THE SELECTIVE ACTION OF GENTIAN VIOLET ON CLOSELY RELATED BACTERIAL STRAINS.*

By JOHN W. CHURCHMAN, M.D., and W. HOWARD MICHAEL.

(From the Pathological Laboratory and the Biological Division of the Laboratory of the Medical Clinic, Johns Hopkins University, Baltimore.)

PLATE 85.

In a previous communication[1] a selective bactericidal property possessed by gentian violet was described, and observations were reported to indicate the nature of this chemical affinity as exhibited in a study of a large number of representative bacteria.

It was shown that bacteria could be sharply divided into two groups by their behavior toward gentian violet, either by staining the organisms directly, and then transplanting them to ordinary media, or by growing the unstained organisms on media containing the dye. In one of the groups of bacteria thus distinguished, gentian violet prevented growth; in the other it was without effect. The first group contained such organisms as *B. subtilis, B. anthracis, B. mycoides, M. aureus, M. albus,* Streptococcus, and *B. diphtheriæ;* in the other group fell *B. typhosus, B. paratyphosus, B. coli communis, B. pyocyaneus, B. prodigiosus, B. choleræ suis,* and many others. Organisms whose growth was inhibited by the dye were referred to as violet positive, while those whose growth was uninfluenced were termed violet negative. The selective action of the dye was well demonstrated by the use of divided plates, so made as to contain plain agar in one half and gentian violet agar in the other. A divided plate across which two violet positive and two violet negative organisms have been stroked is shown in figure 1, which illustrates the selective action of the dye and the technique of its demonstration. The effect of the dye on a violet positive organism is shown in figure 2, which represents a divided plate that has been stroked with seropurulent material from a suppurating parotitis in typhoid fever. On the plain agar side of the plate a number of discrete colonies of *M. aureus* are seen growing, while the gentian violet side of the plate has remained sterile, though the track of the inoculating needle is plainly marked on the plate by a film of dried bloody serum. In smears from the gentian violet half of the plate, no organisms were to be found.

It was not expected that closely related organisms would differ in their behavior toward gentian violet; and it was found, in general, by a study of the effect of the dye on 138 distinct bacterial species,

* Received for publication, August 16, 1912.

[1] Churchman, J. W., *Jour. Exper. Med.*, 1912, xvi, 221.

that nearly allied organisms were similarly affected by the stain. *Bacillus typhosus,* for instance, was uninfluenced in its growth by gentian violet; so also were *Bacillus coli communis, Bacillus paracoli,* and *Bacillus paratyphosus.* Again, *Bacillus prodigiosus* grew luxuriantly in the presence of the dye; so also did *Bacillus pyocyaneus, Bacillus violaceus,* and *Bacillus indicus.* On the other hand, the growth of *Bacillus subtilis* was absolutely prevented by gentian violet; and this was equally true for *Bacillus anthracis, Bacillus anthracoides, Bacillus megatherium, Bacillus pseudoanthracis, Bacillus mycoides, Bacillus mesentericus,* and *Bacillus liodermos.*

In a study, however, of five nearly identical strains belonging to the enteritidis group, the selective affinity of the stain was found to be so specific in its nature as to distinguish between different strains of very closely related organisms. On the growth of one member of this group, the dye exhibited a constant and complete inhibition; on the growth of the other four it was absolutely without effect. It is the purpose of this paper to report a careful study of the behavior of this group of organisms toward the dye.

The organisms included in this study came from the following sources:

(1) E. J. H. H., *B. enteritidis,* Johns Hopkins University stock; originally isolated by Dunham, of Cambridge, in 1900.

(2) E. 18, from the Museum of Natural History, New York; originally obtained from The Rockefeller Institute for Medical Research.

(3) E. 25, from the above Museum; originally isolated by Jordan, from a drainage canal in Chicago.

(4) E. 26, *idem.*

(5) E. 132, from the above Museum; originally from Kral's laboratory.

(6) E. 234, from the above Museum; originally isolated by Dunham, of Cambridge.

The characteristics of the bacteria are shown in table I. None of them was tested for agglutination with a known enteritidis serum. For the purposes of this paper, the important point is that the five organisms were identical, according to the usual tests, and that their characteristics were those described for the enteritidis group. What name is given to them is here immaterial. They were all motile, Gram negative, non-spore-bearing bacilli, which did not coagulate milk or liquefy gelatin. They fermented glucose,

mannite, maltose, and galactose, but did not ferment saccharose, lactose, raffinose, or inulin. E. 26 formed gas a trifle more slowly than the others, and from agar growths of E. 234 and E. J. H. H. homogeneous emulsions in salt solution were less easily made than from agar growths of the other four. But with these very slight exceptions, all six organisms were identical, according to the usual morphological, tinctorial, and cultural tests.

