation (probable accuracy based on results with 48 rabbits)		28	91.3

positive and the rabbit died in thirty-six hours. This suggested that the violet reaction was correct and the coagulase in error.

Bacterial counts presented difficulties which raised doubts as to their accuracy and, since good correlation was obtained with crude suspensions, the strength of which was estimated from their turbidity and viscosity, it was assumed that turbidity or viscosity was a sufficiently precise method for the present purpose. The bacterial counts are recorded in tables 2 and 3.

The effects of strain 5 on rabbit 3 and of strain 20 on rabbit 1 indicate possible errors in the animal experiments. On the basis

of these experiments it would appear that the violet reaction and the hemolysis and coagulase reactions are approximately of the same order of accuracy as the animal inoculation tests.

The correlation of different reactions is summarized in table 4. Some data based on extended experiments have been included.

From these experiments it is concluded that the violet reaction may prove to be a useful addition to hemolysis and coagulase reactions for the study of staphylococci.

SUMMARY

In an effort to improve the correlation between hemolysis and coagulase reactions and animal inoculation tests, a search was made for reactions which might give correlation with hemolysis and coagulase tests, and which might be used to check their accuracy.

When strains were mixed with 1.0 per cent sodium carbonate for two and a half hours and transplants made at the end of that time, growths were obtained from certain strains. There was a 76.8 per cent correlation between the ability of a strain to grow from the carbonate transplant and the presence of hemolysis and coagulase factors. Attempts were made to improve this correlation by substituting other chemical substances for the sodium carbonate. Two strains were selected for comparative tests. One reacted positively to hemolysis and coagulase tests, grew from carbonate transplants and was pathogenic for rabbits; the other gave negative hemolysis and coagulase tests, did not grow from carbonate transplants and was not pathogenic for rabbits. When tested with decimal dilutions ranging from 1:100 to 1:1,562,000 of copper sulfate, crystal violet, mercuric chloride, Metaphen, Merthiolate, ferric chloride, basic fuchsin, Lysol, phenol and hexylresorcinol, growths were obtained from more concentrated solutions of each chemical when the positively reacting strain was used, as compared with similar tests using the negatively reacting strain. The greatest differentiation was obtained with crystal violet. However, exposure of a number of strains to solutions of crystal violet failed to correlate with hemolysis and coagulase reactions. When crystal violet was added

to solid media, it was noted that strains reacting positively to hemolysis and coagulase tests produced violet growths, or golden growths tinged with violet, while those strains which reacted negatively to hemolysis and coagulase tests produced white or pale growths. The correlation between hemolysis and coagulase tests and color on crystal violet agar was 93.0 per cent, on the basis of tests on 1034 strains.

The results of violet agar and hemolysis and coagulase reactions were compared with animal inoculation tests using 5.0 cc. of dense Merthiolated suspensions and the following correlations noted: (1) Between violet agar reactions and toxicity for rabbits, the correlation was 96.4 per cent; (2) there was a correlation of 92.8 per cent between hemolysis and coagulase reactions and the animal inoculation tests. On the basis of 48 tests on 28 strains, the animal inoculation tests were probably correct in 91.3 per cent of the rabbits. This would place hemolysis and coagulase reactions, the violet agar reaction and rabbit inoculation of Merthiolated cultures in the same order of accuracy.

CONCLUSION

The color of growths of staphylococci on crystal violet agar 1:300,000 is a valuable characteristic for the differentiation of staphylococci.

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