THE REVERSE SELECTIVE BACTERIOSTATIC ACTION OF ACID FUCHSIN.

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Recent experiments upon the effects of dyes on cells have raised some interesting questions as regards the use of dyes in bacteriological technique and therapy. There is evidence to show that the majority of Gram-positive bacteria, *both vegetative cells and spores,* are more susceptible to the bactericidal action of gentian violet and other triphenylmethane dyes *than the vegetative cells of many* Gram-negative bacteria.[1,2,3] This selective action of gentian violet is known as selective bacteriostasis.[2] It has been utilized in inhibiting the growth of Gram-positive bacteria in methods of isolating or determining the presence of Gram-negative bacteria. It also delimits the value of these dyes in therapy.[2] Gentian violet has been utilized to check the growth of bacteria in wounds and on infected surfaces. It has not proven very effective in controlling the growth of Gram-negative organisms like *Bacterium coli* and *Pseudomonas pyocyanea*. Recent work suggests that certain dyes, or other substances, such as acid fuchsin, flavines, and sulfanilic acid, can be utilized to destroy the Gram-negative bacteria without affecting the Gram-positive spore bearers, and thus serve as an aid in the isolation of the latter.[3] The selective action of acid fuchsin has been termed reverse selective bacteriostasis. Furthermore, there is the possibility of combining with gentian violet some dye that will destroy the Gram-negative bacteria which are not affected by the gentian violet, thus making possible the use of a disinfectant effective against both types of bacteria.

[1] Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221.
[2] Churchman, J. W., *J. Am. Med. Assn.*, 1922, lxxix, 1657.
[3] Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 1.

The experimental work described in the present paper has been designed, first, to determine the value of acid fuchsin as a disinfectant and the desirability of combining acid fuchsin or some other dye showing reverse selective bacteriostasis with gentian violet for use in the treatment of mixed infections; and, second, to determine the cause of the reverse selective bacteriostasis and of the lack of parallelism between the bactericidal and bacteriostatic properties of acid fuchsin. An examination of the results indicates that acid fuchsin is a very weak disinfectant compared with some of the other dyes, especially in the presence of organic matter and at body temperature. This fact is so evident that little space will be devoted to it, the main discussion being confined to the reverse selective action of acid fuchsin and the lack of parallelism between the bactericidal and bacteriostatic properties.

Reverse Selective Bacteriostatic Action of Acid Fuchsin.

The Gram-positive non-spore bearers are not included in the selective action of acid fuchsin and nothing is known about the resistance of the Gram-negative spore bearers. It is thus seen that the reversed selective action is only partial, certain groups apparently being exempt. The fact that the reversed action applies only to the Gram-positive spore bearers naturally suggests that the apparent reversal is due to spore resistance. Gentian violet destroys both vegetative cells and spores of the Gram-positive bacteria in weaker dilution than it does most of the Gram-negative bacteria.[3] Does acid fuchsin spare both the vegetative cells and spores of the Gram-positive bacteria in weaker dilution than it does the Gram-negative bacteria? We have the resistance of three elements to consider, the Gram-negative vegetative cells, the Gram-positive vegetative cells, and the Gram-positive spores. If only the spores resist the acid fuchsin then we fail to see any significance in the reversed selective action of acid fuchsin. Furthermore, if the Gram-negative bacteria resist the bacteriostatic action of acid fuchsin better than the vegetative cells of Gram-positive spore bearers we have an explanation of the lack of parallelism between the bactericidal and bacteriostatic action of this dye.

For a complete analysis and comparison of the gentian violet reaction with the acid fuchsin reaction it is necessary to determine separately the resistance of vegetative cells and spores of the Gram-positive bacteria to acid fuchsin. A study of the Gram-negative spore-bearing organisms is desirable but not essential. For the acid fuchsin reaction to be comparable with the gentian violet reaction but the reverse thereof, it must be demonstrated that the Gram-positive vegetative cells, freed of spores, resist acid fuchsin in higher dilution than do the Gram-negative vegetative cells. The following experiments demonstrate the comparative resistance of Gram-negative bacteria and the vegetative cells and spores of Gram-positive bacteria.

TABLE I.

Results of an Experiment to Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore-Bearing Organism in the Presence of Organic Matter and at a Temperature of 45°C.