Toward gentian violet, however, a constant and striking difference in the behavior of these organisms was noted. E. J. H. H. was the member of the group first studied; and it was expected, from the previous studies of the colon and paratyphoid group, that gentian violet would have no effect on its growth. On planting it, however, on divided gentian violet plates, we were surprised to find that its growth was absolutely prevented by the dye. This observation was repeatedly made, and was then confirmed by studying the New York descendant of the same original Cambridge stock (E. 234), which was found to behave in exactly the same way. Not only did these two descendants of the same original stock refuse to grow across divided gentian violet plates, but their growth was also much hindered or entirely prevented by staining the organisms and then transplanting them to agar or broth. Moreover, they would not grow in broth containing the dye in a strength of 1 to 80,000. They behaved as all violet positive organisms do.

These findings as to E. J. H. H. and E. 234 led to a study of the four other enteritidis strains. The growth of these (E. 18, E. 25, E. 26, and E. 132) was found to be absolutely uninfluenced by the dye, either when applied directly to the organisms (by the method of vital staining) or when applied to the media on which they were grown. They were, in a word, typically violet negative, in striking contrast to E. J. H. H. and E. 234, which, as has been said, had been found to be violet positive. This specific, selective affinity is well illustrated in figure 3, which shows a divided plate that has been stroked with E. 234, E. 18, E. 25, E. 26, and E. 132. The growth of one (E. 234) will be seen to have been prevented by the dye; on the other four the stain has had no effect whatever. This finding was constant in the large number of transplants made. In other words, of five organisms, identical in all ordinary tinctorial, morph-

TABLE I.

Behavior toward gentian violet.			Ordinary morphological, cultural, and tinctorial characteristics.													
Growth on divided plates.	Growth in gentian violet broth.	Growth after staining with gentian violet.	Organism.	Morphology.	Motility.	Gram stain.	Litmus milk.	Gelatin.	Glucose.	Saccharose.	Lactose.	Mannite.	Maltose.	Galactose.	Raffinose.	Inulin.
Good growth on both sides	Good growth	Good growth	E. 18	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation
Good growth on both sides	Good growth	Good growth	E. 25	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation
Good growth on both sides	Good growth	Good growth	E. 26	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation
Good growth on both sides	Good growth	Good growth	E. 132	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation
No growth on gentian violet side	No growth	No growth, or very faint, slow growth	E. 234	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation
No growth on gentian violet side	No growth	No growth, or very faint, slow growth	E. J. H. H.	Short bacillus; no spores	+	o	Initial acidity; no coagulation	No liquefaction	Fermentation	No fermentation	No fermentation	Fermentation	Fermentation	Fermentation	No fermentation	No fermentation

ological, and cultural characteristics, one (E. J. H. H. or E. 234) was constantly gentian violet positive; while the other four (E. 18, E. 25, E. 26, and E. 132) were constantly gentian violet negative. These facts are recorded in table I.

TABLE II.

Agglutination Reactions with Typhoid Serum.

Organism.	Dilutions of serum.				
	1–100	1–200	1–400	1–800	1–1,600
B. typhosus	+++	+++	+++	+++	0
B. paratyphosus	0	0	0	0	0
E. 18	0	0	0	0	0
E. 25	0	0	0	0	0
E. 26	0	0	0	0	0
E. 132	+	0	0	0	0
E. 234	0	0	0	0	0
E. J. H. H.	0	0	0	0	0

In order to get additional light on the real specificity of this selective action, the organisms were further studied by the method of agglutination. Each one was first tested with a known typhoid serum, and agglutination in no case occurred (table II). Rabbits were then immunized against E. 18, E. 25, E. 26, and E. J. H. H.,[2]

TABLE III.

Agglutination Reactions with the Serum of an Animal Immune to E. 18.

Organism.	Dilutions of serum.				
	1–200	1–400	1–800	1–1,600	1–3,200
B. typhosus	0	0	0	0	0
B. paratyphosus	0	0	0	0	0
E. 18	+++	+++	+++	+++	+
E. 25	0	0	0	0	0
E. 26	0	0	0	0	0
E. 132	+++	+++	+++	+++	++
E. 234	+++	+++	+++	++	0
E. J. H. H.	+++	+++	+++	++	0

and the agglutinative properties of these sera were tested against *Bacillus typhosus, Bacillus paratyphosus,* E. 18, E. 25, E. 26, E. 132, E. 234, and E. J. H. H. The results of these experiments are shown in tables III, IV, V, VI, and VII.

