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Influence of Oxidation and Reduction on the Acid-Fastness of Mycobacteria

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Experiments are described in which the acid-fastness of mycobacteria was destroyed by reduction and restored by oxidation.

In a previous paper (1) it was reported that oxidation enhanced the staining of weakly acid-fast tubercle bacilli and restored that of the chromophobic forms. This finding indicated that oxygen plays an important role in the phenomenon of acid-fastness, hallmark of mycobacteria. It could be postulated, therefore, that reduction of the bacilli should have the opposite effect on their acid-fast staining. To test this hypothesis, a series of experiments were carried out, the results of which are the subject of this publication.

Thirty-one 1-month-old cultures of 7H10 agar, comprising 26 strains of virulent tubercle bacilli, four strains of group I atypical (photochromogenic) acid-fast organisms recovered from patients under treatment for pulmonary tuberculosis, and one culture of *Mycobacterium phlei*, were used for the experiments.

Series of numbered pairs of smears were prepared by spreading between two slides a sufficient amount of culture with enough distilled water to make a suspension which would spread easily and give thin and regular films without injuring the bacilli. The films were placed for the night in a paraffin oven at 60 C to dry and to secure good adhesion of the material to the slides.

The next day, the slides selected for the experiments were stained overnight in carbolfuchsin prepared as follows: basic fuchsin (Allied Chemical), 1 g; absolute alcohol, 10 ml; water, 100 ml; phenol, 5.06 ml. The following day, they were rinsed and decolorized in 5% nitric acid-90% alcohol, washed, counterstained for 30 sec in Loeffler's methylene blue, and examined, and the intensity of their staining was recorded. One slide of each pair was kept for control. The companion slides to be used for the experiment were decolorized with the above mixture to remove the methylene blue, then washed in tap water, rinsed in distilled water, dried, and exposed to the following solution: sodium sulfite (chemical grade), 6 g; 40% formol (chemical grade), 70 ml; absolute alcohol, 30 ml.

Preparation of the solution. The beaker containing the sodium sulfite and formol was placed in a water bath under a hood and heated under stirring until the salt was dissolved and then the alcohol was added by fractions under gentle shaking. Before removing from the hood, the beaker was covered to avoid the pungent fumes of formol. The reducing mixture was transferred into clean Coplin jars preheated in the oven.

Procedure for reduction. (i) The slides under study were transferred into the reducing mixture, and the jars were placed back in the oven. Stained preparations were usually bleached after 5 min when the first check was made, although the bacilli were not yet reduced. They were shaken individually with a forceps and the jars were replaced in the oven. From then onward, the slides were shaken individually every 15 min during the first hour and every 30 min during the next 2 hr. (ii) After 3 hr, they were transferred into jars filled with fresh mixture and left for another 2 hr, interrupted by individual shaking every 30 min. (iii) Then the preparations were washed in running hot water for 30 min, rinsed in distilled water, dried, and examined. Macroscopically, most of the preparations were colorless and a few were faintly pink. Under the microscope, thinly spread bacilli were decolorized; thick agglomerations were frequently faintly pink and occasionally sprinkled with precipitates of fuchsin. No acid-fast organisms were present. (iv) The preparations were tested by steaming with carbolfuchsin and counterstaining as previously indicated. If the reduction was successful, no acid-fast bacilli were visible, but all the organisms were stained blue. (v) The methylene blue was removed with the decolorizing mixture, the preparations were washed in tap water, rinsed in distilled water, and dried.

Next the slides were oxidized in 10% periodic acid overnight and then washed rapidly in tap water, rinsed with distilled water, dried, and stained overnight with carbolfuchsin. After that,

they were decolorized and counterstained as before and examined.

Microscopically, the bacilli had recovered their acid-fastness. The intensity of their staining was compared with that of the companion slides and with that recorded for the original staining of the same slides as shown in the table. In some cases the results were recorded by color photographs taken after the original staining, after reduction, and after oxidation and restaining.

Reduction of stained slides is necessary if photographic evidence of the three steps of the procedure is desired. Otherwise it is preferable to reduce unstained slides, leaving a stained companion slide for comparison. The advantages of reducing unstained material are that the reducing mixture is used on bacterial cells unaltered by the staining procedure, that the elimination of one staining makes the formation of precipitates less likely, and that the terminal staining is frequently better.

Examination of the table shows that, according to their original and experimental acid-fastness, the cultures fall into the following three groups: (i) initial staining equal to terminal staining: 2, 6, 7, 10, 12–14, 17–21, 23, 24, 26, 27, 29; (ii) experimental staining more intense than original: 1, 3, 5, 9, 15, 16, 22, 25, 27B, 31, 32; (iii) original staining more intense than terminal: 4, 28, 30.

It is worth emphasising that the four cultures of atypical acid-fast bacilli (3, 27B, 31, 32) and the grass bacillus (9), originally weakly acid-fast, gave a strong terminal staining. However, when taken from a poorly growing culture, both the initial and terminal staining of the grass bacillus were very weak, and only when taken from a well-developed culture did it react in the same way as the atypical acid-fast organisms.