Organism.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	4 cc. of culture mixed with 1 cc. of saturated aqueous solution of acid fuchsin heated to 45°C. for 1 hr.*	690,000
" " (2).	Control; the same as No. 1 except 1 cc. of broth added in place of acid fuchsin.	48,000,000
" " (3).	The same as No. 2 except heated to 75°C. for 15 min.	720,000

* To make a saturated solution 4 or 5 gm. of the dye were added to 10 cc. of distilled water. Coleman and Bell Co. recommended adding 10 gm. of their dye to 10 cc. of water to get a saturated solution. In most of our experiments the National Aniline and Chemical Company's acid fuchsin was used. This company advises us that their acid fuchsin is soluble to the extent of 4 to 5 per cent.

Experiment 1. To Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore-Bearing Organism in the Presence of Organic Matter and at a Temperature of 45°C.—1 cc. of a saturated aqueous solution of acid fuchsin (Coleman and Bell Co.) was added to 4 cc. of a calcium carbonate dextrose broth culture of *B. subtilis*. The culture of *B. subtilis* was obtained by mixing a 24 hour culture and a 7 day culture. Spores were present. The mixture was heated for 1 hour at 45°C. plated out quantitatively and then incubated at 37°C. for 24 hours. Counts were made in the usual manner. In order to determine whether the vegetative cells as well as spores survived, 4 cc. of the culture were mixed with 1 cc. of broth and heated to 75° C. for 15 minutes. The control consisted of 4 cc. of the culture mixed with 1 cc. of broth and heated to 45° C. for 1 hour.

The results obtained are given in Table I. These indicate that under the conditions of the experiment acid fuchsin destroys the vegetative cells of *Bacillus subtilis* but not the spores.

The presence of organic matter reduces the action of acid fuchsin as well as that of most other dyes except the flavines and favors the resistance of the vegetative cells. It should be noted that in this experiment organic matter was present and yet the vegetative cells failed to survive the exposure to acid fuchsin. The experiment demonstrates that the vegetative cells and spores of a Gram-positive spore bearer differ in their resistance to acid fuchsin. This fact should be taken into consideration in any discussion of reverse selective bacteriostasis.

The experiment was repeated using sulfanilic acid 1 cc. to 1 cc. of the broth culture with comparable results. The vegetative cells were killed and the spores survived.

Experiment 2. To Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore Bearer in the Absence of Organic Matter.—Transplants of *B. subtilis* were made on agar slants at 12 hour intervals for 48 hours, and the growth on the fourth transplant was washed off in saline. This reduced the spores to a minimum. The other conditions of the experiment were the same as those described in Experiment 1.

The results obtained are given in table II and indicate that under the conditions of the experiment acid fuchsin destroys the vegetative cells of *B. subtilis* while the spores survive.

Experiments 1 and 2 suggest that the reverse selective action attributed to acid fuchsin applies only to the spores and not to the vegetative cells of the Gram-positive spore bearers. In broth and saline suspension the spores resist high concentration of acid fuchsin while the vegetative cells are killed. The results of Experiment 3 indicate that *Bacterium coli* in broth is very resistant to acid fuchsin.

Experiment 3. To Determine the Effect of Acid Fuchsin on Bacterium coli in Dilutions Destroying the Vegetative Cells of Gram-Positive Spore Bearers.—The technique of the experiment was the same as in Experiments 1 and 2. Comparison is also made with a Gram-positive non-spore bearer. 24 hour calcium carbonate broth cultures and saline suspensions were used (Table III).

The experiment was repeated, using *Bacterium cloacæ* and *Sarcina lutea* with essentially the same results.

Bacterium coli resisted acid fuchsin in dilutions destroying the vegetative cells of *Bacillus subtilis*. It was destroyed in saline suspension in dilutions of acid fuchin not destroying the spores of *Bacillus subtilis*. *Bacterium coli* appears to be fully as resistant,

TABLE II.

Result of an Experiment to Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore Bearer in the Absence of Organic Matter.

Organism.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	4 cc. of suspension mixed with 1 cc. of saturated aqueous acid fuchsin and heated to 45°C. for 1 hr.	50
" " (2).	Control; the same as No. 1 except 1 cc. of saline added in place of acid fuchsin.	23,000,000,000
" " (3).	The same as No. 2 except heated to 75°C. for 15 min.	53

TABLE III.

Result of an Experiment to Determine the Effect of Acid Fuchsin on Bacterium coli in Dilutions Destroying the Vegetative Cells of a Gram-Positive Spore Bearer.