[2] Attempts at immunization against E. 132 were unsuccessful.

TABLE IV.

Agglutination Reactions with the Serum of an Animal Immune to E. 25.

Organism.	Dilutions of serum.				
	1–200.	1–400.	1–800.	1–1,600.	1–3,200.
B. typhosus	0	0	0	0	0
B. paratyphosus	0	0	0	0	0
E. 18	0	0	0	0	0
E. 25	+++	+++	+++	++	+
E. 26	0	0	0	0	0
E. 132	?	0	0	0	0
E. 234	0	0	0	0	0
E. J. H. H.	0	0	0	0	0

TABLE V.

Agglutination Reactions with the Serum of an Animal Immune to E. 26.

Organism.	Dilutions of serum.				
	1–200	1–400	1–800	1–1,600	1–3,200
B. typhosus	0	0	0	0	0
B. paratyphosus	+	0	0	0	0
E. 18	0	0	0	0	0
E. 25	0	0	0	0	0
E. 26	+++	+++	+++	++	+
E. 132	0	0	0	0	0
E. 234	0	0	0	0	0
E. J. H. H.	0	0	0	0	0

TABLE VI.

Agglutination Reactions with the Serum of an Animal Immune to E. J. H. H.

Organism.	Dilutions of serum.				
	1–200	1–400	1–800	1–1,600	1–3,200
B. typhosus	0	0	0	0	0
B. paratyphosus	0	0	0	0	0
E. 18	+++	++	+	0	0
E. 25	+	0	0	0	0
E. 26	0	0	0	0	0
E. 132	+++	+++	+	0	0
E. 234	+++	+++	+++	++	+
E. J. H. H.	+++	+++	+++	++	+

It will be seen: (1) that none of the sera caused clumping with *Bacillus typhosus* or *Bacillus paratyphosus;* (2) that E. 25 and E. 26 showed no interagglutination, either with each other or with the other organisms studied; (3) that E. J. H. H. and E. 234 were identical in their serum reactions; (4) that E. 18, E. 132, and E. J.

H. H. showed some interagglutination. From these agglutination experiments we may conclude: (*a*) that the organisms studied did not belong to the typhosus or paratyphosus group; (*b*) that two of them (E. 18 and E. 25) were to be distinguished by agglutination from the other three; (*c*) that four of them (E. 18, E. 132, E. 234, and E. J. H. H.) were closely related, as indicated by their interagglutinations (table VII).

TABLE VII.

Summary of Agglutination Reactions.

Organism.	Sera.					
	Typhoid.	Paratyphoid.	E. 18.	E. 25.	E. 26.	E. J. H. H.
B. typhosus	++	0	0	0	0	0
B. paratyphosus	0	++	0	0	0	0
E. 18	0	0	+++	0	0	++
E. 25	0	0	0	+++	0	0
E. 26	0	0	0	0	+++	0
E. 132	0	0	+++	0	0	++
E. 234	0	0	++	0	0	+++
E. J. H. H.	0	0	++	0	0	+++

Briefly, then, this study was concerned with five stock organisms, identical in all morphological, tinctorial, and cultural characteristics. They all failed to agglutinate with known typhoid serum, and to produce serum in animals which would agglutinate either *Bacillus typhosus* or *Bacillus paratyphosus.* Yet one of these organisms (E. J. H. H.) was sharply and constantly violet positive; while on the growth of the other four the dye was absolutely without effect. The studies of the organisms by the method of agglutination showed the gentian violet affinity to be quite as delicate as the serum affinity. The two did not run absolutely parallel. But by the use of gentian violet it was possible to distinguish, with great precision and constancy, one organism from four others identical with it in all ordinary characteristics and possessing similar agglutinative properties.

So far as we are aware, no instance of chemical affinity of this degree of nicety has previously been observed. The observation, as such, is an isolated one; and in itself has only the small value attaching to observations of that kind. As regards, however, its