TABLE 1. *Intensity of natural and experimental acid-fast staining of the 31 cultures examined*

Culture no.	Initial staining	Terminal staining	Culture no.	Initial staining	Terminal staining
2	3-plus	3-plus	21	2-plus	2-plus
6	3-plus	3-plus	1	3-plus	4-plus
7	3-plus	3-plus	3[a]	3-plus	4-plus
10	3-plus	3-plus	5	2-plus	3-plus
12	3-plus	3-plus	9[b]	2-plus	4-plus
13	3-plus	3-plus	15	3-plus	4-plus
14	3-plus	3-plus	16	3-plus	4-plus
17	3-plus	3-plus	22	3-plus	4-plus
18	3-plus	3-plus	25	3-plus	4-plus
19	3-plus	3-plus	27B[a]	2-plus	4-plus
20	3-plus	3-plus	31[a]	1-plus	4-plus
23	3-plus	3-plus	32[a]	2-plus	4-plus
24	3-plus	3-plus	4	3-plus	2-plus
26	3-plus	3-plus	28	3-plus	2-plus
27	3-plus	3-plus	30	3-plus	2-plus
29	3-plus	3-plus			

[a] Atypical acid-fast organism.
[b] *Mycobacterium phlei.*

Trial experiments have shown that lepra bacilli respond to oxidation-reduction in the same way as tubercle bacilli.

Six cultures of *Salmonella*, seven cultures of diphtheroid bacilli, and eight cultures of *Escherichia coli* were treated in the same way as the acid-fast organisms, with no change in their natural staining.

Experiments to get information about the mechanism by which the reducing mixture destroys acid-fastness revealed that neither alcohol nor formol had any effect on it when used either singly or in combination. Sulfite alone, when applied in an aqueous solution, had no appreciable influence on the staining of the bacilli, although in combination with alcohol it has been used in the past for bleaching smears of sputum stained with fuchsin (2). However, when dissolved in hot formol, it did have a clear, though slow, decolorizing action, requiring as long as 24 hr to bleach the bacilli, which suggests that there is some sort of synergistic action between sulfite and formol. Addition of alcohol to the sulfite-formol mixture accelerated its bleaching action, shortening the process to a few hours, probably by removing fuchsin and its leuco compounds from the bacilli. A drawback is that alcohol lessens the ability of formol to dissolve sulfite, which is the active ingredient of the mixture. It was therefore difficult to adjust the proportion of all three ingredients to obtain the optimum results in both quality of terminal staining and speed. The formula given in this paper has been found by trials to be the most satisfactory, particularly since the amount of sulfite can, if necessary, be increased to 10 or even 12%. This flexibility is of great importance in view of the known variability of the acid-fastness of the bacilli depending on the strain, the medium, and the growth rate, to which the concentration of the reducing mixture and the exposure time have to be adjusted.

The chemical reaction by which the reducing mixture obliterates acid-fastness has not yet been studied systematically, but there is sufficient evidence to suggest that it is a reductive process. Measured by a potentiometer (Beckman Instruments, Inc.), the mixture has a reduction potential of about -120 mv and a pH of about 9. The active ingredient of the solution is sodium sulfite, a reducing agent, and its effectiveness is proportionate to the amount of this salt which it contains. Acid-fastness obliterated by reduction can be restored by oxidation.

During the initial phase of this investigation,

when NTS and Lowenstein's egg media were used, growth was good in about one-third of the tubes and less satisfactory in the others, and so was the staining of the bacilli. After reduction and oxidation, the terminal staining in a few tubes was equal to or better than the original staining, but in the majority it was less intense than initially. For this reason the egg media were abandoned and 7H10 agar was used. Growth was irregular during the first subcultures, but it improved with each transfer, and the fourth subculture yielded a homogeneous growth and satisfactory staining with only slight differences between the tubes.

Equally good results have been obtained with cultures grown on Dubos' medium with Tween and albumin which was, however, less frequently used for technical reasons. These observations indicate that the chemistry of the bacilli varies with the medium on which they have been grown and that this is reflected in their acid-fastness, both natural and experimental.

The information presented in this report bears evidence that oxygen plays an important role in the acid-fastness of mycobacteria. In experimental conditions, increase in their oxygen potential results in increase in their acid-fastness, and depletion by reduction is followed by loss of acid-fastness. This suggests that in vivo acid-fastness and its fluctuations are due to reactions of oxidation and reduction accompanying the metabolic acitivity of the cells. The effect of these reactions on the staining affinities of the cells seems to be of a physico-chemical rather than a purely chemical nature. This opinion is supported by trial experiments submitting acid-fast bacilli to ultrasonic vibrations. These showed that the shapeless debris and cellular fragments remaining after the operation, non-acid-fast as was to be expected, could not have their acid-fastness restored by oxidation.

LITERATURE CITED

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