Organism.	Treatment.	Organisms surviving per cc.
Bacterium coli (1).	4 cc. of broth culture mixed with 1 cc. of saturated aqueous acid fuchsin heated to 45°C. for 1 hr.	130
" " (2).	Control; the same as No. 1 except 1 cc. broth used in place of acid fuchsin.	130,000,000
" " (3).	The same as No. 1 except saline suspension organisms used.	None.
" " (4).	Same as No. 3 except 1 cc. of saline used in place of 1 cc. of acid fuchsin.	3,800,000
Staphylococcus albus (5).	Same as No. 1.	680
" " (6).	" " " 2.	88,000,000
" " (7).	" " " 3.	None.
" " (8).	" " " 4.	2,800,000

if not more resistant, to acid fuchsin than the vegetative cells of the Gram-positive spore bearers. This refers to the bactericidal effect. Later experiments demonstrate that the Gram-negative bacteria such as *Bacterium coli* are more resistant than the Gram-

positive spore bearers to the bacteriostatic action of acid fuchsin. And the flavines which are credited with the same properties as acid fuchsin have greater bacteriostatic action on the Gram-positive non-spore bearers than on the Gram-negative bacteria. It follows that the reverse selective action of acid fuchsin indicates only spore resistance.

The results given in Table III indicate variation in the resistance of the individual cells of both species used, 130 *Bacterium coli* cells out of 130,000,000, and 680 *Staphylococcus albus* cells out of 88,000,000 surviving. Whether this variation in resistance to acid fuchsin can be utilized to separate the species into resistant and non-resistant strains to acid fuchsin as done by Churchman with *Bacterium coli* and gentian violet has not been determined. The individual cells of any species of bacteria manifest differing amounts of resistance when exposed to unfavorable conditions. This is well known to be true also of protozoan parasites. Whether the variation in resistance shown in Table III represents simple individual variation, strain variation, or the development of a resistant strain remains to be determined.

As bearing on the general question of reverse selective action it seemed advisable to determine the effect of acid fuchsin on a Gram-negative spore bearer. *Bacillus brevis* was used and the conditions of the experiment were the same as those given in Table II for *Bacillus subtilis*, except that older cultures were used. The results are given in Table IV. Under the conditions of this experiment the vegetative cells of *Bacillus brevis* are killed and the spores survive. The Gram-positive and Gram-negative spore bearers under the conditions of the experiment were affected in the same way by acid fuchsin. By varying the concentration of the dye the results might conceivably have been modified but no experiments of the sort were made. The effect of gentian violet on Gram-negative spore bearers is unknown to us.

We were unable to obtain cultures of *Bacillus subtilis* free of spores. 12 hour transplants were found to contain spores. However, our 24 hour cultures of *Bacillus brevis* were spore-free though spores were present in old cultures. Exposing 24 hour cultures of *Bacillus brevis* to the acid fuchsin was found to destroy all the cells. If the

experiments had been discontinued at this point and the results compared with those obtained with *Bacillus subtilis* the conclusion might have been reached that the reverse selective bactericidal action attributed to acid fuchsin should be extended to include the Gram-negative spore bearers. But the investigation of the effect of acid fuchsin on the spores of *Bacillus brevis* demonstrated that reverse selective action does not apply to the Gram-negative spore bearers.

TABLE IV.

Result of an Experiment to Determine the Effect of Acid Fuchsin on a Gram-Positive and a Gram-Negative Spore Bearer.

Organism.	Age of culture.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	18 hrs. and 30 days.	4 cc. of suspension mixed with 1 cc. of saturated aqueous acid fuchsin, heated 1 hr. at 45°C.	23,000
" " (2).	18 hrs. and 30 days.	Control; 1 cc. of saline used in place of acid fuchsin, treated as above.	8,700,000,000
" " (3).	18 hrs. and 30 days.	The same as No. 2 except exposed to 75° C. for 15 min.	29,000
" *brevis* (4).	18 hrs. and 30 days.	Same as No. 1.	2,300
" " (5).	18 hrs. and 30 days.	" " " 2.	3,700,000
" " (6).	18 hrs. and 30 days.	" " " 3.	1,900
" *subtilis* (7).	24 hrs.	" " " 1.	180
" " (8).	24 "	" " " 2.	59,000,000,000
" " (9).	24 "	" " " 3.	220
" *brevis* (10).	24 "	" " " 1.	None.
" " (11).	24 "	" " " 2.	180,000,000
" " (12).	24 "	" " " 3.	None.

Acid fuchsin is but a weak germicide. Spores of both Gram-negative and Gram-positive bacteria resist it in greater concentration than do the vegetative cells. It follows that the selective action of acid fuchsin is not comparable with the selective action of gentian violet.

Experiment 4. To Compare the Effect of Acid Fuchsin on a Gram-Positive and a Gram-Negative Spore Bearer.—The technique of the experiment was the same as that of Experiment 2. Saline suspensions of the organism were used. To insure

cultures containing both spores and vegetative cells 18 hour and 30 day cultures were mixed. 24 hour cultures were also used to insure either a small number of spores or a lack of spores.