bearing on the general subject of affinity between chemical substances and microörganisms, the instance here reported is of significance for the following reasons: (1) It establishes the fact that chemical substances may be so specific in their selective affinity for microörganisms as to distinguish among strains of bacteria otherwise indistinguishable. The importance of this fact to bacteriology is obvious. None of the closely related organisms which offer difficulty to bacteriologists, as regards isolation in purity, are so nearly identical as the five strains of *Bacillus enteritidis* studied in this investigation; yet an aniline dye picked one of these strains out with great constancy and precision. That a substance will be found possessing a similar selective affinity between such bacteriologically troublesome organisms as *Bacillus typhosus* and *Bacillus coli* does not seem to be out of the question. The observation reported in this paper gives an important lead, and indicates that a systematic study of the anilines by the method of divided plates should be carried out, for light it may well throw on the easy differentiation of closely related bacteria. (2) It indicates also to what extent our ideas of bactericides must be modified by the conception of chemical affinity. The fact that a given bactericide kills a given organism does not justify the conclusion that it will kill all closely related organisms, or even all strains of the same organism. Indeed the behavior of bacteria toward gentian violet, and particularly the instance of it reported in this paper, gives experimental basis for the assumption of chemical fastness which has previously lacked clear confirmation. The members of the general bacterial group to which *Bacillus enteritidis* belongs (*Bacillus typhosus, Bacillus coli, Bacillus paracoli*), and the majority of strains of *Bacillus enteritidis* itself, are naturally fast toward gentian violet: but we have happened to come upon one strain which, though identical with the others in every other respect, lacks this natural fastness and will not grow in the presence of the dye.

EXPLANATION OF PLATE 85.

FIG. 1. The upper half of the plate contains gentian violet agar, the lower half plain agar. The plate has been stroked with *B. typhosus, B. anthracis, M. aureus,* and *B. coli,* from left to right in the order named. The dye has been without effect on the growth of *B. typhosus* and *B. coli,* while it has completely prevented the growth of *B. anthracis* and *M. aureus.*

FIG. 2. The upper half of the plate contains gentian violet agar, the lower half plain agar. The plate has been stroked with the seropurulent exudate from a parotitis occurring during typhoid fever. Many colonies of *M. aureus* are seen growing on the plain agar side; the gentian violet side has remained sterile. The track of the inoculating needle is plainly seen across the whole plate.

FIG. 3. The upper half of the plate contains gentian violet agar, the lower half plain agar. The plate has been stroked with E. 18, E. 25, E. 234, E. 26, and E. 132, from left to right in the order named. It will be seen that on the growth of four, the gentian violet has been without effect, while the dye has completely prevented the growth of E. 234.

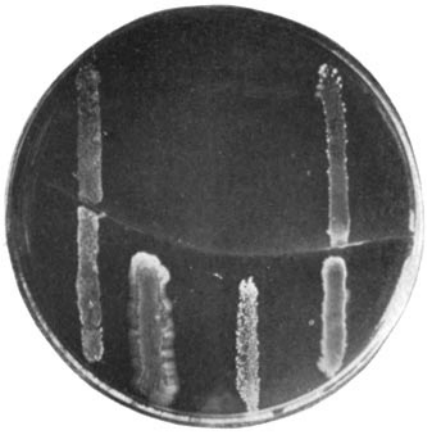

FIG. 1.

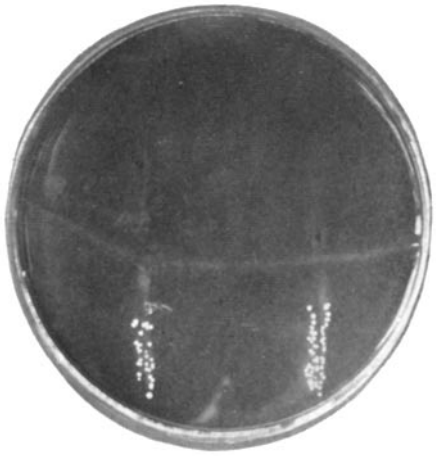

FIG. 2.

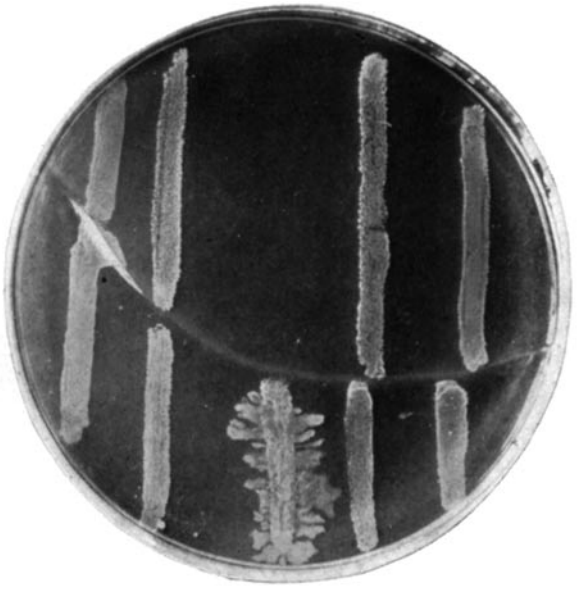

FIG. 3.