In order to determine whether enough acid fuchsin was being carried over to the plates to inhibit growth, sufficient saturated acid fuchsin was added to agar to make a 1:100 saturated solution. This is a more concentrated solution than was present in any of our plates. *Bacterium coli*, *Bacterium cloacæ*, *Staphylococcus albus*, *Sarcina lutea*, *Bacillus brevis*, and *Bacillus subtilis* grew as well on this agar as in agar without the dye, a fact which proves that the action of acid fuchsin in our experiments was bactericidal rather than bacteriostatic. Molds grow readily on the surface of saturated aqueous acid fuchsin.

It has been suggested that the reverse selective action of acid fuchsin may be due to the presence of the SO_3 groups,[3] acid fuchsin being originally produced by the process of sulfonation from basic fuchsin, which has the same selective action as gentian violet. Churchman states that "addition of the sulfonic acid groups not only makes the basic triphenylmethanes more toxic for Gram-negatives but less toxic for Gram-positive spore-bearing aerobes." But if the reverse selective action of acid fuchsin is purely a question of spore resistance, as our experiments indicate, one would expect to find that the process of sulfonation makes the basic fuchsin less toxic for the Gram-negative bacteria as well as for the Gram-positive organisms. In other words, the altered dye should act as an ordinary germicide and a reduction in its action on the Gram-positive spore bearers would be accompanied by a reduction in its action on Gram-negative bacteria.

Experiment. 5. To Determine the Effect of the Addition of SO_3 Groups on the Bactericidal Action of Basic Fuchsin for a Gram-Negative Organism.—In addition to basic fuchsin and acid fuchsin other dyes were used to determine comparative bactericidal powers.

Technique of the experiment: To 4 cc. of a saline suspension of *Bacterium coli* was added 1 cc. of a solution of the dye. The dyes were made in different solutions as given in the table. The tubes were exposed to a temperature of 45°C. At intervals up to 5 hours strokes were made on agar to determine viability. The dye carried over did not produce bacteriostasis.

The results are given in Table V. It is seen that basic fuchsin is a more powerful disinfectant than acid fuchsin for the Gram-negative bacteria as well as for the Gram-positive bacteria. The addition of the SO_3 group to basic fuchsin has decreased the bactericidal action for the Gram-negative as well as the Gram-positive bacteria. The process of sulfonation has reduced or eliminated the specific action characteristic of the basic triphenylmethane dyes for the spores of the Gram-positive bacteria. The staining power is also reduced.

TABLE V.

Results of an Experiment to Determine the Effect of the Addition of SO_3 Groups on the Bactericidal Action of Basic Fuchsin for the Gram-Negative Organism Bacterium coli.

Min.............	0	1	5	10	15	30	60	90	180	300
Basic fuchsin, 1 gm. in 100 cc. of H_2O.......	+++*	+++	+++	+++	++	++	+	+	+	0
Acid fuchsin, 3 gm. in 10 cc. of H_2O......	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Crystal violet, 0.5 gm. in 100 cc. of H_2O	+++	+++	0	0	0	0	0	0	0	0
Mercurochrome 220, 0.5 gm. in 100 cc. of H_2O........	+++	+++	+++	+++	+++	++	++	+	+	+
Neutral acriflavine, 0.5 gm. in 100 cc. of H_2O......	+++	+++	++	++	++	+	0	0	0	0

* +++ indicates as much growth as was shown by the control.

An examination of Table V indicates that under the conditions of this experiment crystal violet is more bactericidal for *Bacterium coli* than neutral acriflavine. We should not expect this to hold true in infected tissues since other experimental work has shown that in serum the bactericidal action of basic triphenylmethane dyes is decreased and that of the flavines increased.

DISCUSSION.

Bactericidal Action of Acid Fuchsin.

Our experiments show that acid fuchsin is not a strong disinfectant, particularly in the presence of organic matter. Churchman's experiments with acid fuchsin were made by combining 5 drops of a saturated aqueous solution of acid fuchsin to 20 drops of an aqueous suspension of bacteria, held at 45°C. for 1 hour, then stroking on agar to determine viability.[3] He expresses the opinion that a weaker solution of the dye would have been effective. It should be noted that 20 per cent of a saturated solution represented a great concentration of the dye, that the experiments were conducted in the absence of organic matter and in the presence of heat. Beckwith has shown that a dilution of less than 1:1,000 of acid fuchsin at 37°C. will kill *Bacterium typhosum* in bouillon in 24 hours;[4] also that the action of the dye increases with an increase in acidity. But it is not advisable to increase the acidity of body fluids in most cases of infection.

Our experiments would seem to indicate that acid fuchsin can have little or no value as a disinfectant in the treatment of wounds, for it is, as just mentioned, a very weak disinfectant in the presence of organic matter. The possibility of combining it with gentian violet or other triphenylmethane dyes for the purpose of inhibiting the Gram-negative bacteria does not appear to be an important one. It is true that the Gram-negative bacteria, such as *Bacterium coli* and *Pseudomonas pyocyanea,* are difficult to eliminate from wounds with gentian violet. However, gentian violet is toxic to these organisms in greater dilution than is acid fuchsin. Apparently nothing would be gained by adding acid fuchsin.

It is doubtful if acid fuchsin can be utilized in the separation of bacterial species to the same extent as gentian violet. The use of heat is a more practical and cheaper method of isolating spore bearers. Neither heat nor acid fuchsin can be used if spores are not present. These statements apply to the bacteria as a group. It is, of course, conceivable that under certain conditions the use of

[4] Beckwith, T. D., *J. Infect. Dis.*, 1921, xxix, 495.

acid fuchsin may serve to separate some Gram-positive bacteria from Gram-negative ones. But it cannot be used in isolating Gram-positive spore bearers directly from wounds since bacteria rarely produce spores in the body.

Bacteriostatic and Bactericidal Power of the Dyes.

A chemical substance may stimulate the growth of bacteria in one strength, inhibit the growth of bacteria in greater concentration, and kill bacteria in still greater concentration. Certain substances may serve very well as inhibiting or bacteriostatic agents but prove to be of little value as bactericidal agents. In general it has been assumed that bacteriostatic and bactericidal properties run parallel; *i.e.*, any substance that will inhibit the growth of one bacillus in greater dilution than it will inhibit the growth of a second bacillus will also kill the first bacillus in greater dilution than it will kill the second bacillus. This refers to vegetative cells. The presence of spores introduces a separate factor. It is evident that a substance may inhibit a spore-bearing organism in weaker dilution than a non-spore-bearing organism but on account of the extreme resistance of spores not kill the spore-bearing organism in any dilution while killing the non-spore-bearing organism. In such a case it might be stated that the bacteriostatic and bactericidal properties do not run parallel.

Recently attention has been drawn to a lack of parallelism between the bacteriostatic and bactericidal properties of acid fuchsin.[3,5] Our experiments indicate that this lack of parallelism is due to spore resistance.

The suggestion made by Churchman that some dye specific for the Gram-negative bacteria might be combined with gentian violet for the preparation of a disinfectant destructive to both groups of bacteria is worthy of consideration. However, every dye so far investigated that will destroy the Gram-negative bacteria such as *Bacterium coli* and *Pseudomonas pyocyanea* in tissues has been found also to destroy or inhibit the Gram-positive bacteria exclusive of the spores. From the evidence of our experiments (Table V) it

[5] Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 543.

seems probable that the SO_3 group will have little value in comparison with the triphenylmethane or flavine dyes in the treatment of infections with either Gram-positive or Gram-negative bacteria. We are investigating the possibility of combining two dyes of different molecular structure without reducing the bactericidal action of either.

Since most of the spore bearers rarely if ever produce spores in the body and since, furthermore, the pathogenic spore bearers are mainly Gram-positive and susceptible to the triphenylmethane dyes, spore resistance is not a weighty clinical problem. It is true that infection often arises from spores introduced into fresh wounds but after the initial stage of infection they cease to be a factor save when they remain dormant and constitute a menace for the future.

CONCLUSIONS.

1. The reverse selective action attributed to acid fuchsin can be explained on the basis of spore resistance. This action does not apply to the vegetative cells of Gram-positive spore bearers. It cannot be extended to include the Gram-negative spore bearers.
2. The selective action of acid fuchsin is that of an ordinary weak disinfectant and is not comparable to the selective action of the triphenylmethane dyes.
3. A lack of parallelism between the bactericidal and bacteriostatic action of acid fuchsin can be explained on the basis of spore resistance and it does not exist when spores are excluded.
4. Acid fuchsin is a weaker disinfectant than basic fuchsin for both Gram-negative and Gram-positive bacteria. The process of sulfonation by which acid fuchsin is made from basic fuchsin results in a reduction in the bactericidal action of the dye for Gram-negative and Gram-positive bacteria.
5. The most significant fact in the action of the dyes on bacteria is the toxic effect of the triphenylmethane dyes on Gram-positive spore-bearing organisms.